William A. Cramer
Toivo Kallas *Editors*

Cytochrome Complexes: Evolution, Structures, Energy Transduction, and Signaling

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Cytochrome Complexes: Evolution, Structures, Energy Transduction, and Signaling
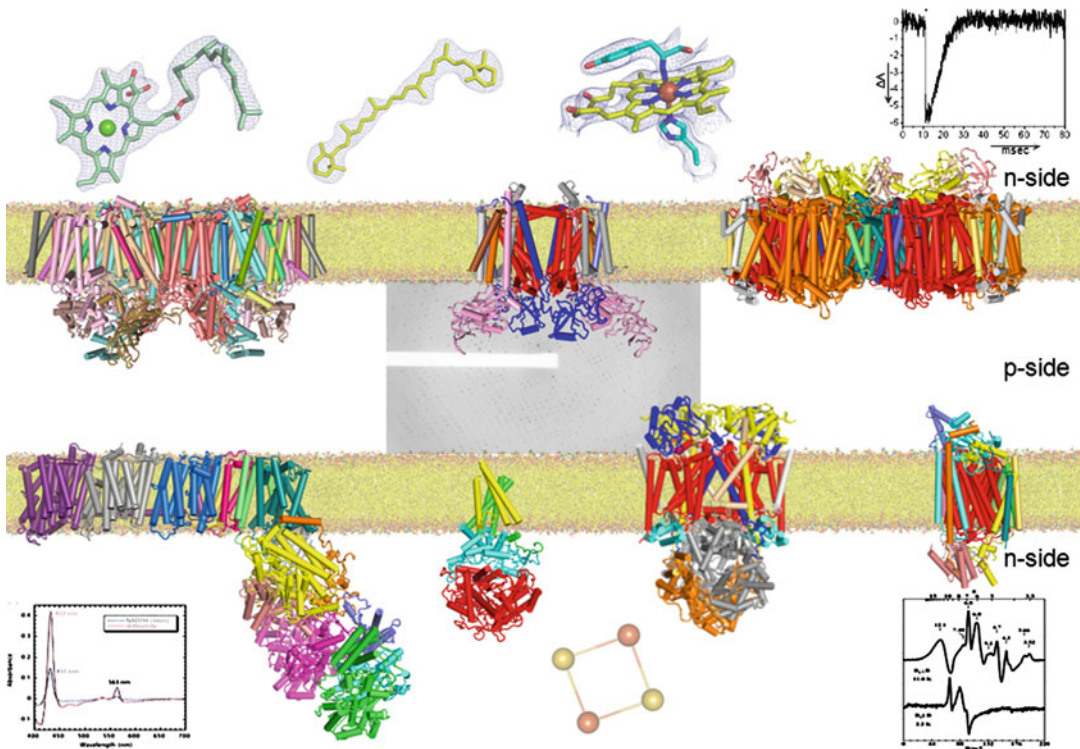
Experimental Information Important for Understanding Properties of Photosynthetic Cytochromes. Along the top are stick diagrams and electron density of pigments involved in light-harvesting – Chl-*a*, β-carotene, and heme of cytochrome *f* with unusual Tyr–His axial ligation (pdb, protein data base code, 4OGQ). This is followed by a flash kinetic trace of cytochrome oxidation-reduction. Along the bottom is a redox difference spectrum of the cytochrome b_6f complex, the [2Fe-2S] cluster in the Rieske iron-sulfur protein (pdb 4OGQ), and electron spin resonance spectra showing the unique $g = 12$ signal for the heme b_n–c_n couple in the cytochrome b_6f complex. In the center is an X-ray diffraction pattern of cytochrome b_6f crystals (pdb 4OGQ). **Protein structures** (*left* to *right*); **upper bilayer**: Photosynthetic electron transfer chain – Photosystem II (pdb 2WU2), cytochrome b_6f complex (pdb 4OGQ), Photosystem I (pdb 1JB0); **lower bilayer**: Mitochondrial electron transport chain – NDH complex (pdb 4HEA), succinate dehydrogenase (pdb 1NEN), cytochrome bc_1 complex (pdb 3CX5), cytochrome c oxidase (pdb 1V54). The lipid bilayer is a homogeneous DOPC bilayer generated with the CHARMM program. *Abbreviations*: p, n-side, electrochemically positive and negative side of the membrane (drawing by S. Saif Hasan)

Advances in Photosynthesis and Respiration
Including Bioenergy and Related Processes

VOLUME 41

The book series ADVANCES IN PHOTOSYNTHESIS AND RESPIRATION *Including Bioenergy and Related Processes* provides a comprehensive and state-of-the-art account of research in photosynthesis, respiration and related processes. Virtually all life on our planet Earth ultimately depends on photosynthetic energy capture and conversion to energy-rich organic molecules. These are used for food, fuel, and fiber. Photosynthesis is the source of almost all bioenergy on Earth. The fuel and energy uses of photosynthesized products and processes have become an important area of study, and competition between food and fuel has led to resurgence in photosynthesis research. This series of books spans topics from physics to agronomy and medicine; from femtosecond processes through season-long production to evolutionary changes over the course of the history of the Earth; from the photophysics of light absorption, excitation energy transfer in the antenna to the reaction centers, where the highly-efficient primary conversion of light energy to charge separation occurs, through the electrochemistry of intermediate electron transfer, to the physiology of whole organisms and ecosystems; and from X-ray crystallography of proteins to the morphology of organelles and intact organisms. In addition to photosynthesis in natural systems, genetic engineering of photosynthesis and artificial photosynthesis is included in this series. The goal of the series is to offer beginning researchers, advanced undergraduate students, graduate students, and even research specialists, a comprehensive, up-to-date picture of the remarkable advances across the full scope of research on photosynthesis and related energy processes. The purpose of this series is to improve understanding of photosynthesis and respiration at many levels both to improve basic understanding of these important processes and to enhance our ability to use photosynthesis for the improvement of the human condition.

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Cytochrome Complexes: Evolution, Structures, Energy Transduction, and Signaling

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From the Series Editors

Advances in Photosynthesis and Respiration Including Bioenergy and Related Processes
Volume 41: Cytochrome Complexes: Evolution, Structures, Energy Transduction, and Signaling

We are delighted to announce the publication of Volume 41 in this series on *Advances in Photosynthesis and Respiration Including Bioenergy and Related Processes*. Oxygenic photosynthesis begins with conversion of light energy into chemical energy at two photochemical reaction centers in two separate photosystems (PS) I and II; this is followed by oxidation of water to molecular oxygen, reduction of pyridine nucleotide $NADP^+$ (nicotinamide adenine dinucleotide phosphate) to NADPH, and synthesis of ATP from ADP and inorganic phosphate by ATP synthase, the latter using the proton motive force produced across the thylakoid membrane during electron transport from water to $NADP^+$. Four protein complexes are essential for the completion of the entire process that leads to the formation of O_2, NADPH, and ATP: (1) Photosystem II (PSII); (2) Photosystem I (PSI); (3) Cytochrome (Cyt) b_6f complex; and (4) ATP synthase. Volume 22 in our Series, edited by T. Wydrzynski and K. Satoh and published in 2005, covered in great depth structure and function of PSII (Water-Plastoquinone Oxidoreductase); and volume 24, edited by J.H. Golbeck and published in 2006, did the same for PSI (Plastocyanin-ferredoxin oxidoreductase). Now, in 2016, we are fortunate to be able to present a complete structure-function description of not only Cyt b_6f complex but related complexes from mitochondria as well as from anoxygenic photosynthesis. This book (volume 41), *Cytochrome Complexes: Evo-lution, Structures, Energy Transduction, and Signaling*, was edited by two international authorities in biology, biochemistry, and biophysics: William A. Cramer and Toivo Kallas. In oxygenic photosynthesis, Cyt b_6f provides the link between PSII and PSI; it takes plastoquinol (PQH_2) made by PSII and reduces plastocyanin, which, in turn, is the source of electrons to reduce feredoxin (and, thus, $NADP^+$) by PSI. In addition, the cytochrome complex functions in the so-called Q cycle that is essential in generating an electrochemical proton gradient sufficient to generate the necessary ATP.

This book starts, appropriately, with a chapter by the late Derek S. Bendall, where he described the historical background of the field as well as the cytochrome notation. The book includes a discussion of the evolution of cytochromes and their functions. Students will benefit by an extensive exposure to both the experiments and the theory underlying electron transfer in proteins, as well as that of the molecular structures of cytochromes and even supercomplexes from both eukaryotes and prokaryotes. Essentially, one has, for the first time, as one would say, "*All we wanted to know about cytochromes, but could not bring ourselves to ask.*" Yes, the book includes authoritative informa-tion even on macromolecular assembly, regulation, and signaling via the Cyt b_6f complex.

Because volume 41 covers an extensive subset of the cytochrome complexes that are involved not only in oxygenic photosynthesis

but also in anoxygenic photosynthesis, as well as in respiration, one is confident that this book will be used in educating undergraduate and graduate students and researchers not only in plant and agricultural sciences but animal sciences as well as in microbiology. And since the book covers in depth the structure and function, the molecular biology, and the biochemistry as well as the biophysics of these cytochrome complexes (see Preface as well as Table of Contents of this book for further details), it can be used by students in molecular & cell biology, biochemistry, chemical biology, and biophysics. Further, one can see its significance in areas of agricultural and chemical engineering and in biotechnology, all of which should contribute to the significance of this book.

Authors of Volume 41

We note with great pride that the current volume is truly an international book; it has authors from 13 countries: Finland (1); France (6); Germany (10); Italy (3); Ireland (3); Israel (2); Japan (8); Lithuania (2); Poland (6); Russia (10): Switzerland (1); UK (8); and USA (25).

We begin by specifically mentioning here two authors, who are also editors of this volume: William A. Cramer of Purdue University, Indiana, USA, and Toivo Kallas of University of Wisconsin Oshkosh, Wisconsin, USA (see their biographies in this volume). Cramer's theme of research is the structure and function of membrane proteins, with a major focus on photosynthetic energy transduction via the cytochrome b_6f protein complex. We recommend that you visit his websites since they provide information on him as well as on his research: https://www.bio.purdue.edu/lab/cramer/; https://www.bio.purdue.edu/People/faculty_dm/directory.php?refID=12 Kallas's theme of research is overall photosynthesis, but in particular, electron transport through the cytochrome b_6f complex. His work has often focused on cyanobacteria; his web page is at http://www.uwosh.edu/facstaff/kallas.

There are 80 authors (including the two editors), who are experts in the field of their research, especially cytochromes. Alphabetically (by last names), they are Jean **A**lric, Eva-Mari Aro, Danas **B**aniulis, Adrian C. Barbrook, Carl E. Bauer, the late Derek S Bendall, Gabor Bernat, Edward Berry, Wojciech Bialek, Robert E. Blankenship, Elisa Bombarda, Martin **C**affrey, William A. Cramer, Fevzi **D**aldal, Miguel A. De la Rosa, Irene Díaz-Moreno, Antonio Díaz-Quintana, Timothy J. Donohue, Anne-Lise Ducluzeau, Seda **E**kici, Robert Ekiert, Lothar Esser, Giovanni **F**inazzi, Maria Luisa **G**enova, Patrice **H**amel, Shigeharu Harada, S. Saif Hasan, Florian Hilbers, Christopher J. Howe, Li-shar Huang, Saheed **I**mam, Daniel Ken Inaoka, Giles N. **J**ohnson, Toivo **K**allas, Bahia Khalfaoui-Hassani, Kiyoshi Kita, Hans-Georg Koch, Piotr Kolesinski, David Kramer, Lev I. Krishtalik, Genji Kurisu, Giorgio **L**enaz, Joseph A. Lyons, Erica L.W. **M**ajumder, Alizée Malnoë**,** Benjamin May, Yuval Mazor, I. Miliute, Jun Minagawa, Anthony L. Moore, Frank Müh, Lars Mueller, Nathan **N**elson, Robert H. Nimmo, Wolfgang Nitschke, Daniel R. Noguera, Artur **O**syczka, Jean-David Rochaix, Matthias Rögner, Marcin **S**arewicz, Georg Schmetterer, Dirk Schneider, Tomoo Shiba, Toshiharu Shikanai, Namita Shroff, Melanie A. Spero, Andrzej Szczepaniak, Petru-Iulian **T**rasnea, G. Matthias **U**llmann, Marcel Utz, Andreia F. **V**erissimo, Di **X**ia, Shinya **Y**oshikawa, Luke Young, Chang-An Yu, Sébastien **Z**appa, Fei Zhou, Francesca Zito, and Athina Zouni.

Our Books

We list below information on the 40 volumes that have been published thus far (see http://www.springer.com/series/5599 for the series website). Electronic access to individual chapters depends on subscription (ask your librarian) but Springer provides free downloadable Preface, sample pages and Table of Content for nearly all volumes. The available websites of the books in the Series are listed below.

- **Volume 40 (2014): Non-Photochemical Quenching and Energy Dissipation in Plants, Algae and Cyanobacteria**, edited by Barbara Demmig-Adams, Győző Garab, William W. Adams III, and Govindjee from USA and Hungary. Twenty-eight chapters, 649 pp, Hardcover ISBN 978-94-017-9031-4, eBook ISBN 978-94-017-9032-1 [http://www.springer.com/life+sciences/plant+sciences/book/978-94-017-9031-4]

- **Volume 39 (2014): The Structural Basis of Biological Energy Generation**, edited by Martin F. Hohmann-Marriott from Norway. Twenty-four chapters, 483 pp, Hardcover ISBN 978-94-017-8741-3, eBook ISBN 978-94-017-8742-0 [http://www.springer.com/life+sciences/book/978-94-017-8741-3]

- **Volume 38 (2014): Microbial BioEnergy: Hydrogen Production**, edited by Davide Zannoni and Roberto De Phillipis, from Italy. Eighteen chapters, 366 pp, Hardcover ISBN 978-94-017-8553-2, eBook ISBN 978-94-017-8554-9 [http://www.springer.com/life+sciences/plant+sciences/book/978-94-017-8553-2]

- **Volume 37 (2014): Photosynthesis in Bryophytes and Early Land Plants**, edited by David T. Hanson and Steven K. Rice, from USA. Eighteen chapters, approx. 342 pp, Hardcover ISBN 978-94-007-6987-8, eBook ISBN 978-94-007-6988-5 [http://www.springer.com/life+sciences/plant+sciences/book/978-94-007-6987-8]

- **Volume 36 (2013): Plastid Development in Leaves during Growth and Senescence**, edited by Basanti Biswal, Karin Krupinska and Udaya Biswal, from India and Germany. Twenty-eight chapters, 837 pp, Hardcover ISBN 978-94-007-5723-33, eBook ISBN 978-94-007-5724-0 [http://www.springer.com/life+sciences/plant+sciences/book/978-94-007-5723-3]

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- **Volume 34 (2012): Photosynthesis – Plastid Biology, Energy Conversion and Carbon Assimilation**, edited by Julian Eaton-Rye, Baishnab C. Tripathy, and Thomas D. Sharkey, from New Zealand, India, and USA. Thirty-three chapters, 854 pp, Hardcover, ISBN 978-94-007-1578-3, eBook ISBN 978-94-007-1579-0 [http://www.springer.com/life+sciences/plant+sciences/book/978-94-007-1578-3]

- **Volume 33 (2012): Functional Genomics and Evolution of Photosynthetic Systems**, edited by Robert L. Burnap and Willem F.J. Vermaas, from USA. Fifteen chapters, 428 pp, Hardcover ISBN 978-94-007-1532-5, Softcover ISBN 978-94-007-3832-4, eBook ISBN 978-94-007-1533-2 [http://www.springer.com/life+sciences/book/978-94-007-1532-5]

- **Volume 32 (2011): C_4 Photosynthesis and Related CO_2 Concentrating Mechanisms,** edited by Agepati S. Raghavendra and Rowan Sage, from India and Canada. Nineteen chapters, 425 pp, Hardcover ISBN 978-90-481-9406-3, Softcover ISBN 978-94-007-3381-7, eBook ISBN 978-90-481-9407-0 [http://www.springer.com/life+sciences/plant+sciences/book/978-90-481-9406-3]

- **Volume 31 (2010): The Chloroplast: Basics and Applications,** edited by Constantin Rebeiz (USA), Christoph Benning (USA), Hans J. Bohnert (USA), Henry Daniell (USA), J. Kenneth Hoober (USA), Hartmut K. Lichtenthaler (Germany), Archie R. Portis (USA), and Baishnab C. Tripathy (India). Twenty-five chapters, 451 pp, Hardcover ISBN 978-90-481-8530-6, Softcover ISBN 978-94-007-3287-2, eBook ISBN 978-90-481-8531-3 [http://www.springer.com/life+sciences/plant+sciences/book/978-90-481-8530-6]

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- **Volume 18 (2005): Plant Respiration: From Cell to Ecosystem,** edited by Hans Lambers and Miquel Ribas-Carbo, from Australia and Spain. Thirteen chapters, 250 pp, Hardcover ISBN978-14020-3588-3, Softcover ISBN 978-90-481-6903-0, eBook ISBN 978-1-4020-3589-0 [http://www.springer.com/life+sciences/plant+sciences/book/978-1-4020-3588-3]

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- **Volume 14 (2004): Photosynthesis in Algae**, edited by Anthony W. Larkum, Susan Douglas and John A. Raven, from Australia, Canada and UK. Nineteen chapters, 500 pp, Hardcover ISBN 978-0-7923-6333-0, Softcover ISBN 978-94-010-3772-3, eBook ISBN 978-94-007-1038-2 [http://www.springer.com/life+sciences/plant+sciences/book/978-0-7923-6333-0]

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Special 25 % discounts are available to members of the International Society of Photosynthesis Research, ISPR http://www.photosynthesisresearch.org/. See http://www.springer.com/ispr.

Future Advances in Photosynthesis and Respiration and Other Related Books

The readers of the current series are encouraged to watch for the publication of the forthcoming books (not necessarily arranged in the order of future appearance):

- *Canopy Photosynthesis: From Basics to Applications* (Editors: Kouki Hikosaka, Ülo Niinemets and Niels P.R. Anten); it is already available; see < http://www.springer.com/us/book/9789401772907>

- *Photosynthesis and Climate Change* (working title) (Editor: Joy K. Ward)

- *Cyanobacteria* (Editor: Donald Bryant)
- *Leaf Photosynthesis* (Editors: William W. Adams III and Ichiro Terashima)
- *Photosynthesis in Algae* (Editors: Anthony Larkum and Arthur Grossman)
- *Plant Respiration* (Editor: Guillaume Tcherkez)

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- Interactions between Photosynthesis and other Metabolic Processes
- Limits of Photosynthesis: Where do we go from here?
- Photosynthesis, Biomass and Bioenergy
- Photosynthesis under Abiotic and Biotic Stress

If you have any interest in editing/coediting any of the above listed books, or being an author, please send an e-mail to Tom Sharkey (tsharkey@msu.edu) and/or to Govindjee (gov@illinois.edu). Suggestions for additional topics are also welcome.

Instructions for writing chapters in books in our series are available by sending e-mail requests to one or both of us; they may also be downloaded from Govindjee's website http://www.life.illinois.edu/govindjee as the fourth item under "Announcements" on the main page.

Acknowledgments

March 15, 2016

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Series Editors

Govindjee is the founding series editor of *Advances in Photosynthesis*, as it was called then, with volume 1 (*The Molecular Biology of Cyanobacteria*, 1994). In addition to being co-series editor (with Tom Sharkey) since volume 31 (The Chloroplast: Basics and Applications, 2010), he has coedited (1) Volume 19 (*Chlorophyll a Fluorescence: A Signature of Photosynthesis*, 2004); (2) Volume 20 (*Discoveries in Photosynthesis*, 2006); (3) Volume 29 (*Photosynthesis In Silico: Understanding Complexity from Molecules to Ecosystems*, 2009); and (4) Volume 40 (*Non-Photochemical Quenching and Energy Dissipation in Plants, Algae and Cyanobacteria*, 2014).

Govindjee, who uses one name only, has been Professor Emeritus of Biochemistry, Biophysics, and Plant Biology at the University of Illinois at Urbana- Champaign (UIUC), since 1999. His short evolving biography can be found in each volume of *Advances in Photosynthesis and Respiration*, and other information on him and his activities is available at his website http://www.life.illinois.edu/govindjee. An interview for Annual Reviews Inc., by Don Ort, is available at https://www.youtube.com/watch?v=cOzuL0vxEi0. His recent publications are listed at http://www.life.illinois.edu/govindjee/recent_papers.html, and earlier publications since 1955 are at http://www.life.illinois.edu/govindjee/pubschron.html.

Govindjee was trained in Plant Physiology (1952–1956) by Shri Ranjan (a student of Felix Frost Blackman of UK), in Biology and Biochemistry of Photosynthesis (1956–1958) by Robert Emerson (a student of the Nobel laureate Otto Warburg), and in Biophysics of Photosynthesis (1958–1961) by Eugene Rabinowitch (a postdoc student of the Nobel laureate James Franck). After his Ph.D. in Biophysics in 1960 from the UIUC, he has learned many concepts and techniques from many others including Louis N.M. Duysens (Leiden, The Netherlands); C. Stacy French (Stanford, California, USA); Herbert S. Gutowsky (Urbana, Illinois, USA); Bessel Kok (Baltimore, Maryland, USA); Jean Lavorel (Gif-syur-Yvette, France); Gregorio Weber (Urbana, Illinois); and Horst Witt (Berlin, Germany).

Govindjee's discoveries, with his coworkers and graduate students, include the participation of chlorophyll (Chl) *a* (in what we now call Photosystem (PS) II); proof of the existence of two light reactions in NADP reduction, and in Chl a fluorescence; temperature dependence of excitation energy transfer down to 4° K in algae and cyanobacteria; molecular understanding of both the fast and slow Chl a fluorescence (both prompt and delayed) changes—that includes participation of membrane potential, pH gradient, "traffic jam" in PSI, and the so-called state changes; first picosecond measurements on the primary photochemistry of PSI and PSII; unique role of bicarbonate in electron transfer and protonation events at the Q_B binding site; first comprehensive theory of thermoluminescence in plants; and the first use of lifetime of Chl a fluorescence measurements in understanding photoprotection in plants.

Govindjee's activities include, besides research on "Light Reactions of Photosynthesis," honoring others (Robert Blankenship; Bob Buchanan; Andre Jagendorf; Wolfgang Junge; Hartmut Lichtenthaler; George Papageorgiou; William Ogren; Vladimir Shuvalov; and Diter Von Wettstein; see Govindjee's website) in the field, as well as participating in writing and editing obituaries and tributes (Lou Duysens; Colin Wraight; Al Bassham; Rene Marcelle; V.S.R. Rama Das; Prasanna Mohanty; Jalal Aliyev; Al Frenkel; and Andy Benson). In addition, he enjoys lecturing on the history of photosynthesis research and in teaching photosynthesis by having students act as molecules and demonstrating the path of electrons in photosynthesis by having them enact a drama. He has had many honors, but he cherishes five of them dearly: 2006 Lifetime Achievement Award of the Rebeiz Foundation for Basic Biology; 2007 Communication Award of the International Society of Photosynthesis Research; 2008 Liberal Arts & Sciences Lifetime Achievement Award of UIUC; 2015 Dr. B.M. Johri Memorial Award for Excellence in Plant Biology, from the Society of Plant Research, India; and a 2016 Distinguished Alumnus Award from his High School (Colonelganj Inter College) in Allahabad. In addition, he has enjoyed what others have written on him at his 75th and 80th birthdays (see Photosynth Res **93**:1–5 (2007); **94**: 153–178 (2007); **100**: 49–55 (2009); **116**: 107–110; 111–144 (2013); **122**: 113–119); also see <https://www.linkedin.com/pulse/govindjee-living-legend-i-met-dr-ravi-sharma>. (To see this site, click on "living-legend".) A rather useful effort in teaching others is through the article "Photosynthesis Web Resources" <http://www.life.illinois.edu/govindjee/photoweb/>. Also see a collection of education books at http://www.life.illinois.edu/govindjee/g/Books.html. He is always delighted to respond to questions on both photosynthesis research and education (e-mail: gov@illinois.edu).

Thomas D. (Tom) Sharkey obtained his Bachelor's degree in Biology in 1974 from Lyman Briggs College, a residential science college at Michigan State University, East Lansing, Michigan, USA. After 2 years as a research technician, Tom entered a Ph.D. program in the Department of Energy Plant Research Laboratory at Michigan State University under the mentorship of Klaus Raschke and finished in 1979. Postdoctoral research was carried out with Graham Farquhar at the Australian National University, in Canberra, where he coauthored a landmark review on photosynthesis and stomatal conductance. For 5 years he worked at the Desert Research Institute, Reno, Nevada. After Reno, Tom spent 20 years as Professor of Botany at the University of Wisconsin in Madison. In 2008, Tom became Professor and Chair of the Department of Biochemistry and Molecular Biology at Michigan State University. Tom's research interests center on the exchange of gases between plants and the atmosphere and carbon metabolism of photosynthesis. The biochemistry and biophysics underlying carbon dioxide uptake and isoprene emission from plants form the two major research topics in his laboratory. Among his contributions are measurement of the carbon dioxide concentration inside leaves, an exhaustive study of short-term feedback effects in carbon metabolism, and a significant contribution to elucidation of the pathway by which leaf starch breaks down at night. In the isoprene research field, Tom is recognized as the leading advocate for thermotolerance of photosynthesis as the explanation for why plants emit isoprene. In addition, his laboratory has cloned many of the genes that underlie isoprene synthesis and he has published many important papers on the biochemical regulation of isoprene synthesis. Tom's work has been cited over 21,000 times according to Google Scholar in 2015. He has been named an Outstanding Faculty member by Michigan State University and in 2015, he was honored, and named as a University Distinguished Professor. Tom has coedited three books, the first on trace gas emissions from plants in 1991 (with Elizabeth Holland and Hal Mooney), volume 9 of this series (with Richard Leegood and Susanne von Caemmerer) on the *Physiology of Carbon Metabolism of Photosynthesis* in 2000, and volume 34 (with Julian Eaton-Rye and Baishnab C. Tripathy) entitled *Photosynthesis*: *Plastid Biology*, *Energy Conversion and Carbon Assimilation*. Tom has been co-series editor of this series since volume 31.

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Preface

Why study cytochrome complexes? An answer is provided by the range of subtopics in the book, "evolution, structures, energy, and signaling," which are described in the book title. Studies on the cytochrome family of proteins encompass a uniquely wide area of basic and applied research. Research in this field utilizes a range of theoretical and computational approaches, as well as a broad cross section of experimental techniques. Understanding obtained on the structure and function of the cytochromes and cytochrome complexes utilizes an extraordinary range of experimental approaches, including computational biology, genetics, macromolecular biochemistry, molecular biology, physics of charge transfer reactions, structure analysis using x-ray and electron diffraction, and ultrafast spectroscopy.

As reflected in the book title, the information and understanding gained in the field has an influence on a wide range of subjects, including evolution, mechanisms of membrane-based respiratory and photosynthetic energy transduction, theory of charge transfer in proteins, structure-function of large hetero-oligomeric membrane proteins, including lipid-protein interactions, and transmembrane signaling.

A special aspect of cytochromes, cytochrome complexes, as well as other proteins involved in bioenergetics and charge transfer is that they allow function to be quantitatively analyzed. Thus, in this group of proteins, it is possible to determine that a protein or protein complex is functional before committing a large amount of time to crystallization and analysis of structure. Dating back to the 1988 Nobel Prize in Chemistry, given to J. Deisenhofer, H. Michel, and R. Huber for determination of the crystal structure of the bacterial photosynthetic reaction center, the majority of the crystal structures of hetero-oligomeric membrane proteins obtained in the subsequent 10 years were of energy-transducing proteins. Of these, a substantial fraction involved cytochrome complexes.

The Logic of the Collection   The book starts with an Introduction by Derek Bendall describing cytochrome notation, which is connected to the history of the field, focusing on research in England in the pre-World War II era. An *ab initio* "start with the beginning" logic then leads to a discussion of the evolution of cytochromes and hemes. Before presentation of the many individual cytochrome systems, the fundamentals of the theory of electron transfer in proteins are presented, followed by an extensive description of the molecular structures of cytochromes and cytochrome complexes from eukaryotic and prokaryotic sources, including those derived from photosynthetic reaction centers. The presentation of atomic structure information has a major role in these discussions, including the relatively new subject of "supercomplexes." This structure information has a major niche in the broad field of membrane structure-function. Expanding the perspective beyond structure-function applied to charge transfer and energy storage, the problems of protein and macromolecule assembly, regulation, and signaling, including transmembrane signaling, which have conceptual connections to central areas of biochemistry, biophysics, and cell biology, are considered. Regarding subjects related to cutting-edge areas of biology and plant biology, the up-to-date presentation of the topics of *Regulation* and *Signaling* is noted here.

The broad extent of fundamental intellectual and research areas that are represented in this book makes it a useful resource for teaching of academic courses and presentation of seminars on fundamental and

broad aspects of biological energy transduction to advanced undergraduates and graduate students with interests in biology, biochemistry, biological engineering, chemistry, and biophysics.

As a last entry to the Introduction to this book, we note, sadly, the passing of Derek S. Bendall and Bernard L. Trumpower, whose achievements in this field are substantial and of fundamental importance. The Remembrances in this volume that are dedicated to them are of historical importance and note their many contributions to the subjects discussed herein.

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Editors

William A. Cramer (WAC) received a B.S. (Physics, 1959) from the Massachusetts Institute of Technology (MIT), Boston, Mass., USA. From MIT, WAC went to the University of Chicago, where he obtained his M.S. in Physics; he initially worked and published in muon physics and birefringence of muscle proteins, and obtained a Ph.D. in Biophysics (1965), studying with R.B. Uretz. Subsequently, he carried out postdoctoral studies on photosynthetic electron transport with Warren L. Butler at the University of California/San Diego. He is currently the Henry Koffler Distinguished Professor in the Department of Biological Sciences at Purdue University, West Lafayette, Indiana. His research is focused on the relationship between structure and function of membrane proteins, including those involved in photosynthetic energy transduction. He is an authority on the cytochrome (cyt) b_6f complex, a hetero-oligomeric lipoprotein complex in the cytochrome bc family that functions in the photosynthetic and respiratory electron transport chains. The b_6f complex generates a major fraction of the transmembrane electrochemical potential and proton gradient; it is also responsible for significant generation of superoxide, and for a unique redox-controlled mechanism of transmembrane signaling that activates a kinase responsible for phosphorylation of the light-harvesting chlorophyll protein. Cramer also studies the membrane-mediated mechanism of cellular import of bacterial cytotoxins.

Some of the conceptual and experimental bases for his chapters in this book have been discussed in the 1991 textbook by him and the late David B. Knaff, entitled *Energy Transduction in Biological Membranes,* published by Springer-Verlag, as well as reviews on the cytochrome complex and the mechanism of cytotoxin import, including those in the Annual Reviews (Biophysics, Biochemistry, Plant Biology, and Genetics). WAC has served as the Chair of the Gordon Research Conferences on Photosynthesis (1990) and Bioenergetics (2001). He has served as the Chair of the Bioenergetics

Subgroup, and as the Program Chair (1996), of the National Meeting (1996) of the American Biophysical Society. He has been a recipient of the Charles F. Kettering Award of the American Society of Plant Physiology for "Excellence in Photosynthesis" (1996), a Guggenheim Fellow (1992), and is a Fellow of the Alexander von Humboldt Foundation and of the Biophysical Society.

Toivo Kallas is a Distinguished Professor of Microbial Genetics and Biotechnology at the University of Wisconsin (UW) – Oshkosh, USA. He holds a B.A. from Carleton College, Minnesota, and a Ph.D. in Microbiology from the University of Oregon where he worked with Richard Castenholz on thermophilic cyanobacteria. He did postdoctoral work with Germaine Cohen-Bazire (Stanier), Institut Pasteur (Paris, France), on aerobic nitrogen fixation by unicellular cyanobacteria and with Richard Malkin, University of California (UC), Berkeley, on the cytochrome b_6f electron transfer complex of photosynthesis. He spent sabbaticals with Francis-André Wollman and Catherine de Vitry, Institut Biologie Physico-Chimique (Paris, France), on regulation and electron transfer kinetics in *Chlamydomonas* and cyanobacteria and Michael Sussman, UW Madison Biotechnology Center, on proteomics of silica cell wall biogenesis in diatoms. His research at UW Oshkosh has focused on electron transfer proteins, energy transduction, gene expression, and metabolic engineering of cyanobacteria. Some 80 undergraduate students, 24 Master's students, and several postdoctoral and visiting scientists have participated in this research funded by grants from the US National Science Foundation, US Department of Agriculture, UW Oshkosh, and UW WiSys Technology Foundation.

Over the past several years, Kallas, Matthew Nelson (UW Oshkosh), and Eric Singsaas (Natural Resources Research Institute, University of Minnesota, Duluth, USA) have worked on metabolic engineering of cyanobacteria and photobioreactor design to convert solar energy and waste CO_2 into high-value isoprene-terpene bioproducts and biofuels. This project has reached several milestones, and the team recently founded Algoma Algal Biotechnology (AABT), a limited liability company (LLC), to develop both biomass conversion and microalgal carbon capture as avenues for sustainable hydrocarbon production. Kallas has published two detailed authoritative reviews on the cytochrome *bf* complex for the *Advances in Photosynthesis and Respiration* series (volumes 1 and 34) and has coedited (with Anne Ruffing, of Sandia National Labs, Albuquerque, New Mexico, USA) a recent special issue on Cyanobacteria, the Green *E. coli* for *Frontiers in Bioengineering and Biotechnology*. He co-organized (with Jack Meeks, UC Davis) the 2007 Cyanobacterial Workshop and has co-organized two Midwest-Southeast Photosynthesis Conferences (in 2001 and

2010). His current work on CO_2 capture, isoprene production, and AABT business development is funded by Small Business Technology Transfer (STTR) Phase I and IB grants from the US National Science Foundation and a supplement from the UW Center for Technology Commercialization. Further information on Toivo Kallas is available at his website http://www.uwosh.edu/facstaff/kallas.

Remembrances

Remembering Professor Derek S. Bendall

The distinguished biochemist Derek S. Bendall, whose last written scientific work is presented in this volume, passed away in December 2014 at the age of 84 years. We would like to contribute to a celebration of Derek's life with personal reflections on Derek and his scientific legacy and our experiences working closely with him on a collaborative project.

Derek's research passion was redox reactions of biological systems, a topic that he pursued for nearly 60 years. In particular, he was fascinated by the problem of how solar energy is transduced into chemical energy in green plants, and on the mechanism of photosynthetic oxygen evolution. His research journey began with his scientific training as an undergraduate in Cambridge in the UK, where he was awarded a first class degree in 1953. His evolving interests in photosynthesis led him to studies of plant microsomes and mitochondria for a dissertation project with Robin Hill in the Biochemistry Department. He was awarded a Ph.D. in 1957

from Cambridge University and then spent a year working in Louvain, Belgium, where he studied methods for preparing mammalian mitochondria fractions for characterization. Derek then worked for the Nyasaland Tea Association in both Africa and Cambridge before becoming a demonstrator (1960) and lecturer (1965) in the Biochemistry Department in Cambridge. His early work demonstrated that mitochondria have a cytochrome oxidase, and he analyzed cytochromes in chloroplasts. He studied the cycling of electron transfer around photosystem I, and how this helps to protect against the effects of rapid changes in light level. Under this condition, electrons are transferred from the plastoquinone pool or terminal oxidase rather than photosystem I to reduce backup of photosynthetic electron transfer.

The mechanism of biological electron transfer and, in particular, transient protein-protein interactions mediating such reactions was of special interest to him, and he edited and contributed to an excellent book on the

subject, entitled *Protein Electron Transfer*, which was published in 1996 before Derek's official "retirement." For Jonathan Worrall, this book holds special memories and was a source of continuous background reading during his Ph.D. and early postdoc years. It was with great pleasure therefore that upon attending a meeting in Denmark focused on transient protein-protein interactions, Jonathan was awarded the poster prize and received from Derek a signed copy of his book.

Not content to follow retirement in the traditional sense, Derek joined Chris Howe's laboratory in the Biochemistry Department in Cambridge, where he continued earlier collaborative work that they had started on various projects. He continued studies on protein targeting in cyanobacteria, and in particular on the distribution of photosystem proteins in the membrane, following up earlier studies in which they had found evidence for the localization of some proteins in the cytoplasmic membrane. In addition he continued his interest in weak protein-protein interactions, particularly those of the electron transfer complex formed between cytochrome f and plastocyanin.

His interest in cytochromes attracted him to a mysterious cytochrome c_6 homologue, encoded in the genome of several land plants and algae, but possessing a more negative redox potential than its bacterial counterparts. To distinguish this homologue from the bacterial proteins, Derek suggested that it should be named cytochrome c_{6A}. We worked with Derek to initially characterize the crystal structure of cytochrome c_{6A} in reduced and oxidized forms. Derek's driving enthusiasm to understand why cytochrome c_{6A} possessed a redox potential some 250 mV lower than bacterial cytochrome c_6 proteins led to further collaboration with us and to experimental support for a mechanism of redox potential control by the interactions of side chains around the heme. The chapters presented in this book written with his long-term collaborator, Chris Howe, describe his last work on cytochrome c_{6A}.

Derek was also involved in translational projects during his retirement. He worked on renewable energy projects using cyanobacteria, studying direct electron transport from cyanobacteria and algal cells onto electrodes to make direct photovoltaic devices. He was also involved in engineering cyanobacteria to explore terminal oxidases in the process of direct electron transfer.

Derek had a long-standing interest in tea, both as a tea-drinker connoisseur and as a subject for scientific investigation. He studied the biochemical processes of tea fermentation, and his work, including preparation and characterization of catechol oxidase, resulted in several reports on the fermentation process. His passion for botany was also expressed in his activities as a keen gardener.

Through our work with Derek on the cytochrome c_{6A}, we had an opportunity for extensive interactions that involved hypothesis formulation and testing, and these were stimulating interactions. We were struck by Derek's knowledge and scientific imagination, by his gentle character and modest nature. Like many other colleagues, we feel very fortunate to have had the opportunity to work with him and to have benefited from these interactions.

For further information and references to Bendall's key papers. see C.J. Howe, P.R. Rich and M. Ubbink (2015) Derrek Bendall (1930–2015) Photosynth Res 124: 249–252.

March 15, 2016

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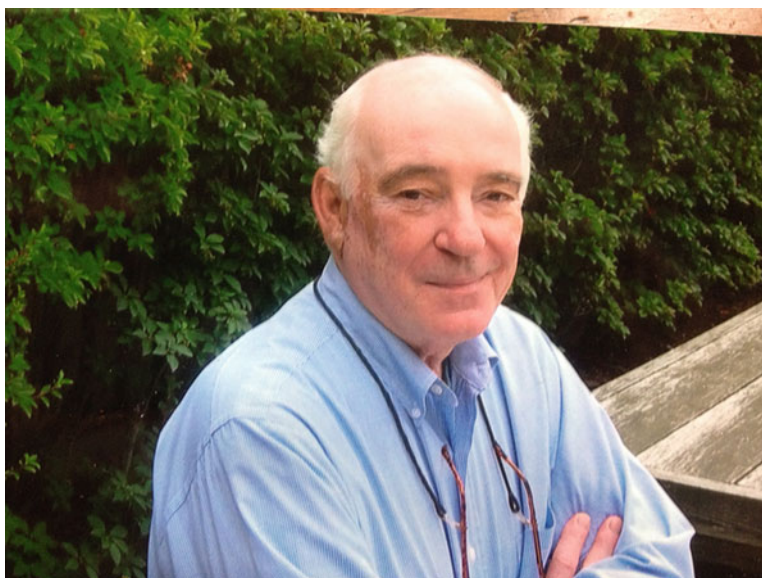
Remembering Professor Bernard L. Trumpower

The news that Bernard had passed away on the Christmas day of 2014 spread rapidly among his friends, colleagues, former Ph.D. students, and postdocs. Together with the personal sorrow that inevitably comes with such a message, I immediately felt struck by the gap created within our scientific community. "Bernie," as we used to call him, when I had the privilege to work as a postdoc in his laboratory, was clearly a leading figure in the field of bioenergetics for many years. Here, I can only try to give an account of his fundamental contributions to our understanding of the structure and function of the mitochondrial cytochrome bc_1 complex.

Bernard L. Trumpower studied Chemistry at the University of Pittsburgh and completed his Ph.D. in Biochemistry at St. Louis University, Missouri, USA, in 1969, where he worked with Robert E. Olson on the biosynthesis of ubiquinone-9. During his postdoctoral studies with Efraim Racker at Cornell University, Ithaca, N.Y. (1969-1972), he became acquainted with the membrane protein complexes of the oxidative phosphorylation system that use ubiquinone as a substrate. This set the stage for his successful career of more than 40 years studying the structure and function of the cytochrome bc_1 complex that made him a leader in the field of Molecular Bioenergetics.

In 1972, when Bernard was appointed Assistant Professor of Biochemistry at Dartmouth Medical School, the molecular mechanism of this central complex of the respiratory chain was still enigmatic. Early on, he had realized the key role of the Rieske iron-sulfur protein, and the usefulness of specific inhibitors for studying the intricate mechanism of the enzyme. This allowed him to provide decisive experimental proof for the proton-motive Q-cycle, soon after Peter Mitchell published this hypothesis for the redox-driven proton translocation by the cytochrome bc_1 complex in 1975. Shortly after Bernard was promoted to Associate Professor in 1978, he published a series of landmark papers showing the activation of cytochrome bc_1 complex function by reconstitutively active Rieske iron-sulfur protein (Trumpower and Edwards 1979), establishing the use of Electron Paramagnetic Resonance (EPR) spectroscopy with Tomoko Ohnishi to study catalytic intermediates (Ohnishi and Trumpower 1980,

Trumpower et al. 1980), and introducing novel ubiquinone analogues as specific inhibitors of the cytochrome bc_1 complex (Bowyer and Trumpower 1980). When he became a full Professor of Biochemistry at Dartmouth in 1983, he was just performing groundbreaking functional studies together with Gebhard von Jagow using myxothiazol, the first representative of another new class of specific inhibitors. In a classical experiment, later known as the "double-kill," they were able to demonstrate unambiguously and characterize functionally the two distinct ubiquinone-binding sites of the cytochrome bc_1 complex, thereby confirming a central postulate of the proton-motive Q-cycle hypothesis (von Jagow et al. 1984).

Following Efraim Racker's pledge "*Don't waste clean thinking on dirty enzymes*." Bernard developed simple and efficient protocols to purify cytochrome bc_1 complexes from mitochondria and bacterial membranes still applied today that were among the first to demonstrate the usefulness of the now widely used detergent dodecyl-maltoside to purify membrane protein complexes (Ljungdahl et al. 1987). This paved the way for a series of important studies on the kinetic properties of the bacterial enzyme seminal to understanding central aspects of the proton-motive Q-cycle.

Again leading the field, Bernard recognized soon the enormous potential of molecular biology that became widely applicable in the 1980s. In his quest to understand all aspects of structure and function of the cytochrome bc_1 complex, he spent time with Gerry Fink at Harvard University (Cambridge, Massachusetts) to learn yeast genetics. Using this powerful approach, Bernard's laboratory subsequently cloned several genes of all so-called supernumerary subunits of the complex from yeast mitochondria and characterized their function by genomic deletion and structure/function studies (see Zara et al. (2009) for a review). Exhaustive site-directed mutagenesis of the Rieske iron-sulfur protein were a straightforward extension of his early work and led to the identification of the functionally critical domains (Graham et al. 1993).

Returning to the roots of his early research, Bernard then used the X-ray structures of the cytochrome bc_1 complex that finally became available in the late 1990s as a guide for fundamental studies to understand the molecular basis of the proton-motive Q-cycle. Intrigued by the fact that the structures showed the cytochrome bc_1 complex to be a constitutive dimer, he indeed defined a functional role for this remarkable structural feature (Covian and Trumpower 2008).

In the years before retirement and becoming Professor Emeritus from the Dartmouth Medical School in 2013, he also dived into an important medical aspect demonstrating the societal impact of his research: The molecular target of the widely used antimalarial Atoquavone is the ubiquinol oxidation site of the cytochrome bc_1 complex. Bernard applied his profound knowledge on structure and function of enzymes, to design quinone derivatives binding to this site that are less susceptible to drug resistance (Hughes et al. 2011).

The groundbreaking research contributions by Bernard L. Trumpower were honored through a number of national and international awards including the Humboldt Prize in 1984 and the Merit Award of the National Institute of Health (NIH) in 1994. He served the scientific community in many ways, as editor of several leading biomedical journals, member of NIH Study Sections, and organizer of a number of major national and international conferences.

Reaching far beyond the dry facts that can be documented in a list of his publications, Bernard had a truly lasting impact as a guide and a role model for his students and young colleagues. Never content with a superficial answer, he taught us to stay focused, to be thorough, persistent, and self-critical. I will never forget that when we finally reached a conclusion in an intense and controversial scientific discussion, his next sentence would usually start with "But..." to express that there is always another side of the problem to explore.

For me, his unconditional quest for perfection best characterizes Bernard not

only as a scientist. It can be experienced nicely by looking at the series of wonderful photographs he made after his retirement (https://www.dartmouth.edu/~grnhouse/brout.php) showcasing the beauty and elegance of orchids.

March 15, 2016

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Part I

Diversity and Evolution

Chapter 1

Keilin, Cytochrome and Its Nomenclature

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Summary

D. S. Bendall describes the micro-spectroscopic studies on bacteria, tissue, and plant samples that took place in Cambridge in the years just preceding World War II, which provided the rationale for the modern-day notation for cytochromes and hemes. This history includes the discovery of 'cytochromes' by D. Keilin and his original description of a respiratory chain that occurred in the background of the studies on respiration and fermentation by O. Warburg. Bendall notes the subsequent development of the high resolution difference spectrophotometry of turbid samples by B. Chance and W. L. Butler that has been crucial in studies of biological electron transport chains.

I. The Cytochromes and History

Cell respiration was still a black box in the decade following the end of the First World War. Attempts to throw light on the mechanisms involved mainly made use of the interplay between the concepts of specific enzymes and unspecific surface forces associated with colloids or membranes. The outstanding problem became how to reconcile two opposing notions (Keilin 1966; Kohler 1973). On the one hand, there was activation of hydrogen in metabolic substrates followed by an unspecific reduction of oxygen (Wieland and Thunberg), as could be beautifully and simply demonstrated by the reduction of methylene blue in a 'Thunberg tube'. On the other hand there was Warburg's theory of the '*atmungsferment*' which postulated an iron compound that could be reduced by unspecific surface forces but required a specific activation to be reoxidized by oxygen. An elegant series of experiments by Warburg, in which he studied the reversal by light of the effect of carbon monoxide on respiration and fermentation in yeast, led eventually to an absorption spectrum with maxima at 578, 546 and 436 nm which suggested to him that the *atmungsferment* was related to haemoglobin, which also forms a light-sensitive compound with carbon monoxide (Warburg 1927).

David Keilin's discovery of cytochrome, which was to resolve this problem, was the serendipitous result of a study of haemoglobin in the horse bot fly, *Gas-*

terophilus intestinalis, and depended absolutely on his use of a simple but powerful instrument, the microspectroscope ocular, invented by Sorby in the late 1860s. The singular advantage of this instrument was that by virtue of its low dispersion, which sharpened absorption bands, and the fact that it allowed an intense beam of light to be focused on the sample placed on the microscope stage, absorption spectra of intact tissues or of turbid suspensions could be observed. Conventional spectroscopes and spectrophotometers, already available in the 1920s, were confined to the study of clear solutions, and even today this ability of commercial instruments is the exception rather than the rule. A variety of techniques were tried for overcoming this; opal glass was the key to one, but the most successful was a large end-window photomultiplier to collect the maximum amount of scattered light. This was used by Warren Butler, for example, but the most accessible were the dual-wavelength (double-beam) and split beam spectrophotometers developed by Britton Chance and colleagues at the Johnson Research Foundation in Philadelphia, which led to commercial instruments. The microspectroscope, however, remains a quick and simple way of getting a good idea of what you have in a coloured sample. It is also a curious fact that the way the eye interprets the absorption bands means that a shoulder on an otherwise symmetrical band is immediately obvious in the microspectroscope, but could easily be missed in a cursory examination of the spectrum given by a conventional spectrophotometer—try looking at the α-band of cytochrome f with a microspectroscope.

Keilin was a parasitologist, inspired by his association with Maurice Caullery in Paris. He became interested in the life cycles of parasitic insects and especially the evolutionary adaptions of respiration in dipterous larvae. In 1915 he moved to Cambridge to join George Nuttall who had been elected as the first Quick Professor of Biology. Keilin's work became concentrated on the behaviour of *Gasterophilus*. Larvae of this fly find their home in the stomach of the horse in an environment low in oxygen and in compensation their tracheal cells become rich in haemoglobin. Eventually they are voided in the faeces, burrow into the soil and pupate, and several weeks later adult flies emerge. The problem to be solved was the fate of the high concentration of haemoglobin in the larvae as the mature fly develops, and without his use of the microspectroscope the solution, and the discovery of cytochrome, would have been long delayed. The spectrum of haemoglobin gradually disappeared during pupation and was replaced in the thoracic wing muscles of the adult by a distinct four-banded spectrum. The obvious conclusion would have been that the pigment responsible for this spectrum was derived from haemoglobin. Keilin, however, was a biologist and before jumping to a conclusion looked at the spectra of intact thoracic muscles from several insects, especially *Galleria mellonella*, the wax moth which parasitises bees' nests. These moths were cultured in the laboratory and showed no haemoglobin at any stage. All his samples showed the same four-banded spectrum, and he also found it in cultures of *Bacillus subtilis* which had been used for culturing the insects, and eventually in yeast and plant tissues (chlorophyll free). Thus *Gasterophilus*, *Galleria* and the microspectroscope pointed the way, in Keilin's hands, to the discovery of cytochrome.

At this point, Keilin's literature searches led to his unearthing of the papers of MacMunn published between 1880 and 1890 in which he reports the occurrence of the characteristic four-banded spectrum in a variety of animal tissues (MacMunn 1886). MacMunn gave the names myohaematin and histohaematin to the pigment responsible and concluded that it was a respiratory pigment distinct from haemoglobin and its derivatives. Hoppe-Seyler would not accept MacMunn's conclusion, claiming that the spectrum observed by MacMunn, which he had not looked for himself, was in fact derived from haemoglobin in different ways (ignoring MacMunn's finding of it in invertebrates which lack haemoglobin) (MacMunn 1890). MacMunn's work was gradually forgotten.

Keilin's first paper *On Cytochrome* (Keilin 1925), was outstanding in various ways. To a novice scientist entering the field in the mid 1950s it seemed like the ideal model for a scientific paper. The whole of the Introduction is devoted to a discussion of MacMunn's observations and the way in which they had been misunderstood and eventually ignored. He then gives an account of his own observations, starting with the occurrence of the characteristic spectrum in a wide range of animal tissues and also, most significantly, in some bacteria, yeast and plant tissues. This is followed by a series of simple and elegant experiments from which he was able to argue conclusively about the physiological function of the pigment and its chemical constitution. The pigment was introduced under the name "*Cytochrome*, signifying merely 'cellular pigment', pending the time when its composition shall have been properly determined." The change of name was justified by the much wider distribution of the pigment than had been envisaged by MacMunn. Moreover, Keilin showed that cytochrome was not a single pigment, as MacMunn had thought, but the superposition of three. Thus the names cytochrome *a*, *b* and *c* became established from the beginning, corresponding to the α-bands at about 605, 560 and 550 respectively. The fourth band in the cytochrome spectrum at around 520 nm was the fused β-bands of the three components (in case anyone should think that this arbitrary name system was in the wrong order, note that in a direct-vision spectroscope long wavelengths appear towards the left and short wavelengths to the right). The three components were haemochromogen-like (haemochrome-like) substances, giving slightly different haemochromogens when denatured with KOH. Cytochrome *c* was obtained separate from the others in an aqueous extract of yeast. Anson and Mirsky had established that the characteristic haemochromogen spectrum (sharp α-band, more diffuse β-band) is formed by the following reaction

Reduced haem

$\quad$ + nitrogen compound $\rightleftarrows$ haemochromogen

(Anson and Mirsky 1925a). The three components could be related to haemoglobin only by the common possession of haem groups (Anson and Mirsky 1925b).

The most important observation (Keilin 1925) was that cytochrome could be reversibly oxidized and reduced in vivo depending on the availability of oxygen. MacMunn had demonstrated the existence of oxidized and reduced states because the four-banded spectrum only appeared in the reduced state and disappeared when oxidized, but he had only shown this using chemical reagents. Thus Keilin had demonstrated that cytochrome is involved in cell respiration in virtually all aerobic organisms. By 1929 he was able to state clearly that the role of cytochrome in cell respiration is to connect the reducing systems of Wieland and Thunberg with an oxidase, as in Fig. 1.1, this being the first formulation of the respiratory chain (Keilin 1929). The oxidase was equated with what had previously been described as indophenol oxidase and also with Warburg's *atmungsferment*. The more appropriate name, first suggested by Malcolm Dixon, was of course cytochrome oxidase (Dixon 1929). The arrangement of the individual cytochrome components, especially cytochrome *b*, was not clear at that time.

The term cytochrome could then logically be applied to any intracellular haemochromogen-like substance the function of which is to undergo reversible oxidation and reduction in an electron transfer process. Thus, as new cytochrome components were discovered that did not readily fit into Keilin's *a*, *b*, *c* system for respiration, it became natural to extend this arbitrary lettering system. Cytochromes *e*, *f*, *h* and *o* were soon described.

Cytochrome *e* is now forgotten, but was the name given by Keilin and Hartree (1949) to a component revealed in their particulate cytochrome preparation from heart muscle by application of their new technique

leucoindophenol

Indophenol

narcotics

Dehydrase ⇅ — Substrate

H
H
{ Cytochrome
a (b) c }
← 0 - oxidase.

+2H   +O₂

+Nadi

KCN
H₂S
CO

O₂

substrate ←
as
direct catalysts
{ Haematin
Cytochrome b. }

{ Haematin
Cytochrome, a.b.c. }
as
peroxidase +H₂O₂
→ substrate
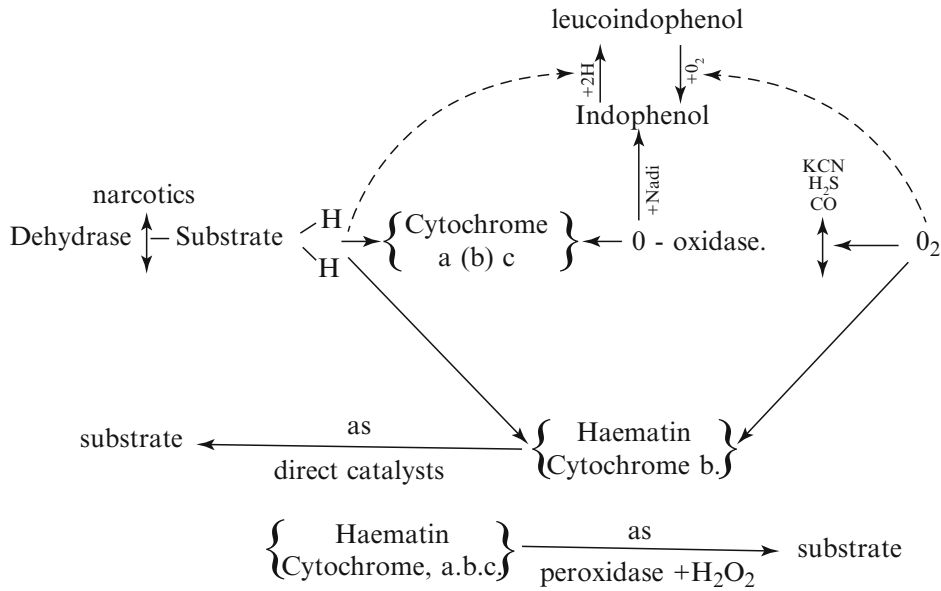
Fig. 1.1. The first formulation of the function of cytochrome in a respiratory chain (From Keilin 1929).

for sharpening and intensifying absorption bands by cooling a suspension in 50 % glycerol down to 77 K with liquid air or liquid nitrogen. They later realised, however, that cytochrome *e* was identical with a component previously discovered by Yakushiji and Okunuki in heart muscle preparations after extraction of all the cytochrome *c* and to which they had given the name cytochrome c_1. The name cytochrome *e* was therefore abandoned (Keilin and Hartree 1955).

Cytochrome *f* we are all familiar with. Robin Hill worked in the Biochemistry Department in Cambridge, but was closely associated with Keilin, a few yards away in the Molteno Institute of Parasitology. Hill had done original work on the chemistry of haem compounds and had published with Keilin on the nature of the haem of cytochrome *c*. His real passion was the chemistry of plants, and following Keilin's work he was keen to discover whether cytochrome also had a role in photosynthesis. Looking for cytochrome in leaves was not straightforward because of the high concentration of chlorophyll which masked the spectra of cytochrome components. Hill and Kamala Bhagvat had succeeded in isolating a partic-

ulate preparation from plants that possessed succinate and cytochrome oxidase activities (Bhagvat and Hill 1951; Hill and Bhagvat 1939) and, around this time and immediately following the end of the war, the generality of the cytochrome system in plant respiration was being demonstrated in several laboratories, concentrating mostly on chlorophyll-free tissues. Rosenberg and Ducet (1949), however, found cytochrome oxidase activity in impure preparations of chloroplasts while Hill and Scarisbrick were able to extract cytochrome *c* from leaves (Hill and Scarisbrick 1951; Scarisbrick 1947). Hill and Scarisbrick first sought to observe cytochrome in leaves by examining acetone powders, free of chlorophyll, and found that the spectrum was dominated by a strong band centred at 554 nm. They were soon able to extract this component into solution with ethanol-ammonia as solvent. It was clearly different from cytochrome *c* both in its absorption spectrum and redox potential, and was also incapable of being oxidized by cytochrome oxidase. Furthermore, its concentration in leaves was an order of magnitude higher than that of cytochrome *c* and, unlike cytochrome *c*, it was confined to the green tissues of

plants. To emphasise its distinctive association with leaves they named it cytochrome *f* (Latin *frons* as stated by Hill and Scarisbrick, or *folium*), a name which has turned out to be too convenient to lose, even though an attempt was later made to rename it cytochrome c_6 once its formal classification as a *c*-type cytochrome became apparent.

Cytochrome *h* is no longer well known, but is closely related to helicorubin which is like an extracellular *b*-type cytochrome occurring in the intestinal tract of snails and some other molluscs and crustaceans. Cytochrome *h* occurs in the digestive gland of snails and was purified by Joan Keilin (1956, 1957) from *Helix pomatia*, hence the name. Although it appears to be a protein similar to helicorubin, the exact relationship is still not clear, and unlike helicorubin the haem group is not released by treatment with acid acetone. Thus cytochrome *h* is more likely to turn out to be a *c*-type cytochrome.

Cytochrome *o* is the name given by Castor and Chance (1959) to a bacterial oxidase which was clearly distinct from *a*-type oxidases and which they had discovered with their new technique for measuring the photochemical action spectrum of CO-inhibited respiration. This turns out to be the oxygen-reacting component of a quinol oxidase occurring in a number of bacterial species (Poole 1983). It has often been assumed to be a *b*-type component despite the strong reservations of Castor and Chance, and the Nomenclature Committee of the IUB even goes so far as to recommend that the name cytochrome *o* should be abandoned in favour of cytochrome *b*', the prime indicating it is not strictly a haemochromogen as the 6th ligand position of the iron is free for combination with CO or O_2. The situation has been clarified by Wikström and colleagues (Puustinen and Wikström 1991; Wu et al. 1992) who have demonstrated unequivocally that haem *o* differs from protohaem in that one of the vinyl sidechains has become farnesylhydroxyethyl as in haem *a*, but differs from haem *a* in not possessing a formyl sidechain. Nevertheless, the high-spin haem *o* is associated with a second, low-spin

haem and also a copper atom, suggesting that the enzyme belongs to the cytochrome aa_3 family of oxidases. The low-spin haem can be either protohaem or haem *o* depending on the source of enzyme, and both function equally well. The enzyme is usually referred to as cytochrome *bo* or bo_3, the subscript indicating homology with the cytochrome aa_3 oxidase as suggested by Puustinen and Wikström (1991), although no cytochromes o_1 or o_2 are currently known. It is one of two ubiquinol oxidases occurring in *Escherichia coli*, and has a relatively low affinity for oxygen.

The arbitrary assignment of letters to different cytochrome components was based on the position of the α-band of the reduced form, but it soon became apparent that since they were all haem proteins a more satisfactory classification could be based on their respective pyridine haemochromogens. The pyridine haemochromogens would give more consistent spectra, based on the chemical nature of the haem group. Anson and Mirsky had shown that cytochrome preparations contained the same haem as haemoglobin, that is protohaem, and this could be assigned to cytochrome *b*. The pyridine haemochromogen of cytochrome *c*, which could be extracted into solution free of the other components, was clearly different. Hill and Keilin (1930) obtained porphyrin *c* and showed that it was related to haematoporphyrin in which the vinyl sidechains of protoporphyrin are replaced by hydroxyethyl groups, but the nature of its attachment to the protein was not established until Theorell (1938) demonstrated that it was bound through thioether linkages to cysteine residues. The distinctive feature of haem *a*, which shifts its α-band to the red end of the spectrum, is the possession of a formyl group as sidechain in place of the methyl group at position 8 of the porphyrin ring, and it also contains a long alkyl sidechain at position 2 in place of vinyl (Caughey et al. 1975).

The classification of cytochromes was thus based upon structure as well as function, which is the case for the formal classification of enzymes. Newly discovered cytochromes,

if they did not fit readily into Keilin's respiratory chain, could be named as being type a, b or c according to the position of the α-band of the pyridine haemochromogen (587, 557 and 550 nm respectively) and given a subscript number. Hence we have, for example, cytochromes a_1, a_2 and a_3. Cytochromes a_1 and a_2 are bacterial components initially so named because their α-bands lie at the red end of the spectrum, but different from cytochrome a. Castor and Chance (1959) showed that they are oxidases from the action spectra for photodissociation of CO complexes. Subsequent analysis of the pyridine haemochromogens, however, has shown that in many cases the assignment to type a was a mistake, although cytochrome a_3 is correctly assigned.

Cytochromes a_1 and a_2, with α-bands around 590 and 630 nm respectively (Keilin 1934, 1966; Poole 1983), have had somewhat chequered careers. Cytochrome a_1 has been purified from *Acetobacter aceti* and shown to be a ubiquinol oxidase that possesses one haem a and one haem b (Matsushita et al. 1990), and thus should be named cytochrome ba or perhaps more appropriately ba_3. On the other hand in *Escherichia coli* a_1 and a_2 occur together in the second ubiquinol oxidase (the other being cytochrome bo_3) which has a high affinity for oxygen and contains no haem a (Castor and Chance 1959). In this case what was previously described spectroscopically as cytochrome a_1 actually contains a high-spin b-type haem with an α-band in the reduced form at 595 nm and now referred to as cytochrome b_{595} (Lorence et al. 1986). The haem of so-called cytochrome a_2 was shown by Barrett (1956) to be of a new type which contains a dihydroporphyrin or chlorin in which the porphyrin ring is partially reduced. This is now known as haem d, the structure of which was established in detail by Timkovich et al. (1985). This second oxidase contains another b-type haem which is low-spin and has its α-band at 558 nm, but there is no copper to provide a binuclear centre to react with oxygen. Evidence has accumulated that cytochrome d and cytochrome b_{595} together

form a binuclear centre but no crystal structure is yet available. These enzymes are usually referred to as cytochrome bd oxidases. The distinguishing feature of haem d is that the porphyrin ring is partially saturated with one reduced pyrrole ring, and so is a chlorin as shown by Barrett. In addition to the oxidases, however, a bacterial nitrite reductase also contains a partially reduced haem group, but with a different set of sidechains and a pair of carbonyl groups attached to adjacent pyrrole rings. This has been named haem d_1. The enzymes containing haem d_1 carry out the one electron reduction of nitrite to nitric oxide, but they are also capable of reducing oxygen to water, which is a four-electron reaction (Timkovich et al. 1984).

One more type of enzyme that has not conventionally been described as a cytochrome, but in fact conforms to the general definition of a haem protein involved in intracellular electron transfer, is the assimilatory sulphite reductase of sulphate reducing bacteria which carries out the six electron reduction of sulphite to sulphide. The haem prosthetic group has been given the name sirohaem to indicate its involvement in sulphite reduction, and like haem d_1 has a pair of saturated pyrrole rings (although with different sidechains) making it an isobacteriochlorin (Murphy and Siegel 1973). It could thus be regarded as a type d cytochrome with the name cytochrome d_2.

The use of the wavelength maximum of the α-band to describe a cytochrome component was first suggested by Scarisbrick (1947) and was normally written on the line hyphenated to the haem type, for example cytochrome c-553. This was convenient for a new component which had not been clearly established in terms of either structure or function, but suffered from the disadvantage that it might not represent a unique component. Cytochrome c-553 (sometimes c-552) was the term being used to describe the so-called soluble cytochrome f to distinguish it from the bound cytochrome f but when it became clear that the soluble component and the true

membrane-bound cytochrome f have distinct functions in the photosynthetic electron transport chain (Wood 1977) the name c-553 was relinquished in favour of the more definitive c_6. The wavelength maximum has frequently been written as a subscript, as b_{558} and b_{595}, but this notation is best reserved for those cases, as here, in which it describes a well-established component.

The naming and numbering of cytochrome components often seems abitrary, but has been determined by the history of their discovery, and well known names tend to be perpetuated, even if strictly erroneous. The many different kinds of b-type cytochromes illustrate this point. Cytochrome b-559 associated with the reaction centre of photosystem II is an example. It is frequently referred to as cytochrome b_{559}, which is preferable, but even so the true wavelength maximum is at 559.6 nm, so a more accurate name would be cytochrome b_{560} (Cramer 2004). It would have been possible to avoid the nomenclature problems by defining this component as cytochrome b_9, but the name b_{559} seems too well established for change to be accepted.

Cytochrome b_5 is well known as a distinctive component with widespread occurrence in microsomal membranes and the outer mitochondrial membrane. Cytochrome b_6 has proved a useful name for the component of the cytochrome b_6f complex to distinguish its properties from those of the mitochondrial cytochrome b occurring in complex III, the cytochrome bc_1 complex. Previously the name cytochrome b_2 had been used to describe the flavocytochrome which is the yeast lactate dehydrogenase. These numbers, however, leave puzzling gaps for b_1 and b_3. Cytochrome b_1 was used by Keilin (1934) to describe the principal component of *Escherichia coli*, which did not belong to his classical a, b, c group. More recently cytochrome b_1 has been shown to be a multisubunit protein identical with bacterioferritin, an iron-storage protein (Smith et al. 1988). On the other hand, little is known about cytochrome b_3. In their initial study of the cytochrome components of leaves Hill

and Scarisbrick (Scarisbrick 1947) found two cytochromes in aqueous extracts which had been allowed to stand overnight to allow all chloroplast fragments to aggregate. These were cytochromes c and a new b-component which they named b_3, both of which also occurred in non-green tissues. Cytochrome b_3 had an α-band at 559 nm, was autoxidisable and could be reduced by ascorbate. Martin and Morton (1955) suggested that it is derived by autolysis from a component of the microsome fraction of certain plant tissues on the basis of the similarity in properties of the two, which is plausible but has not been directly demonstrated. Rich and Bendall (1975) showed that the main components of plant microsomes are cytochromes b-559.5 and b-562 with similar properties, b_5 and P450, but that the relative amounts varied widely between different tissues. Nothing is known about the function of cytochrome b-559 (nor b_3).

This volume celebrates the fact that cytochromes often occur in relatively large protein complexes containing more than one polypeptide and frequently more than one haem group with possibly additional redox centres. The first example of this was the mitochondrial cytochrome aa_3, first described by Keilin and Hartree (1938). The first clear expression of the concept of protein complexes with catalytic activity in electron transfer was made by Hatefi and other colleagues of David Green in the Institute for Enzyme Research at the University of Wisconsin in 1962 (Hatefi et al. 1962). This was the origin of the idea of the mitochondrial respiratory chain being divided into four discrete complexes which could be individually purified, hence complex I (DPNH—coenzyme Q reductase), complex II (succinic—coenzyme Q reductase), complex III (QH$_2$—cytochrome c reductase) complex IV (cytochrome c oxidase). The emphasis was on the proteins and the enzymic activities, rather than the cytochromes, but complex IV was equivalent to Keilin and Hartree's cytochrome oxidase and complex III was what we now know alternatively as a cytochrome bc complex.

The activities of these complexes were linked by two diffusible components, the lipid soluble coenzyme Q (ubiquinone) and the water soluble cytochrome c. Since that time the concept of cytochrome complexes has burgeoned, especially with regard to photosynthetic systems, both eukaryotic and prokaryotic, the complex processes occurring in cytochrome bc complexes, and the rich field of bacterial oxidases.

This brings us to the last example concerning cytochrome nomenclature. At present bacterial oxidases fall into two broad classes, those containing a haem-copper binuclear centre with a high spin haem, and those that contain haem d together with a second high-spin haem (b_{595}) instead of the copper. A low spin haem compound, usually of type a, b or sometimes o, is always associated with the binuclear centre as electron donor, and occasionally there is an additional bound cytochrome c as well. The high spin haem of haem-copper enzymes may be type a, b or o, and is usually given the subscript 3, following a_3 of the mitochondrial enzyme. There is no known example of a high spin haem c in this role, and the name cytochrome c_3 has been appropriated by a low-potential, low spin, multihaem cytochrome in sulphate reducing bacteria (Postgate 1956). Nevertheless, a high-spin cytochrome c, originally described as pseudo-hemoglobin and later RHP (Rhodospirillum heme protein) but now designated cytochrome c', was discovered in aqueous extracts of *Rhodospirillum rubrum* by Vernon and Kamen (1954). Its function has long been a puzzle, but it may play a role in redox poising to prevent over reduction or over oxidation of the cyclic electron transfer system (Meyer and Cusanovich 2003).

In 1925 Keilin justified use of the new term cytochrome by the fact that it is much more widely distributed than had been envisaged by MacMunn and established the defining feature that it is involved in intracellular electron transfer. The second and crucial point demonstrated by Keilin is that there is more than one cytochrome compo-

nent (hence the respiratory chain) and that they are all haem compounds. But at the same time he thought of cytochrome as a temporary term "pending the time when its composition shall have been properly determined". He could not have imagined (as early as 1925) how the numbers of known cytochrome components would expand in subsequent years, nor how much detailed information about their composition would accumulate. Incomplete as our knowledge and understanding is, we can nevertheless say that they are all proteins with no common structure or evolutionary origin. In other words Keilin's two defining characteristics are the only ones we can apply to all cytochromes. So we end with names, established by custom and usage, of a series of cytochrome components each with its own type of haem, a, b, c, d or o which may have a subscript number defining its function. Recognizing the importance of habit in the use of names we make an honourable exception to this rather crude formulation for cytochrome f, and as we have seen, a strictly consistent nomenclature is difficult to achieve and probably undesirable.

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When Did Hemes Enter the Scene of Life? On the Natural History of Heme Cofactors and Heme-Containing Enzymes

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Summary

Heme proteins are almost ubiquitous both in Archaea and in Bacteria. The last universal common ancestor (LUCA) of the two prokaryotic domains was, therefore, assumed until recently to already have made use of heme cofactors, a notion bolstered by molecular phylogenies of several heme-bearing enzymes. The discovery of a second pathway for heme biosynthesis, predominantly present in Archaea, was subsequently interpreted to indicate independent origins of heme biosynthesis in each of the two prokaryotic domains (Lane and Martin (Cell 151:1406–1416, 2012)), implying that the LUCA might have been entirely devoid of hemes and heme proteins. In this contribution, we outline the presently

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available evidence in favour of either scenario and propose a new model reconciling the seemingly contradictory messages sent by molecular phylogeny of heme-bearing enzymes and the biosynthesis pathway dichotomy. A possible relation to other biosynthesis pathway dichotomies is suggested, and the far-reaching repercussions of the ultimate resolution of the controversy on our understanding of free energy conversion in the LUCA and at life's origin are emphasized.

I. Introduction

From the point of view of thermodynamics, living organisms fundamentally are free energy converting systems transforming environmental redox disequilibria into the extraordinary entropy decrease that characterizes cellular life (Branscomb and Russell 2013). One of the quintessential attributes of life therefore is its ability to perform redox reactions and to channel reducing equivalents from the environmental electron donating to the accepting substrates. Redox compounds therefore can safely be assumed to be crucial elements of life in general and thus must have already played a paramount role at its origin. Obvious candidates for such redox compounds at life's inception are centres made up from environmental transition metals such as iron, nickel, molybdenum, tungsten, manganese, cobalt etc.

Indeed, several redox cofactors found in extant life have been proposed based on molecular phylogeny to be as old as life itself, that is, iron sulphur clusters (Eck and Dayhoff 1966), nickel-iron centres (Vignais et al. 2001) and molybdenum/tungsten compounds (Schoepp-Cothenet et al. 2012). Iron-sulphur clusters and Mo/W-centres are indeed basically ubiquitous in extant life. However, another iron-based redox centre, i.e. heme, likely is almost as widely distributed in living organisms as are iron-sulphur and Mo/W-centres.

Abbreviations: Ahb-pathway – Alternative heme biosynthesis pathway; (B)Chl – (Bacterio)Chlorophyll; FePPIX – Iron-protoporphyrin IX; GOE – Great oxidation event; HGT – Horizontal gene transfer; LUCA – Last universal common ancestor of Archaea and Bacteria; Mo/W – Molybdenum/tungsten; NOR – NO reductase; O_2R – O_2 reductase

The heme cofactors in heme proteins overwhelmingly serve as electron transferring redox centres operating in an impressively wide range of electrochemical potentials. The major part of contributions to this volume indeed deals with electron transferring heme proteins. However, hemes can also play the roles of small-molecule transporters (e.g. Suzuki and Imai 1998) and sensors (Cutruzzolà et al. 2014; Martinkova et al. 2013; Pokkuluri et al. 2008) or even directly participate in catalysis (e.g. P_{450}, O_2–reductase *alias* cytochrome oxidase or cd_1–type nitrite reductase). The listed examples imply that in the context of this contribution, the term "heme" will refer to substituted Fe-tetrapyrroles in general, encompassing not only Fe-protoporphyrin IX (FePPIX), i.e. heme *b* and its derived heme *c*, but also differently substituted Fe-tetrapyrroles such as sirohemes or heme *a*, heme *o,* etc. This extension of scope becomes ineluctable in the evolutionary context since, as we will see, the synthesis pathways of differently substituted protoporphyrins are heavily convoluted and individual pathways can therefore not be dealt with separately.

Until a few years ago, the occurrence of heme proteins and hence necessarily of hemes in the oldest cellular entity which we can define, i.e. the last universal common ancestor (LUCA) of Bacteria and Archaea, was tacitly taken for granted by a large part of the heme protein community. A number of recent articles have challenged this view (Lane and Martin 2012; Sousa et al. 2013, Sousa and Martin 2014), and the previously held opinion on the evolutionary ancestry of all kinds of heme proteins are consequently called into question. Our contribution aims at providing the readers with a better understanding of the pros and cons of both

the "heme-bearing" and the "heme-free LUCA" paradigms and of the evolutionary consequences at stake in either scenario.

II. Hemes in LUCA; Pros and Cons

A. Does Ubiquity Automatically Translate into Deep Ancestry?

In the realm of the prokaryotes, only a small number of taxonomic groups are known thus far, the members of which are devoid of heme and the most conspicuous examples likely are certain methanogenic Archaea, the homoacetogenic Bacteria from the clostridial phylum or strict fermenters such as the Thermotogales (Sousa et al. 2013). Pervasive presence of a specific trait in both Archaea and Bacteria is frequently taken as indicating the presence of this trait already prior to the Archaea/Bacteria divergence (see for example the case made for aerobic respiration in the LUCA by Brochier-Armanet et al. 2009). As we have argued in the past (van Lis et al. 2011; Nitschke and Russell 2013; Ducluzeau et al. 2014a), we consider this line of reasoning as fraught with problems. The existence of horizontal gene transfer (HGT) between the prokaryotic domains is a well-established fact (haloarchaeal genomes provide an extreme example, see Nelson-Sathi et al. 2012) and a novel trait providing a substantial increase in evolutionary fitness is likely to be widely distributed within the prokaryotes via this mechanism. The evolutionary driving force for trait-dissipation is further augmented if the novel trait is specifically adapted to altered ambient conditions following large-scale environmental transitions. Such transitions have almost certainly occurred several times during the roughly four billion years of life's history on planet Earth and the most conspicuous and best-studied example is the so-called Great-Oxidation-Event (GOE) occurring about 2.3 billion years ago. The GOE has indeed turned the basically O_2-free primordial planet into an "aerobic" world. Whether the GOE had resulted in a persistent oxygenation of the biosphere or was followed by a substantial drop in O_2-levels between 1.9 and about one billion years ago is presently debated (Partin et al. 2013). While the "phylogenomic distribution argument" thus considers that scarce traits have a poor chance of having been present in the LUCA whereas ubiquitous ones are good candidates, we would argue that the undisputable occurrence of profound, one-way, changes of the planet's geo-environment substantially diminishes the logical basis of this argument. In our view, evolutionary traits favourable during life's infancy have a non-negligible chance of becoming outcompeted following major changes in the environment by traits evolving as a result of such environmental overturning and subsequently being widely dispersed through HGT.

All this thus means that the mere pervasive presence of heme cofactors in both Archaea and Bacteria likely is insufficient to conclude on a heme-bearing LUCA. A more stringent approach, albeit prone to a number of experimental difficulties (as discussed in Ducluzeau et al. 2014a) consists in the application of molecular phylogeny (Zuckerkandl and Pauling 1965). The obvious difficulty here is that hemes as organic, transition-metal bearing, cofactors are not (directly) encoded by genes and therefore don't feature (gene- or amino acid-) sequences. However, enzymes using hemes as crucial cofactors as well as the proteins involved in heme biosynthesis do. Which kind of messages can be extracted from the molecular memories of these systems?

B. Phylogenies of Heme-Bearing Bioenergetic Enzymes Suggest a Red LUCA

In the following, we will restrict our discussion to cover only enzymes participating in bioenergetic electron transfer. To the best of our knowledge, heme-dependent enzymes from other types of cellular processes have so far not been studied in great detail with

respect to their deep evolutionary history. Furthermore, the paramount importance for life of free energy converting mechanisms guarantees a good species coverage for individual enzymes and thus improves the reliability of derived phylogenetic trees. Last but not least, the bioenergetic cytochrome systems are what readers of this volume will be most interested in.

Examples for bioenergetic enzymes which involve heme cofactors in a functionally crucial manner are provided by the Rieske/cyt$b$ complexes (encompassing the bc_1 and b_6f complexes as well as their homologs), the superfamily of O_2- and NO-reductases and the cd_1-type nitrite reductases. Other enzymes feature heme-containing subunits involved in electron transfer from or to the catalytic centre while their reaction turnover is not or only marginally dependent on the presence or absence of these heme proteins. Representatives of the latter class are for instance Group 1 [Ni-Fe]-hydrogenases (Pandelia et al. 2012), the superfamily of Mo/W-bisPGD-enzymes (Schoepp-Cothenet et al. 2012), succinate dehydrogenases/fumarate reductases (Lemos et al. 2002), certain types of heterodisulphide reductases (Thauer et al. 2008) or several enzymes involved in the bioenergetics of sulphur compounds (Grein et al. 2013). The heme subunits in these enzymes typically serve to link redox reactions in the soluble phase (i.e. the peri- or cytoplasm) to membrane-integral lipophilic hydrogen carriers such as quinones and methanophenazines (Schoepp-Cothenet et al. 2013). The vast majority of these heme-subunits belong to one of three structural groups (Baymann et al. 2003; Schoepp-Cothenet et al. 2013; Grimaldi et al. 2013).

Only a few of the proteins mentioned have been studied by molecular phylogeny so far. Prominent examples are the Rieske/cyt$b$ complexes (Schütz et al. 2000; Dibrova et al. 2013; Kao and Hunte 2014), the O_2R/NOR superfamily (Pereira et al. 2001; Ducluzeau et al. 2009, 2014; Gribaldo et al. 2009; Sousa et al. 2012), the cytochrome subunits of Group 1 [Ni-Fe]-hydrogenases (Pandelia et al. 2012) and cd_1-type nitrite reductases

(van Lis et al. 2011). For the majority of these heme proteins, the reconstructed phylogenetic trees support their presence prior to the Archaea/Bacteria divergence and they have consequently been proposed to be "pre-LUCA" enzymes. For the case of the O_2R/NOR superfamily, a controversy persists as to which of the subfamilies is the most ancient (Gribaldo et al. 2009 vs. Ducluzeau et al. 2014b) while all studies advocate the presence of ancestral members of the superfamily in the LUCA (as discussed in Ducluzeau et al. 2014a). For the case of the Rieske/cyt$b$ complexes, one article (Dibrova et al. 2013) challenges the conclusion on a pre-LUCA presence repeatedly arrived at over the last two decades (Castresana et al. 1995; Schütz et al. 2000; Lebrun et al. 2006; Ducluzeau et al. 2009; ten Brink et al. 2013) as well as most recently (Kao and Hunte 2014).

As we have pointed out in the past (Ducluzeau et al. 2014a), molecular phylogeny is prone to a number of methodological and database problems and the results obtained (i.e. the phylogenetic trees) from this approach represent likelihoods rather than certainties. With this caveat having been clearly stated, we would nevertheless hold that molecular phylogenies of heme-carrying proteins and enzymes are by and large in favour of the presence of heme proteins in the LUCA.

C. The Dichotomy of Heme Biosynthesis Enzymes

Two distinct pathways have been found enabling prokaryotes to synthesize FePPIX (heme b) (Fig. 2.1). The earlier discovered pathway was elucidated mainly in crown-group proteobacteria (and in particular in $E.$ $coli$) and in Firmicutes (Bacilli) (for a recent review, see Bali et al. 2014). More recently, a distinct pathway was discovered in sulphate-reducing δ-proteobacteria (Ishida et al. 1998; Bali et al. 2011) and was shown to be abundant among Archaea (Bali et al. 2011). The earlier deciphered pathway is now frequently referred to as the
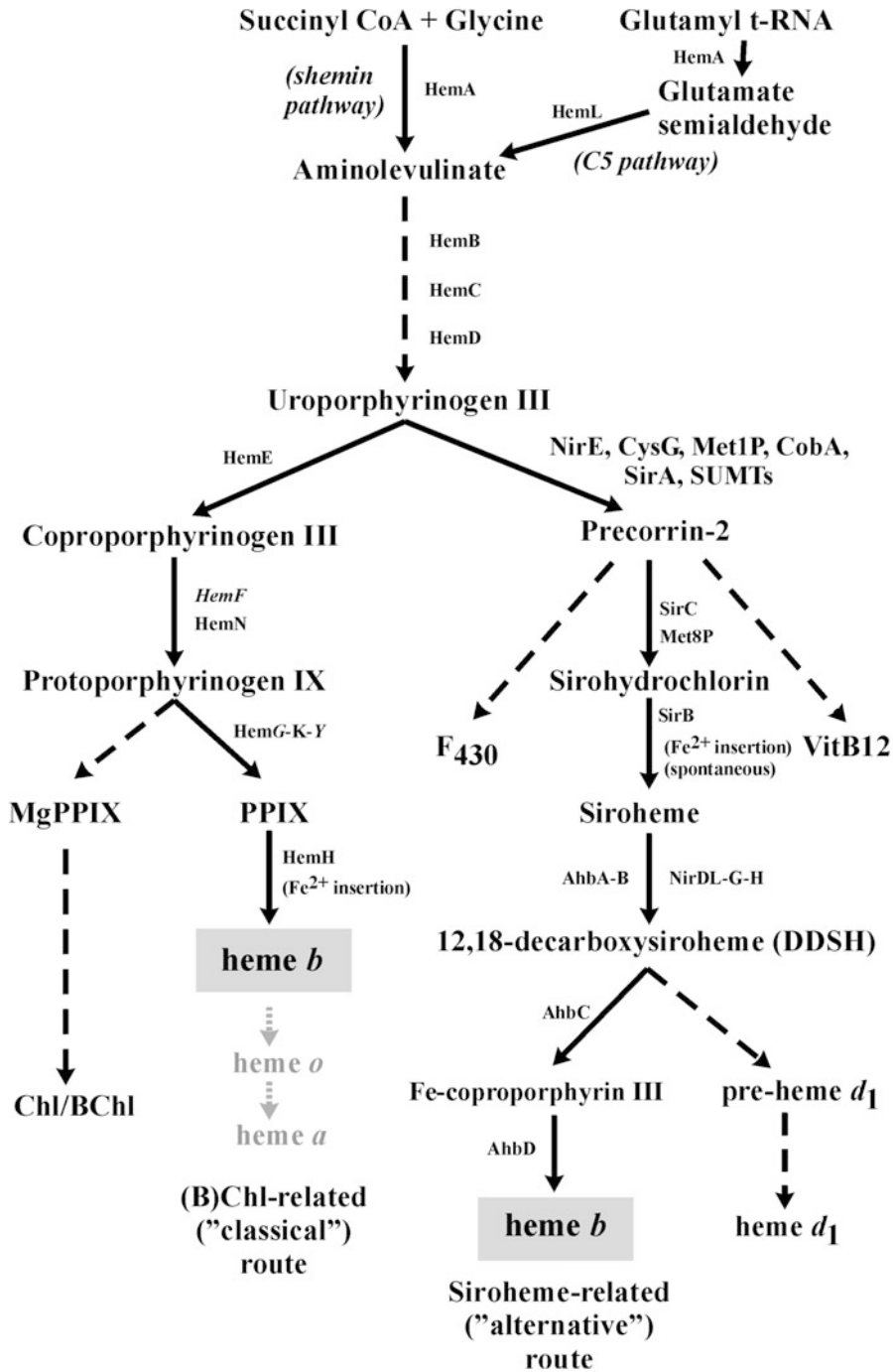
Fig. 2.1. Schematic representation of the two biosynthetic pathways yielding heme *b* and its close relatives together with their relationship to the biosynthesis routes for (B)Chl- and corrin-derived cofactors. *Dashed arrows* denote shortcuts summarising several consecutive steps. The corresponding enzymes catalysing specific conversions are marked *next to the arrows*. O_2-dependent enzymes are *highlighted in italics*.

"classical" route (Bali et al. 2011, 2014). It is closely connected to the biosynthesis of (bacterio)chlorophylls and branches from the (bacterio)chlorophyll-pathways at the level of protoporphyrinogen IX (Fig. 2.1, left branch). The second route, now generally termed the "alternative heme biosynthesis" (Ahb-) pathway (Fig. 2.1, right branch), sequentially features corrins and sirohemes as intermediates on the way to heme *b* (Bali et al. 2011) rather than porphyrins as in the traditional pathway. Both routes share the common intermediate uroporphyrinogen III and all steps leading up to this compound (see Fig. 2.1).

1. Does the Existence of Two Distinct Pathways Necessarily Argue for an Anemic LUCA?

Several recent articles have interpreted the dichotomy of heme biosynthesis pathways as indicating that LUCA had not yet invented hemes and hence must have been devoid of heme-carrying enzymes (Lane and Martin 2012; Sousa et al. 2013; Sousa and Martin 2014). In the line of these authors' argu-

ments, the fact that Archaea mainly synthesize hemes via the Ahb-pathway while the majority of Bacteria utilize the classical one, means that the two routes must have evolved independently, one in Archaea and the other one in Bacteria (Fig. 2.2a) and that the LUCA therefore must have operated on types of metabolism not calling upon the catalytic or electron transfer properties of hemes. An inventory of genes coding for enzymes involved in one or the other pathway over all available genomes (Sousa et al. 2013) confirmed the predominance of the Ahb-route in Archaea and the classical pathway in Bacteria. Exceptions to this rule detected in this study, some of which have already been pointed out previously (Bali et al. 2011), were taken to be due to HGT events.

The apparent overall correlation between type of prokaryotic domain and type of biosynthesis pathway certainly is intriguing. However, are these observations sufficient to unambiguously draw a conclusion about a heme-free LUCA? A number of general considerations and a few details of the pathways indicate that the story may be more complicated:
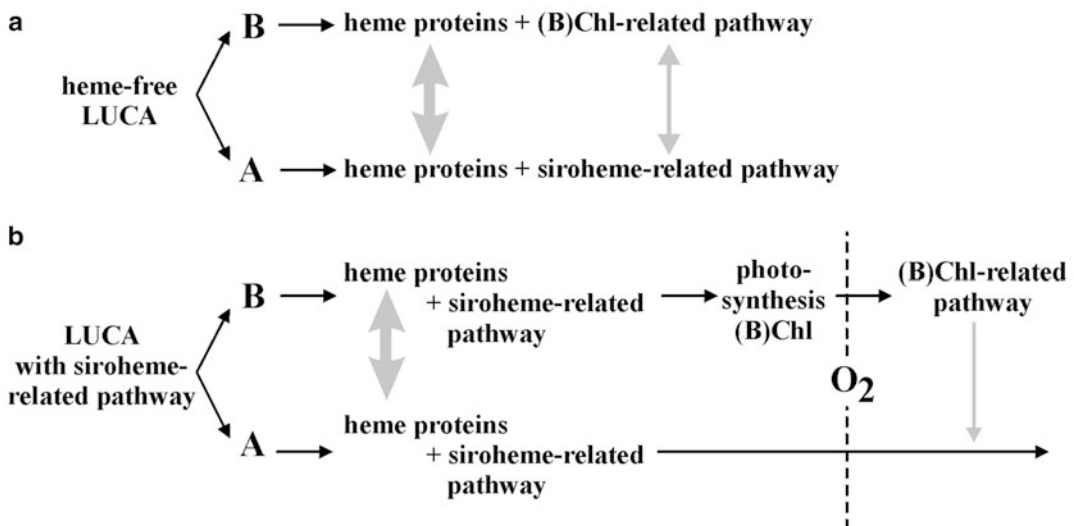
Fig. 2.2. Schematic representation of (**a**) the evolutionary scenario accounting for pathway dichotomy as proposed by Lane and Martin (2012), Sousa et al. (2013) and (**b**) our "alternative" model as described in the text. A and B stand for Archaea and Bacteria, respectively. The (B)Chl- and the siroheme-related pathways correspond to the "classical" and the "Ahb"-routes as defined by Bali et al. (2014), respectively.

– The Ahb-pathway appears substantially more widely distributed over both Archaea and Bacteria whereas the traditional pathway, abundant in several bacterial phyla, is only very rarely present in Archaea (see Fig. 7 in Sousa et al. 2013).

– The classical pathway is related to the synthesis of (B)Chl. In the past, the standard interpretation of this relationship was that (B)Chl synthesis evolved from the heme-biosynthetic pathway. This was based on the consideration that hemes are likely to be evolutionarily older than (B)Chls. Photosynthesis indeed appears to have originated "relatively" late within the bacterial domain (Baymann et al. 2001). This scenario appeared inevitable while the classical pathway was still thought to be life's only way to make hemes, i.e. before the discovery of the Ahb route. The present picture of the topology of reaction schemes shown in Fig. 2.1, however, allows alternative, plausible scenarios. Given the existence of a parallel heme-biosynthesis pathway, the evolutionary sequence of a (B)Chl route growing out of an ancestral Ahb-pathway, and a second route for heme synthesis evolving only later as a derivative of the (B)Chl scheme, is at least as plausible as the original "(B)Chl-from-heme" model. The plausibility of this latter scenario is reinforced by the fact (Fig. 2.1) that the Ahb-pathway is part of the route providing not only heme b but also vitamin B_{12}, siroheme, and cofactor F_{430} (a molecule involved in crucial reaction steps of archaeal methanogenesis, Thauer et al. 2008). The credentials of a "good-for-everything" pathway as the more ancestral appear much stronger to us than those of a system specialized to making only heme b (plus a and o) such as the classical route. An analogous set of arguments has already been considered by Bali et al. (2014).

– Counting from their common precursor, that is, uroporphyrinogen III, the classical pathway goes through three major intermediates before reaching FePPIX (heme b) while the Ahb pathway passes through five such reaction intermediates.

– Intriguingly, the classical pathway features several steps which are dependent on the presence of O_2 (the respective enzymes are denoted by italics in Fig. 2.1). In the absence of O_2, alternative enzymes catalyse these conversions through chemically differing reaction schemes (for a review, see Bali et al. 2014).

III. An Alternative Scenario

A. A Scenario Potentially Reconciling Molecular Phylogeny of Heme Enzymes and Pathway Dichotomy

In addition to the above detailed "independent and domain-inherent origins"-model, at least one additional distinct scenario is thus in principle possible. In this scenario, one of the two pathways would have been present in the LUCA and consequently been inherited by both Archaea and Bacteria. The other pathway would have been a later innovation featuring some kind of evolutionary advantage over the ancestral pathway. This novel pathway might have appeared either in Bacteria or in Archaea and then have supplanted the ancestral pathway in many species due to its evolutionary edge over the other route. An analogous sequence of events has, for example, been invoked to rationalize the evolutionary history of O_2- and NO-reductases (Ducluzeau et al. 2014b).

In the light of the above listed pathway idiosyncrasies we will in the following tentatively outline a more detailed version of this alternative scenario of the evolutionary history of heme biosynthesis. In this scenario, the Ahb-pathway is the ancestral route for the biosynthesis of heme b, siroheme, Vit B_{12}, F_{430} and likely further related cofactors (Fig. 2.2b). This pathway was already present in the LUCA and operated under strictly anaerobic conditions which likely characterized the primordial planet. It was subsequently vertically inherited into both Archaea and Bacteria. At some point in evolutionary history, certainly prior to 2.5 billion years ago, i.e. prior to the GOE, certain Bacteria evolved photosynthesis with tetrapyrroles as light-responsive cofactors (in contrast

to archaeal photosynthesis which is based on light-induced conformational changes of opsins and which eventually gave "vision" to life). Optimization of tetrapyrrol-based photosynthesis towards higher absorption efficiency for solar photons in the wavelength window not filtered out by the atmosphere led to the emergence of the (B)Chl branch of Fig. 2.1. Hemes, by contrast, were still synthesized via the Ahb-route. The ultimate advent of non-negligible amounts of O_2 in the biosphere (Fig. 2.2b) induced by oxygenic photosynthesis (although probably only after a significant time lag due to geochemical buffering of the O_2 produced) allowed the emergence of a plethora of biochemical pathways making profitable use of the newly available, highly reactive and strongly oxidizing molecular oxygen (Raymond and Segrè 2006). Among them was an energetically more economical pathway for heme *b* biosynthesis. Since the oxygenic photosynthesizers likely were among the very first to encounter increasing levels of O_2, it seems plausible that this novel heme biosynthesis pathway evolved within these species and was related to the (B)Chl route due to co-regulation. When O_2 had become pervasive in the biosphere (i.e. after 2.3 billion years ago), this pathway became strongly favourable even for non-O_2-producers generating an evolutionary driving force for its dissemination towards other aerobic organisms throughout the prokaryotes. The Ahb-route was increasingly overgrown by the more modern "classical" pathway except in organisms that remained confined to the shrinking anaerobic niches and never ventured out into oxygenated environments. Aerobic species having entirely lost the Ahb-route, however, were obliged to develop alternative O_2-independent enzymes if they were to re-colonise O_2-poor or anaerobic habitats, which rationalizes the presence of O_2-dependent and O_2-independent enzymes at certain biosynthesis steps in the (B)Chl-related pathway (Fig. 2.1).

The wording of the preceding paragraph highlights the psychological hurdles entailed by the current pathway nomenclature. Referring to a "classical" and an "alternative" route subliminally opposes the notion that the "alternative" pathway might have been THE ancestral route while the "classical" one might in fact represent the actual alternative route that had emerged only at a later time. As will be discussed below, the same nomenclature scheme is in use in the field of menaquinone biosynthesis (Hiratsuka et al. 2008). We do think that it is time to adopt less suggestive terms to identify these pathways. For obvious reasons (see Fig. 2.1), we propose the terms "(B)Chl-related pathway" for the "classical" route and "siroheme-related pathway" for the "alternative" one. We have used this scheme to denote pathways in Fig. 2.2.

It seems obvious to us that both the above sketched "heme-free-LUCA" and the "Ahb-in-LUCA" scenarios are generally in line with presently available evidence. To decide between these two alternatives, global phylogenies of enzymes involved in all (i.e. encompassing heme-, (B)Chl-, siroheme-) biosynthesis pathways are required. Such a study would represent an impressive and highly time-consuming task, but we would argue that it will be indispensible for progressing on the question of the evolutionary history of hemes and heme proteins.

B. Is the Heme-Biosynthesis-Pathway Dichotomy Related to Other Major Pathway Dichotomies?

The scenario of an Ahb-pathway in the LUCA which was later outcompeted (likely after the onset of oxygenic photosynthesis some 2.7 billion years ago) by a more recently emerged system was stimulated by the fact that several other intriguingly analogous pathway dichotomies may find an explanation in the same manner. Two prominent ones are those of the menaquinone- and the iron-sulphur cluster biosynthesis systems. Menaquinones (MK), likely the most ancestral of respiratory quinones (Schoepp-Cothenet et al. 2009, 2013) are

synthesized in prokaryotes via two distinct routes, i.e. the (once again) "classical" Men-pathway (predominantly studied in *E. coli* (Bentley and Meganathan 1983) and the "alternative" futalosine-pathway (Hiratsuka et al. 2008). The dichotomy of MK-biosynthetic routes has been argued to again indicate the absence of quinones in the LUCA (Lane and Martin 2012; Sousa et al. 2013). Phylogenies of several enzymes involved in both pathways have been reconstructed and appear to show that the futalosine pathway is more ancestral than the Men-route (Zhi et al. 2014). However, when it comes to deciding whether the futalosine pathway was present in the LUCA, the published data so far are inconclusive. Phylogenetic tree reconstruction based on different algorithms yields divergent results and no clear answer is therefore possible at present.

A further pathway dichotomy has been found for iron-sulphur cluster biosynthesis. The quite distinct "Isc"- and "Suf"- pathways are scattered over the realm of the prokaryotes with some species containing either one or the other, while others use both pathways differentially as a function of growth conditions and predominantly of oxidative stress (Roche et al. 2013). To the best of our knowledge, no detailed and evolutionarily deep molecular phylogenies of the involved enzymes have been reported so far. However, while heme- and quinone-biosynthesis may indeed be argued to have been absent from the LUCA, we would hold that an iron-sulphur-cluster-free LUCA does not figure in any of the presently proposed scenarios. Parallel, independent, origins of the Isc- and Suf-systems in Archaea and Bacteria, as proposed by Lane and Martin (2012) for heme and quinone biosynthesis, therefore seem highly unlikely. Both in the Fe-S and in the MK-biosynthesis pathways, O_2-dependent/resistant enzymes are asymmetrically distributed and seem to be more frequent in the Men- and the Suf-routes. Since Fe-S centres are highly O_2-labile, it seems more likely to us that emergence of an "aerobic" system (likely the Suf-pathway) was driven by the need to harden the Fe-S-cluster transporting biosynthesis enzymes against the deleterious effects of O_2 rather than by favourable O_2-mediated biosynthetic steps.

The occurrence of all these pathway dichotomies therefore may well indicate the late origins of O_2-dependent routes supplanting anaerobic ones instead of the independent emergences of two pathways in Archaea and Bacteria, respectively. We have proposed in the past that a very similar scenario perfectly rationalizes the convoluted evolutionary history of O_2- and NO-reductases (Ducluzeau et al. 2014). The advent of molecular O_2 on planet Earth certainly represented one of the most profound geochemical revolutions of the environment. It seems to make perfect sense to us that the appearance of the novel and powerful reactant O_2 would drive the emergence of pathways that reap benefits from the opportunity of unprecedented reaction schemes. If the mentioned pathway dichotomies should indeed reflect the late emergence of O_2-related systems, a better understanding of the source organisms, i.e., the species wherein these novel pathways emerged, will substantially further our understanding of life's ways to weather the biochemical turmoil that must have been associated with the GOE.

IV. Why All the Fuzz?

The controversy concerning the detailed evolutionary scenario accounting for the origin of heme cofactors may appear purely academic and of interest only to a very restricted community of molecular phylogeny geeks. However, as we have discussed recently (Ducluzeau et al. 2014a), the resolution to this problem will have substantial consequences for our understanding of energy conversion at the origin of life. Approaches attempting to retrodict bioenergetic mechanisms operating in the LUCA from what we see in extant life to our mind represents a much more "empirical" way to study the origin of life then the conceiving of "*ab-initio*" hypotheses based

solely on chemical and geochemical plausibility arguments (discussed in Schoepp-Cothenet et al. 2013). A scenario remarkably successful in rationalizing the emergence of chemiosmotic free energy conversion, while being perfectly in agreement with thermodynamic requirements, was proposed by Martin and Russell (2003, 2007) as a biochemical incarnation of the seminal hypothesis stipulating alkaline hydrothermal vents as the cradle of life (Russell and Hall 1997). This scenario stipulates Wood-Ljungdahl-type energy conversion (Martin and Russell 2007) and carbon fixation as the ancestral types of metabolism in the LUCA and inorganic versions thereof back to the very origin of life. The Wood-Ljungdahl pathways mainly rely on Fe-S- and Ni-containing cofactors for catalysis but are devoid of quinones and hemes (Lane and Martin 2012). More recently, a variant of this scenario was proposed based on a re-evaluation of the inventory of likely redox substrates present in presumed locales for life's origin (Nitschke and Russell 2013). This scenario still maintains the importance of catalytic steps featuring in the Wood-Ljungdahl pathway (if partially in the reverse direction of catalysis), but adds an oxidative branch resembling anaerobic respiration necessarily implicating quinones and hemes. Whereas the first scenario stipulates only H_2 and CO_2 as providing the environmental redox disequilibrium which drove the emergence of life, the second one envisages a wider cocktail of substrates involving at least H_2 and CH_4 as reductants and CO_2, nitrate, nitrite and potentially Fe^{3+}, Mn^{4+} etc. (Russell et al. 2014) as oxidants resulting in a substantially stronger and multifaceted environmental redox disequilibrium tapped by nascent life. Whereas quinones and hemes are conceivable in the LUCA, they are more difficult to envisage at life's very origin. Inorganic metal-complexes able to have played the roles later taken over by quinones and hemes have therefore been proposed (Nitschke et al. 2013). However, if the LUCA had been devoid of hemes and quinones altogether, the types of energy metabolism proposed to have fuelled the LUCA in the more recent scenario (Nitschke and Russell 2013) are precluded and by extension have little chance of having operated at life's origin.

The question whether the LUCA was red or "anemic" therefore by far exceeds the mere evolutionary history of heme proteins and has crucial repercussions for our understanding of life's origin.

Acknowledgments

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Chapter 3

The Diversity of Photosynthetic Cytochromes

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Summary

This chapter explores the myriad of cytochromes involved in the photosynthetic process that generates the most energy and oxygen on Earth. All photosynthetic organisms rely on cytochromes to move electrons around the cell and drive the photosynthetic mechanism. Although cytochromes have many jobs in many environments, only a few types of cytochromes are involved in these electron transport chains. The hemes vary in axial ligation to meet the needs and reduction potential of each specific role. Some cytochromes

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diverge from the typical alpha helical structure, such as cytochrome f with beta sheet secondary structure. Whether in membranes or in the cytoplasm, diverse cytochromes keep photosynthesis pumping in many ecosystems.

I. Introduction

The photosynthetic process, in both anoxygenic and oxygenic phototrophs, is the major producer of available energy on Earth. Photosynthesis converts sunlight into stable chemical energy through a series of excitation energy transfer, electron transfer and enzymatic steps (Blankenship 2014). Proteins in the cells perform these functions, and the activity is often centered on the metal atom housed in the proteins. A major class of electron transport metalloproteins ubiquitous across the tree of life is cytochrome proteins. Cytochrome proteins contain at least one heme molecule and move electrons via redox reactions. Phototrophic organisms use cytochromes as subunits in membrane protein complexes as well as free soluble proteins to transfer electrons around the cell (Cramer and Whitmarsh 1977).

Phototrophs belong to taxa from the smallest bacteria up through the giant redwood trees. The first photosynthetic organisms were bacteria. Bacterial phyla that contain phototrophic representatives include: Green Sulfur Bacteria, Purple Bacteria, Heliobacteria, Chloroacidobacteria, Cyanobacteria, Filamentous Anoxygenic Phototrophs and Gemmatimonadetes (Blankenship 2014; Zeng et al. 2014). All of these except cyanobacteria carry out anoxygenic (non-oxygen-evolving) photosynthesis. Cyanobacteria developed the

ability to perform oxygenic photosynthesis, and the cyanobacteria were the source of the chloroplast of photosynthetic eukaryotes by endosymbiosis (Margulis 1992). Eukaryotic phototrophs range from algae to plants of all sorts. Cytochromes persisted in the evolutionary jump from anoxygenic to oxygenic photosynthesis because, in order to accommodate the new surroundings, the organisms incorporated different types of cytochromes into the photosynthetic machinery including both b and c type cytochromes (Moore and Pettigrew 1990) (Fig. 3.1a, b). Table 3.1 summarizes all of the cytochromes discussed in this chapter in the order of appearance.

II. Cytochromes in Anoxygenic Photosynthetic Electron Transport Chains

The earliest forms of bacterial photosynthesis emerged before the presence of oxygen in the Earth's atmosphere. These pioneering organisms found a way to use solar energy to drive cellular processes without oxygen as an electron source (Blankenship 2010). Anoxygenic phototrophs continue to thrive today in extreme or niche environments such as the under-layers of microbial mats, sulfur springs or deep sea hydrothermal vents (Lau and Pointing 2009) because they utilize a variety of electron sources and electron shuttles (Meyer and Donohue 1995; Ritchie and Runcie 2012). This variety of electron donors and acceptors is seen in Fig. 3.1a. The basic components of a photosynthetic electron transport chain, whether anoxygenic or oxygenic, are a light harvesting antenna, a pigment-protein reaction center, a proton pumping complex, intermembrane electron shuttles and soluble

Abbreviations: ACIII – Alternative Complex III; CEF – Cyclic Electron Flow; CFX – *Chloroflexus aurantiacus*; cyt – Cytochrome; ETC – Electron Transport Chain; FAP – Filamentous Anoxygenic Phototroph; GSB – Green Sulfur Bacteria; LEF – Linear Electron Flow; OEC – Oxygen Evolving Complex; PB – Purple Bacteria; PSI – Photosystem I; PSII – Photosystem II; Q_i – Quinone Reduction Site; Q_O – Quinone Oxidation Site; RC – Reaction Center; RFX – *Roseiflexus castenholzii*

electron carriers. As explored throughout this chapter, cytochromes form key subunits of the reaction center and proton pumping complexes. They are also the most common form of soluble electron carrier used. Intermembrane carriers are quinols or other small molecules. Anoxygenic phototrophs rely on a diverse collection of cytochromes to survive in their challenging environments.

A. Cytochromes in Type I and II Reaction Centers

The Reaction Center (RC) is the pigment-protein complex to which excitation energy is transferred to drive charge separation, thereby converting solar energy into redox free energy. The primary charge separation process is carried out by pigments, known as the special pair, and does not involve cytochromes in the primary process. Cytochromes, however, function in RCs to reduce the pigments after the primary electron transfer (Parson 1968). RCs are classified into type I and type II as determined by their final electron acceptors, and the iron sulfur clusters or quinones that they carry, respectively, but are thought to all have a common ancestor (Hohmann-Marriott and Blankenship 2011). The cytochromes associated with type I and type II RCs in anoxygenic phototrophs are found on the periplasmic side of the RC and have the function of special pair re-reduction.
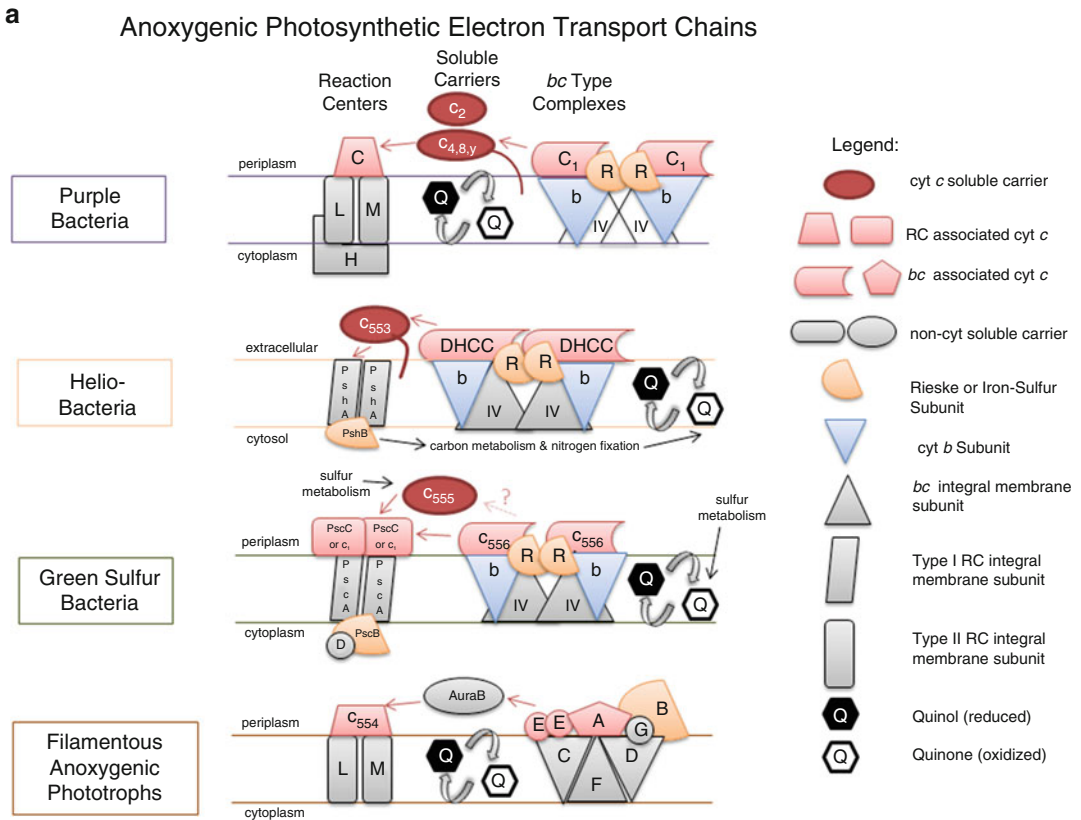
Fig. 3.1. Photosynthetic electron transport chains. Schematics of electron transport chains in representative families of (**a**) anoxygenic and (**b**) oxygenic photosynthetic organisms. Legend is included in part A. *Arrows* represent the flow of electrons. Cytochrome and FeS subunits are in *color* and labeled with their common name, see Table 3.1 for full names.
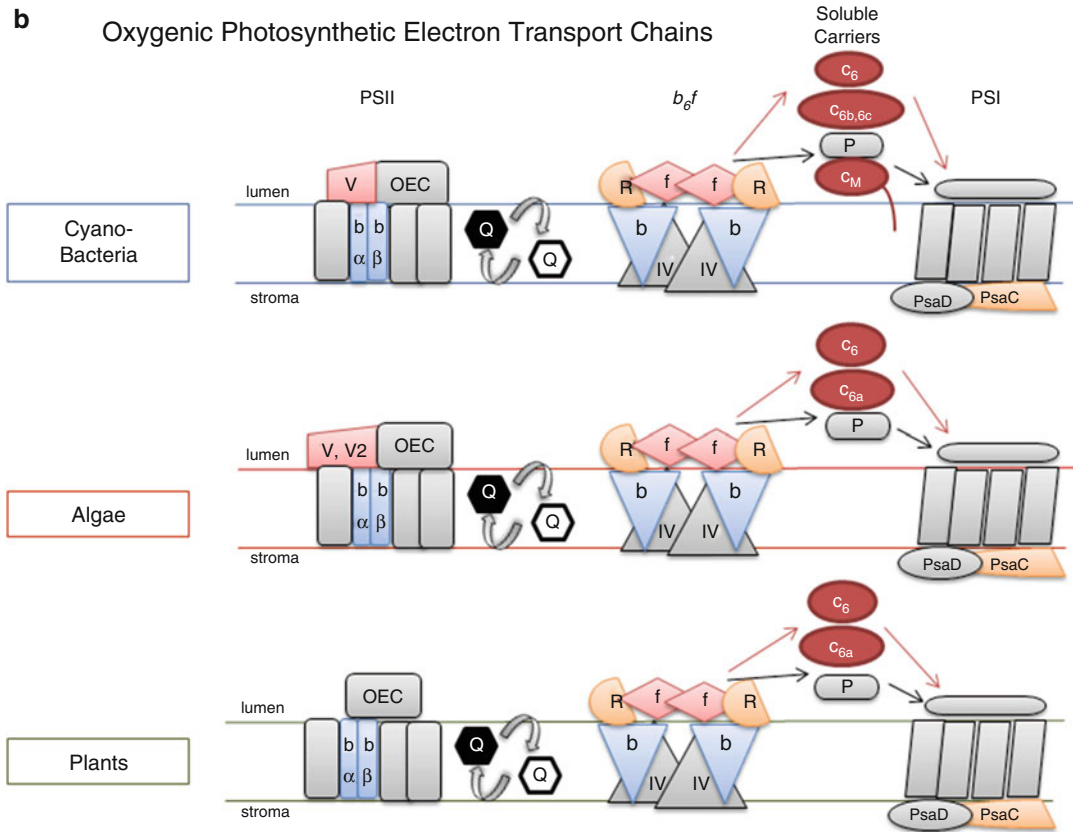
Fig. 3.1. (continued)

1. Cytochromes in Type I Reaction Centers

Prokaryotic anoxygenic type I photosynthetic Reaction Centers are found in Green Sulfur Bacteria (GSB), Chloroacidobacteria and Heliobacteria (Azai et al. 2010). Type I RC's exist as homodimers and are more primitive than their oxygenic counterparts, appearing early in the evolution of photosynthesis (Williamson et al. 2011). Light energy striking the special pair pigments, P800 or P840, causes charge separation and subsequent electron transfer through the RC iron-sulfur clusters, designated Fe-S_x, Fe-S_A, and Fe-S_B, to a soluble carrier, typically a member of the ferredoxin family, small iron-sulfur cluster proteins. The c-type monoheme cytochrome c_z of Green Sulfur Bacteria and c_{553} of Heliobacteria

function in the periplasm to re-reduce the special pair bacteriochlorophylls after charge separation has occurred (Oh-oka et al. 1998; Azai et al. 2010). While performing the same basic function, the differences of these two monoheme c-type cytochromes represent the diversity of anoxygenic type I RC associated cytochromes.

For GSB, the RC associated cytochrome is c_z, also known as PscC or by its absorption peak c_{551}. Formal identification of c_z spanned several decades from initial observation in the 1970s by membrane flash kinetics to more sophisticated techniques in the 1990s (Fowler et al. 1971; Prince and Olson 1976; Okkels et al. 1992; Oh-oka et al. 1995). Cyt c_z is held in place with three N-terminal helices that anchor the cytochrome to the RC complex. This three helix structure on the N-terminus is unique among cytochromes and

Table 3.1. Representative cytochromes in photosynthesis.

Cytochrome	Other names	Major organism Family/ies	Location in cell & attachment	Complex or Shuttle	Function	Special features
c_z	PscC, c_{551}	GSB	Periplasm, n complex	T1 RC	Re-reduce special pair	Interacts with c_{555} & c_{556}
c_{553}	PetJ	Helio	Periplasm, soluble/ complex, anchor	T1 RC	bc to RC	Anchor tail, direct reduces special pair
c_{554}	tetraheme cyt c, RC cyt c	PB, FAP	Periplasm, in complex	T2 RC	Re-reduce special pair	Surprise in crystal structure, uphill energy transfer
Cyt b anoxy	b_H & b_L, b_p & b_n, b_O & b_i	All	Integral membrane	bc	Q cycle	Helio version is PsbE precursor
c_1		PB & mitochondria	Periplasm, in complex	bc_1	Rieske to soluble acceptor	Interacts with far moving Rieske
DHCC	diheme	Helio	Periplasm, in complex	bc	Rieske to soluble acceptor	Has two hemes
c_{556}		GSB	Periplasm, in complex	bc	Rieske to soluble acceptor	Can directly interact with cz
ActA	pentaheme	FAP	Periplasm, in complex	ACIII	FeS to soluble acceptor	has five hemes
ActE	monoheme	FAP	Periplasm, in complex	ACIII	FeS to soluble acceptor	Two copies of the subunit
c_2		PB	Periplasm, soluble	shuttle	bc to RC	Rapid kinetics
c_y		PB	Periplasm, soluble, anchor	shuttle	bc to RC	Alternative c_2, anchor tail
c_4		PB	Periplasm, soluble	shuttle	bc to RC	Alternative c_2
c_8		PB	Periplasm, soluble	shuttle	bc to RC	Alternative c_2
c'	CycP	Anoxygenic	Soluble	De-nitrification	Binds NO & CO	Sequence similarities to c_{556}
c_{555}	CycA	GSB	Periplasm, soluble	shuttle	bc to RC	Can be reduced by sulfur metabolism
b_{559}	PSII cyt b, PsbE&F	Oxygenic	Integral membrane	PSII	unknown	α & β with heme bridged, 4 potentials

(continued)

Table 3.1. (continued)

Cyto-chrome	Other names	Major organism Family/ies	Location in cell & attachment	Complex or Shuttle	Function	Special features
V	PsbV, cyt c_{550}	Cyano, red algae	Lumen, in complex	PSII	Ion gating	Near OEC
V2	PsbV2	Cyano, red algae	Lumen, in complex	PSII	Replace V under stress	Alternative to PsbV, His/Cys axial ligation
Cyt b oxy	b_H & b_L, b_p & b_n, b_O & b_i	Oxygenic	Integral membrane	b_6f	Q cycle	Has pigments
c_i	c_n, c_x	Oxygenic	Integral membrane	b_6f	Q cycle	Surprise in crystal structure
Cyt f		Oxygenic	Lumen, in complex	b_6f	Rieske to soluble acceptor	Beta barrel, unique structure, unknown evolution
c_6	PetJ, Cyt c_{553}	Oxygenic	Lumen, soluble	shuttle	bc to RC	
c_{6a}		Green algae & plants	Lumen, soluble	shuttle	bc to RC, forms disulfide bridges?	Alternative c_6, di-thio cyt
c_{6b}	Pet J1	Cyano	Lumen, soluble	shuttle	bc to RC	Alternative c_6
c_{6c}	Pet J2	Cyano	Lumen, soluble	shuttle	bc to RC	Alternative c_6
c_M		Cyano	Lumen, soluble, anchor	shuttle	bc to RC & donation to COX	Alternative c_6

was assigned its own class, V, of cytochrome protein structures (Azai et al. 2010). Cytochrome c_z reduced the special pair pigments on the 40 µs timescale (Oh-oka et al. 1998). The anchoring allows for the rapid re-reduction of the special pair by keeping the cytochrome in an ideal place for electron transfer rather than letting the dynamics be diffusion controlled.

Besides the unique structure of the three helix anchor on the N-terminus, the $cyt\ c_z$ C-terminus domain also contained structural surprises. The soluble C-terminal portion of cyt c_z has been overexpressed, characterized, crystallized, and its structure solved (Hirano et al. 2010). Despite the low amino acid sequence similarity, this domain of cyt c_z surprisingly showed structural similarity to class I cytochromes (Higuchi et al. 2009; Hirano et al. 2010). Class I cytochromes are c-type low spin hemes with heme attachment near the N-terminus histidine and a sixth coordinating axial ligand, methionine. The unique structure may contribute to its second proposed role of accepting electrons.

In addition to special pair reduction, cyt c_z has been implicated in direct electron transfer from the cyt bc_1 complex. It was shown to enhance the 150 µs time-scale of electron transfer between the RC and the cyt bc_1 complex by interacting directly with the periplasmic subunit of the bc_1 complex, cytochrome c_{556} (Oh-oka et al. 1998). In other bacterial cyclic electron transport chains, an additional free soluble electron carrier would shuttle electrons from the bc_1 to the RC. However, in GSB, no such additional carrier has been observed. One possibility to fill this functional role was the GSB soluble carrier cyt c_{555}, also known as CycA when involved in sulfur metabolism, but it was unable to shuttle electrons between c_{556} and the c_z. This implied that the two peripheral cytochrome subunits of the RC and bc_1 complex may directly contact each other to move electrons without an intermediate (Oh-oka et al. 1998; Tsukatani et al. 2008). Although CycA, cyt c_{555}, was not shown to shuttle between RC and bc_1, in the GSB *Chlorobium tepidum,* cyt c_{555} donated electrons to

cyt c_z on the same timescale as cyt c_{556} (Azai et al. 2008; Tsukatani et al. 2008). This finding does not detract from the direct electron transfer between RC and bc_1 hypothesis. Cyt c_z of the GSB type I RC's is a unique cytochrome structurally and in choice of reaction partners.

Apart from cyt c_z, another type I RC cytochrome has been characterized, cyt c_{553} (PetJ) in Heliobacteria. Unlike GSB, Heliobacteria are gram-positive, lacking a periplasmic space and restricting mobile cytochromes to the cytoplasmic space. Cytochrome c_{553} is therefore found in the cytoplasm and thought to be linked to the membrane via the terminal cysteine residue forming covalent bonds with lipid fatty acids (Azai et al. 2010). When the full Heliobacterial RC complex was purified, cyt c_{553} was integrated as a subunit (Sarrou et al. 2012). The subunit was also expressed and characterized. It displayed with similar function and a redox potential, + 217 mV, to the native RC preparation (Kashey et al. 2014).

Functionally, GSB cyt c_{553} donates electrons to the RC special pair, P800, with an electron transfer donation rate about 5×10^3 s^{-1}, similar to that of cyt c_z (Tsukatani et al. 2008). This rate is ten times larger than in purple bacteria, which have type II RCs and are discussed in the next section (Azai et al. 2010). The tenfold rate enhancement of type I to type II suggests that the hemes and special pairs of the type I RC's are better aligned for efficient electron transfer. Also like GSB, cyt c_{553} can shuttle electrons directly from the cyt bc_1 complex to the RC (Azai et al. 2010; Sattley and Blankenship 2010). Periplasm type I RC cytochromes transfer electrons to re-reduce the RC special pair. Cyt c_z and c_{553} are doing the same job at about the same rate with different structures and attachments showing how organisms can use a variety of cytochromes conditioned to their habitat. Cyt c_z and c_{553} of prokaryotic type I RC's demonstrate the similarity and differences amongst this large family of proteins and reflect the diverse environments in which the proteins function.

2. Cytochromes in Type II Reaction Centers

Type II Reaction Center protein complexes have been characterized more extensively than their type I counterparts due to enhanced stability and relative ease of purification. Type II RCs typically have a Light Harvesting (LH) subunit attached to or surrounding the Reaction Center complexes, unlike type I's the only have the charge separation and electron transfer subunits (Westerhuis 1996). Type II RC cytochromes are usually involved in cyclic rather than linear electron transfer in prokaryotes (Blankenship 2014). The special pair, P870 will receive the excitation energy from light and completes charge separation by quickly passing the charge down bacteriochlorophyll and bacteriopheophytin pigments. The electron is passed through two quinones, from Q_A to Q_B and then to another quinone that will carry the electron through the membrane. The special pair is re-reduced by a cytochrome so that the process can start again.

Anoxygenic Type II RCs, as exemplified by Purple Bacteria (PB), contain three integral membrane subunits: L, M, H and, in many cases, a fourth periplasmic cytochrome subunit. As in the type I RC's, they utilize a cytochrome to re-reduce the special pair of pigments responsible for charge separation (Allen et al. 1987). Many of the purple bacteria contain a tightly bound tetraheme cytochrome that reduces the RC special pair after photo-induced electron transfer (Jones 2009). However, some purple bacterial RCs do not have a bound cytochrome c subunit. Instead, they depend on a soluble cytochrome c_2 that is discussed below or other soluble electron transfer protein (Ortega et al. 1999; Axelrod and Okamura 2005).

The tetraheme cytochrome c RC subunit in Purple Bacteria is a periplasmic subunit with four hemes responsible for accepting electrons from a soluble carrier and re-reducing the RC special pair. It belongs to class III of cytochrome proteins, which are defined by their multihemes with low redox potentials (Coutinho and Xavier 1994).

Early biochemical work on PB RC's had focused on *Rhodobacter sphaeroides* or *Rhodobacter capsulatus*, which do not have the tetraheme cytochrome subunit (Allen et al. 1987; Bauer et al. 1988). Thus, it had been thought that the PB RC's lacked an attached cytochrome. Additionally, the RC cyt c subunit can remain firmly attached during purification, but it is sometimes lost, which made it difficult to detect or characterize. However, an important advance in the knowledge and understanding of the PB RC, and in particular the cytochrome subunit, was the crystal structure of the RC of *Blastochloris viridis,* (formerly known as *Rhodopseudomonas viridis*) (Fig. 3.2a) (Deisenhofer et al. 1985). This was the first photosynthetic RC crystallized and revealed a surprise, the tetra-heme cytochrome c. The discovery and characterization of the tetraheme cytochrome in the *Bl. viridis* structure altered the perception not only of PB RC's, but of all cytochrome donors of reaction center complexes. The discovery spurred questions such as: Why are there four hemes? Does each heme have a distinct redox potential? and How do electrons flow through the hemes?

This tetraheme subunit, known as cyt c_{554} or RC cyt c, accepts electrons from a periplasmic soluble electron donor, and transfers an electron to the special pair of pigments to re-reduce the pair after charge separation has occurred (Hara 1998; Roszak et al. 2012) Cyt c_{554} is analogous to cyt c_z in type I RC's, but has four hemes and no transmembrane anchor helix. The crystal structure revealed a bound tetraheme cytochrome c subunit with a roughly vertically stacked heme alignment (Fig. 3.2b) that had not previously been observed in any cytochrome protein (Deisenhofer et al. 1985). This arrangement was postulated to direct the flow of electron transfer and was confirmed with the assignment of the redox potentials. The redox potentials are heme 1 at -60 mV, heme 2 at $+310$ mV, heme 3 at $+20$ mV, and heme 4 at $+380$ mV in *Bl. viridis* (Dracheva et al. 1988). The subunit has two low potential and two high potential
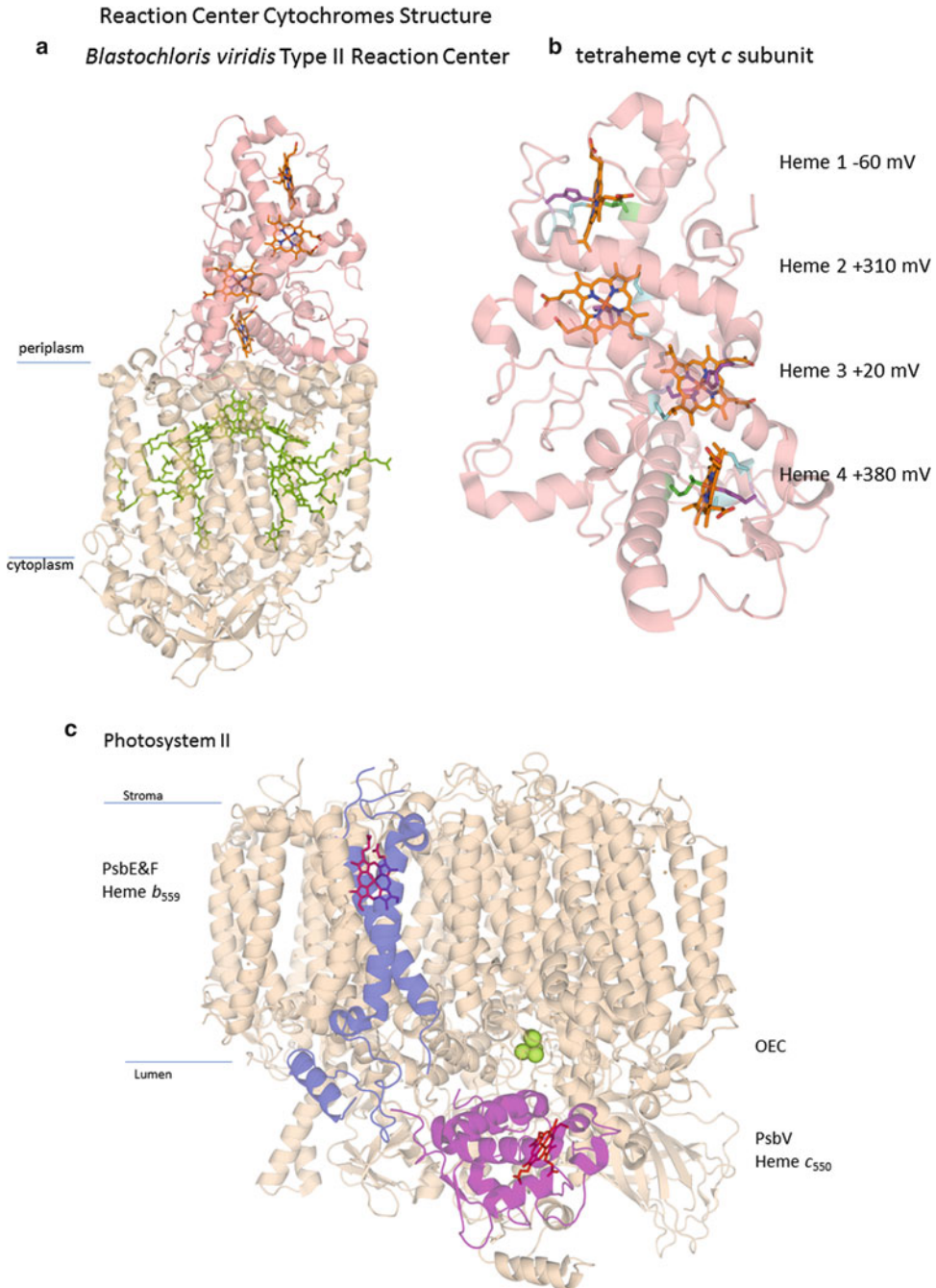
Fig. 3.2. Crystal Structures of photosynthetic reaction center cytochromes. (**a**) *Blastochloris viridis* RC (PDBID 3T6D). The tetraheme subunit is in *red* and the integral membrane subunits are in *tan*. Heme cofactors are *orange* and special pair pigments are *green*. (**b**) The tetraheme subunit alone. Chain is in *red* and hemes are in *orange*. Heme binding amino acid side chains are shown as cysteine in *cyan*, histidine in *purple* and methionine in *green*. (**c**) *Thermosynechococcus elongatus* Photosystem II (PDBID 3BZ1). PSII integral membrane subunits are *tan*, cyt b_{559} subunit is in *blue*, PsbV is in *purple*, *b*-type hemes are *pink*, *c*-type hemes are *red* and OEC is in *green*.

hemes (Nogi et al. 2005). The function of each heme and order of electron transfer within this subunit corresponds to the redox potential. A Low Potential-High Potential-Low Potential-High Potential-Special Pair electron flow pathway was established (Dracheva et al. 1988).

Another unique feature of cyt c_{554} is the ability of the subunit to move electrons uphill against the redox potential gradient. Overall in cyclic electron transport, the distal heme of the tetraheme cytochrome has a much lower redox potential (-60 mV) than donor, cyt c_2 ($+345$ mV), yet donates within 60 μs to the electron acceptor special pair, as characterized in *Bl. viridis* (Blankenship 2014). Somehow the tetraheme subunit is able to overcome this energy barrier; likely from overall downhill thermodynamics of the electron transfer from cyt c_2 to the RC.

Attached tetraheme cytochromes are not only found in PB, but they are observed in other anoxygenic photosynthetic prokaryotic families such as the Filamentous Anyoxygenic Phototrophs (FAPs) (Collins et al. 2010). Many FAPs differ from PBs by growing in warmer environments, being filaments versus rods and having different light harvesting antenna (Tang et al. 2011). Their RCs also lack the H subunit but are still type II RC's (Collins et al. 2009). FAPs are represented by the closely related *Chloroflexus aurantiacus* (CFX) and *Roseiflexus castenholzii* (RFX), but differ in that CFX has a large peripheral light harvesting complex, the chlorosome, and RFX does not (Yamada et al. 2005; Cao et al. 2012). Additionally, the tetraheme cytochrome c subunit of RFX stays firmly attached to the RC during purification but that of CFX does not (Meyer et al. 1989). Despite the loss during purification, the CFX subunit was characterized, and re-reduction of the RC cyt c_{554} was observed in vitro (Meyer et al. 1989). Likewise, the intact RFX RC complex has been purified and characterized (Collins et al. 2010). The re-reduction of the RC special pair by the tetraheme cyt c kinetics in RFX were similar to that of CFX (Xin et al. 2011). The structure of the FAP RC's has not been determined, but a high sequence similarity between the FAP and PB RCs suggests that structure would be similar (Gardiner et al., unpublished data).

In addition to promoting uphill electron transfer, the presence of an attached cytochrome c subunit has been shown to increase the photocurrent detected for a Light Harvesting Reaction Center (LHRC) complex versus an LHRC with no cyt c subunit during in vitro photochemical cell assays (Friebe & Majumder, unpublished data). This increase in photocurrent is attributed to the attached cytochrome c subunit because then the electron donor binding is not the rate limiting step and the assay is not dependent on diffusion and binding of the carrier cyt c. The tetraheme cyt c enhances function of the RC by contributing to the reaction rate and overcoming an energy barrier. The tetraheme cyt c of PB and FAPs adds to the structural and functional diversity of anoxygenic photosynthetic RC cytochromes.

B. Cytochromes in bc Complexes

After RCs complete charge separation, electrons move through the cyclic or noncyclic electron transport chain. The electron movement generates a proton-motive force that ultimately drives ATP synthase forming chemical energy such as ATP (Crofts et al. 2013a). In the RC, two electrons and two protons are donated to a quinone, reducing the quinone to quinol. The reduced quinol diffuses through the membrane to the second large complex of the anoxygenic cyclic electron transport chain, the cytochrome *bc* complex (See Chaps. 9 (Cramer & Hassan) and 10 in this edition) (Hunte 2003). This complex oxidizes the quinol, sending the electrons through the *bc* complex via a process known as the Q-cycle and simultaneously translocates protons to the positive side of the membrane periplasmic or P side (Cape et al. 2006). The proton translocation against the electrochemical gradient contributes to the proton motive force. The electrons, after going through the Q-cycle are loaded onto a soluble carrier, typically a cytochrome (Kramer et al. 2009) which is discussed in the next section.

The cytochrome *bc* complexes are named for the their major cofactors, *b* and *c*-type hemes. A few key differences distinguish *b* and *c* type hemes: *c* type hemes are covalently linked to the protein via thioether bonds while *b* type hemes have vinyl groups in those positions. In typical cytochrome *bc* complexes the *b* and *c* heme cofactors are localized in different subunits. The *b* subunit is usually involved in quinone redox reactions and the *c* subunit in electron transfer with soluble carriers. The proton pumping ability of cyt *bc*-type complexes makes them well suited to electron transport chains for cellular respiration. Cyt *bc* complexes are consequently found in phototrophic and non-phototrophic taxa of prokaryotes including proteobacteria and the in mitochondria of eukaryotes including humans (Cape et al. 2006). The cyt *bc* complexes of anoxygenic photosynthetic prokaryotes discussed in this section are small and simple compared to their eukaryotic counterparts (Esser et al. 2008). However, despite being present on nearly the whole tree of life, the fundamental catalytic components of this protein complex have relatively few forms compared to the variety of species and electron transport chains in which they exist (Berry et al. 2000). This section explores the canonical cyt *bc$_1$* complex, the *bc* complex of Heliobacteria, and the novel functional replacement the Alternative Complex III (ACIII).

1. Cytochrome bc$_1$ Complex

The proton pumping complex used in most anoxygenic phototrophic bacteria is the cytochrome *bc$_1$* complex, which functions to preserve energy within the cell through the Q cycle (Trumpower 1990; Cape et al. 2006). The establishment of the Q cycle mechanism has been the focus of effort in *bc* complexes in recent years and relied heavily on understanding the cytochrome subunits. Typical cyt *bc$_1$* complexes have a cytochrome *b* subunit, a cytochrome *c$_1$* subunit and a Rieske iron-sulfur cluster subunit (Fig. 3.3a) (Khalfaoui-Hassani et al. 2012). The cytochrome *b* and Rieske

protein subunits start the reactions in the Q cycle by providing the sites for quinol oxidation.

The cyt *b* subunit of the cytochrome *bc$_1$* complex has two *b*-type hemes in a classical *b*-type fold with the heme plane parallel to the helix bundle axis (Berry et al. 2013). They belong to the cyt *b* family of proteins defined by their two *b*-type hemes and presence in *bc* complexes (Berry et al. 2000). The subunit has an elongated structure that spans the membrane with about eight transmembrane helices. It is also predicted to be the precursor of the PSII RC cyt *b$_{559}$* discussed in later sections (Shinopoulos and Brudvig 2012). The two *b* hemes within the subunit are named *b$_L$* and *b$_H$* for their respective low and high redox potential. They can also be called *b$_p$* and *b$_n$*, corresponding to *b$_L$* and *b$_H$*, in reference to their locations near the positive and negative sides of the membrane. The hemes are the major cofactors near to the Q$_o$ and Q$_i$ sites, also known as Q$_p$ and Q$_n$ in the same fashion as the hemes, where quinol is oxidized and quinone is reduced, respectively, during the Q cycle (Czapla et al. 2013). Heme *b$_L$* is found near the Qo site to match the redox potential needs of quinol oxidation, and heme *b$_H$* is found near the Q$_i$ site because of the redox potential needed to reduce quinone. The specific chemistry at each of these sites and the mechanism of electron transfer through each heme is part of the Q cycle.

To start the Q cycle, the Q$_o$ site facilitates bifurcated electron transfer. Here we give only a brief description, but see the chapters in this volume by Sarewicz et al. (Chap. 14) and Fisher et al. (Chap. 21) for more information. Two electrons and two protons are extracted from the quinol sequentially (Crofts et al. 2013b). One electron moves onto the Rieske subunit and the other to heme *b$_L$*. The now reduced lower potential heme passes its electrons down the energy gradient to heme *b$_H$*. In the Q$_i$ site a quinone is bound and receives the electron from *b$_H$* generating a semiquinone radical (Cooley 2010). Another quinol binds to the Q$_o$ site and the process repeats generating a reduced quinol in the Q$_i$ that then diffuses into the membrane.

After a quinol is oxidized at the Q_o site, the second arm of the bifurcation event loads one electron onto the Rieske Fe-S subunit, which moves almost 20 angstroms to dock with the cytochrome c_1 on the periplasmic face of the bc_1 complex (Esser et al. 2008; Osyczka and Cooley 2013). The cytochrome c_1 subunit has a typical cytochrome c fold around the monoheme and is anchored to the membrane with a C-terminal helix (Fig. 3.3b). It belongs to class I cytochrome c's. Cyt c_1 transfers electrons to a soluble cytochrome c_2 or sometimes interacts with a different soluble cytochromes c_8 or c_y (Fig. 3.1a). The cyt c_1 subunit has a hydrophobic surface with positive and negative patches complementary

to the soluble acceptor cyt c_2 (Figs. 3.3c and 3.4b). The complementary electrostatic surfaces permits c_1 to bind transiently but tightly with the soluble acceptor. Cyt c_2 or the other soluble carriers, as discussed in the next section, serve to re-reduce the RC, thereby completing the Q-cycle (Sanders et al. 2009). Unlike the cyt b subunit of bc complexes, which are conserved across nature, there is more variation in the cyt c_1 subunits.

2. Cytochrome bc Complexes

Heliobacteria are strictly anaerobic phototrophs that belong to the Firmicutes family, which are Gram positive bacteria and to the class of Clostridia which are anaerobic
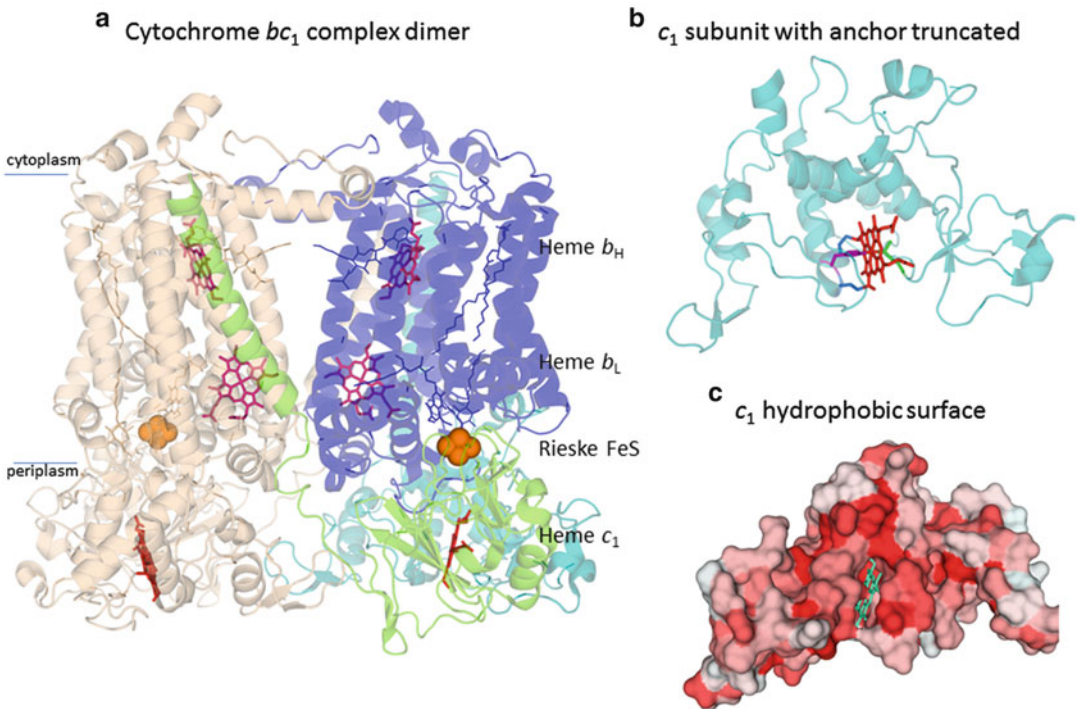
Fig. 3.3. Crystal structures of photosynthetic *bc* complex cytochromes. (**a**) Cytochrome bc_1 complex from *Rhodobacter sphaeroides* (PBDID 2QJK). The complex is shown as the biological dimer with one half color-coded. The cytochrome *b* subunit is in *blue*, the cytochrome c_1 subunit is in teal and the Rieske subunit is in *green*. *b*-type hemes are *pink*, *c*-type hemes are *red* and FeS is in *orange*. The chains of the second half of the dimer are in *tan*. (**b**) The cytochrome c_1 subunit alone with anchor helix truncated. Chain is in teal and heme is in *red*. Heme binding amino acid side chains are shown as cysteine in *cyan*, histidine in *purple* and methionine in *green*. (**c**) Hydrophobic surface of the cytochrome c_1 subunit. The *darker the red*, the more hydrophobic the area is. Heme is shown in *cyan* and shown in same orientation as 3B.
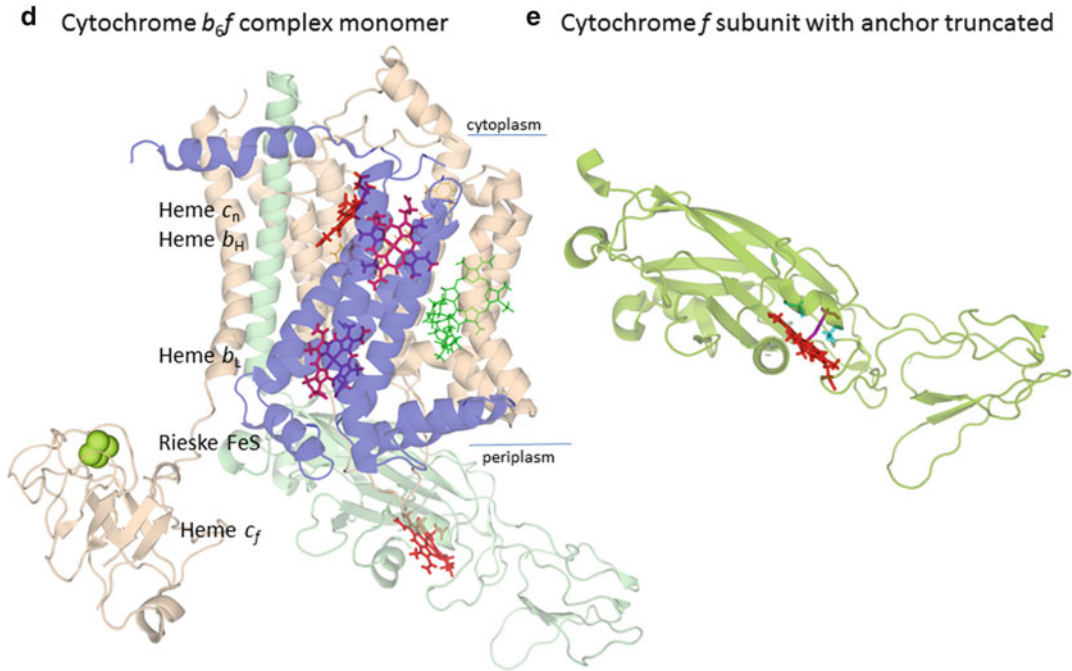
Fig. 3.3. (continued) (**d**) Cytochrome b_6f complex from *Nostoc sp. PCC7120* (PBDID 4HH4). The complex is shown as half of biological dimer. The cytochrome *b* subunit is in *blue*, the cytochrome *f* subunit is in *green*. *b*-type hemes are *pink*, *c*-type hemes are *red* and FeS is in *green*, carotenoid is in *orange* and chlorophyll is in *green*. The other subunits are in *tan*. (**e**) The cytochrome *f* subunit alone with anchor helix truncated. Chain is in *green* and heme is in *red*. Heme binding amino acid side chains are shown as cysteine in *cyan*, histidine in *purple* and methionine in *green*.

(Heinnickel and Golbeck 2007). For photosynthetic machinery, they employ Type I Reaction Centers and a cyt *bc* complex that is comparatively simple or smaller than the previously discussed complexes (Redding et al. 2013). From the cyt *bc* complex, the *b* subunit in Heliobacteria is the postulated evolutionary precursor for both cyt b_6f and cyt bc_1 cyt *b* subunits (Dibrova et al. 2013). The deviation from the conventional pattern is in the periplasmic cytochrome subunit. Instead of a monoheme cytochrome *c*, heliobacteria have a diheme cytochrome *c*. This protein is predicted to have resulted from a gene duplication event and subsequent fusion (Baymann and Nitschke 2010).

Unlike other DHCCs, in the heliobacterial DHCC, both hemes have equal reduction potentials (Yue et al. 2012). Recent work predicts a structure and mechanism for this complex using homology modeling in combination with Hydrogen Deuterium Exchange Mass spectrometry (Zhang et al. 2014). In the absence of a crystal structure, the modelled protein has two similar *c*-type cytochrome domains with an N-terminal helix anchor. In the predicted mechanism, the electron arrives at DHCC via the iron-sulfur cluster. The N-terminal heme would accept the electron from the iron-sulfur cluster. The electron would then pass to the heme of the C-terminal domain. The C-terminal domain is more flexible and solvent accessible and therefore should donate the electron to the soluble electron carrier. This mechanism involving both hemes in the DHCC subunit would allow the complex to store electrons or be able to turn over even if the soluble carrier has not retuned yet (Zhang et al. 2014).

As in Heliobacteria, the complex in Green Sulfur bacteria is also called a *bc* complex because it also an alternative to cyt c_1. GSB

Soluble Cytochromes Structures

a   Cytochrome c_2

c   Cytochrome c_6

b   Cytochrome c_2 hydrophobic surface
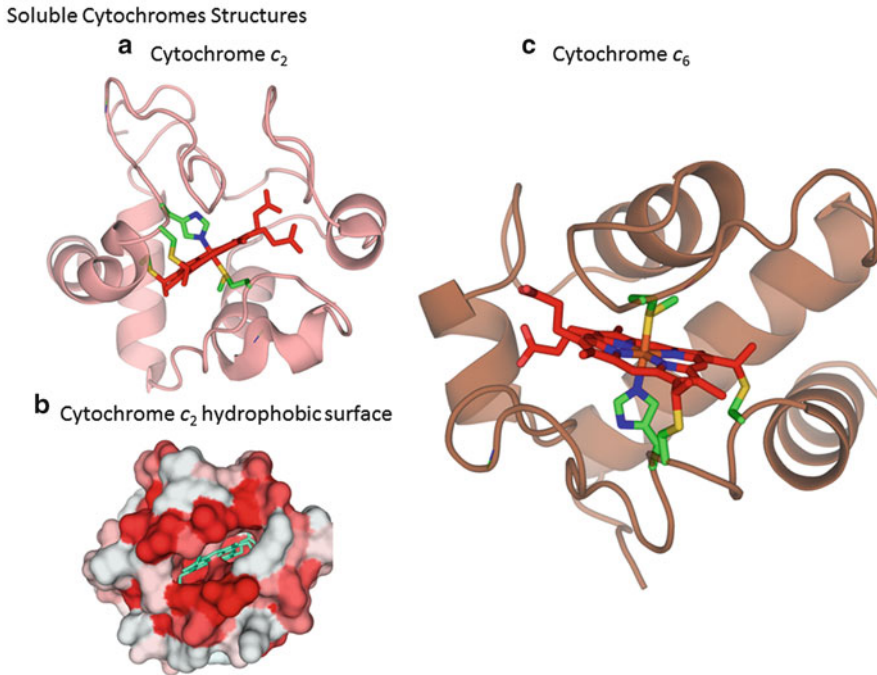
Fig. 3.4. Crystal structures of photosynthetic soluble cytochromes. (**a**) Cytochrome c_2 from *Blastochloris viridis* (PDBID 1IO3) that interacts with the RC in Fig. 3.2a. Chain is *light brown*, heme is in *red*, and the heme binding amino acid side chains are colored by element. (**b**) Hydrophobic surface of the cytochrome c_2. The *darker the red*, the more hydrophobic the area is. Heme is shown in cyan and shown in same orientation as 4A. (**c**) cyt c_6 from *Chlamydomonas reinhardtii* (PDBID 1CYI) that interacts with the RC in Fig. 3.2c. Chain is *brown*, heme is in *red*, and the heme binding amino acid side chains are *colored by element*.

have a cytochrome c_{556} that is structurally much like cyt c_1, but differs in that c_{556} is predicted to directly donate to the RC cyt c_z without a soluble intermediate (Oh-oka et al. 1998). There is diversity in the structure and mechanism of anoxygenic photosynthetic cyt *bc* complexes.

3. Alternative Complex III

Some anoxygenic phototrophs contain novel integral membrane cytochrome complexes that do not fit into either the bc_1 or b_6f category. They fulfill the same functional role as a major enzyme of the electron transport chain of organism but do not share sequence homology or an evolutionary relationship with the conventional *bc* complexes.

An unconventional cytochrome complex that stands in contrast to the cytochrome *bc* complex protein family, belonging instead to the molybdopterin oxidoreductase family, is the Alternative Complex III (ACIII) (Yanyushin et al. 2005; Gao et al. 2009; Majumder et al. 2013). ACIII complexes are found in several families of non-phototrophic bacteria and in photosynthetic prokaryotes, in the Filamentous Anoxygenic Phototrophs (FAPs) family (Refojo et al. 2013). ACIII is predicted to have the function of the *bc* complex, but does not resemble the typical complex, having more homology to polysulfide reductase (Majumder et al. 2013). It has seven subunits, two of which contain *c*-type hemes and does not contain *b*-type cytochromes.

The two heme subunits of ACIII that have been characterized in the photosynthetic prokaryote *Chloroflexus aurantiacus* are ActA and ActE. ActA has five hemes, and ActE is a monoheme subunit with two copies present in the full complex. ActA and ActE

are both found in the periplasm. The 5 hemes in ActA have 3 redox potentials of -228 mV (3 hemes), -110 mV (1 heme) and $+94$ mV (1 heme). ActE has a redox potential of $+390$ mV (Gao et al. 2010). ActA is predicted to donate electrons to ActE, and ActE is thought to be the final electron acceptor within the complex, much like cyt c_1 in the bc complexes. ActE has been shown to be necessary for oxido-reductase function (Gao et al. 2013). Furthermore, it is thought to be a dimer (Gao et al. 2010). A homologous protein to ActE has not been identified in the Protein DataBank, and therefore suggests that ActE does not have the typical cytochrome c fold. The amino acid sequence of ActA, however, aligns with other multiheme proteins such as the *Escherichia coli* hexaheme nitrite reductase, but shows differences that arise from the presence of a N-terminal trans-membrane helix in ActA (Majumder et al. 2013).

Overall, ACIII contains seven hemes to carry out a one-electron process. Other proteins with mutlihemes carry out chemistry that requires more electrons, which poses the question of the mechanism of ACIII (Clarke et al. 2004, 2007). There is no evidence for a Q-cycle-like mechanism in ACIII. A Q_o site has been identified by inhibitor assays by a HQNO fluorescence assay, but there is no Q_i site nor b-type hemes (Refojo et al. 2010). The mechanism of ACIII is expected to be quite different from that of typical cyt bc complexes. In mechanism and subunit composition, ACIII is a novel member of the photosynthetic cytochromes.

C. Soluble Cytochromes in Anoxygenic Cyclic Electron Transport

Soluble cytochromes are the mobile components of photosynthetic electron transport chains, shuttling electrons between the large membrane proteins. All known soluble cytochromes in photosynthesis are monoheme c-type cytochromes, most with the usual c-type cytochrome protein fold and are located in the periplasm (Azai et al. 2010). Examples of other non-cytochrome soluble electron carriers are two small blue copper proteins: plastocyanin in oxygenic photosynthetic organisms and auracyanin in the FAPs (King et al. 2013). While some soluble cytochromes are restricted by an anchoring tail that attaches these cytochromes to the membrane surface, other soluble cytochromes are neither attached nor associated with the membrane (Dutton et al. 1975; Kashey et al. 2014). Regardless of membrane attachment, soluble cytochromes proteins are promiscuous binders allowing them to bind two different protein complexes transiently (Myllykallio et al. 1998; Bernroitner et al. 2009).

A canonical soluble anoxygenic cytochrome is cyt c_2, which shuttles electrons from the cyt bc_1 complex to the RC in Purple Bacteria (Dutton et al. 1975; Sponholtz et al. 1976). This protein belongs to the class I c-type cytochromes having histidine and methionine as axial ligands and the heme attachment near the N-terminus of the protein (Lin et al. 1994). The crystal structures of cyt c_2 from several purple bacterial species have been solved (Fig. 3.4a). The solved cyt c_2 structures all contain an alpha-helical c-type cytochrome fold. The heme is buried except for one exposed edge surrounded by positively charged lysine residues (Fig. 3.4b) (Axelrod et al. 2009). This feature defines the binding site to the RC and cyt bc_1 which have corresponding regions consisting of complementary negatively charged residues on their surface (Blankenship 2014). This electrostatic interaction allows for tight but transient binding since c_2 must deliver electrons quickly to the two different partners. The flash-induced kinetics for c_2 donation to the RC special pair are multiphasic, representing each step in the electron transfer process: first the diffusion of the reduced c_2 and binding to the RC is seen in the slower kinetic phase and secondly, the fast phase, 1 μs, reflects the rapid oxidation of a bound c_2 (Daldal et al. 2001; Gong et al. 2003).

In Purple Bacteria, cytochromes c_y, c_8, c_4, and a HiPIP (High-Potential Iron-Sulfur Protein) have been implicated as alternatives

cyt c_2, the donor to the RC. Cytochromes c_4 and c_8 are also c-type soluble cytochromes (Verméglio et al. 2002). The ability of cyt c_8 to donate to the RC in several PB species was established in the 1970s (Tatsuhiko 1970; Samyn and Smet 1996; Hochkoeppler et al. 1997). It has also been observed to function during autotrophic growth (Verméglio et al. 2002). The origin of c_8 may be in the nitrite reduction pathway (Nagashima et al. 2011). Cyt c_y is unique as one of the few anchored soluble cytochromes, also found in PB (Hochkoeppler et al. 1995). The membrane anchor is a tail that attaches to a membrane lipid moiety. It therefore has a pendulum type motion in shuttling electrons between the large integral membrane complexes. The electron transfer rates in c_2-less mutants that only use c_y still showed efficient photosynthetic growth suggesting that c_y is functional replacement for c_2 (Jenney et al. 1994; Hochkoeppler et al. 1995; Myllykallio et al. 1998). The alternative donors provide PB the flexibility to survive in changing conditions such as light intensity and nutrient concentration.

Another unique class of cytochromes found in phototrophic and aerobic bacteria is cytochrome c' (Ambler et al. 1981). They are soluble c-type cytochromes whose function is not well understood. They are designated as c-type because of the covalent bonds from the heme to cysteines but differ in most other ways. Cytochrome c' has the heme in a high spin environment, but does not have typical ligands or a c-type cytochrome fold and is thus pentacoordinate (Weber et al. 1980). This class of alternative cyt c proteins has their heme binding sites near the C-terminal end of the protein, which similar to c_{556} from GSB, but has other structure features of cyt c_1 (Ambler et al. 1981). A study of cyt c′ in PB *Rhodobacter sphaeroides* showed that cyt c′ is expressed most during photosynthetic denitrification growth and binds NO and CO (Choi et al. 2005; Russell et al. 2013). Further work will reveal the function of cyt c'.

The story of soluble cytochromes in other anoxygenic species is not as straightforward. Green Sulfur Bacteria were originally thought to use a soluble cyt c_{555}, but it is now known that there are other sources of electrons from sulfur metabolism that enter the photosynthetic cyclic electron transport chain, but they that are not well characterized (Sakurai et al. 1996). As seen in Fig. 3.1a, cyt c_{555} can take electrons from sulfur metabolism and potentially donate them to c_z of the RC. As discussed above, Heliobacteria are now believed to have no soluble cytochromes as part of their CET chain and rely solely on membrane-bound cytochromes (Blankenship 2014). The Filamentous Anoxygenic Phototrophs, as mentioned above, rely on the small blue copper protein auracyanin to shuttle electrons between ACIII and the RC (Fig. 3.1a) (Majumder et al. 2013). Whether typical or atypical, many cytochromes are key components in the photosynthetic electron transport chain of anoxygenic bacteria. These cytochromes persisted in their same basic forms during the evolutionary transition to oxygenic photosynthesis.

III. Cytochromes in Oxygenic Photosynthetic Electron Transport Chains

As the evolution of the Earth progressed, organisms developed the ability to split water and produce oxygen approximately three billion years ago as part of their photosynthetic electron transport (Iverson 2006; Falkowski and Raven 2007). This process developed as part of the great oxygenation event released oxygen into the atmosphere and made possible complex, multicellular life as we know it. This was a phenomenal achievement billions of years in the making. Even in oxygenic eukaryotic organisms, cytochromes perform many of the same functions as in their prokaryotic counterparts (Fig. 3.1b).

A. Cytochromes in Photosystems I and II

The oxygenic versions of the Type I and Type II RCs are Photosystem I and Photosystem II. These are very large protein complexes

with around 20–30 subunits and associated light-harvesting proteins (Drop et al. 2014). They function together in linear electron transport with PSII extracting electrons from water and passing them onto PSI (Fig. 3.1b). Cyclic electron transport flow has also been observed around PSI (Iwai et al. 2010; Shinopoulos and Brudvig 2012; Trubitsin et al. 2014). Cytochromes in the Photosystems are found more as integral membrane components with fewer examples of soluble cytochromes being used than their anoxygenic counterparts.

1. Photosystem I

Cyanobacteria, algae and plants have a type I Reaction Center Photosystem I, and a type II RC, Photosystem II that will be discussed below. PSI, unlike the anoxygenic type I RCs, contains no cytochromes. PSI is distinct from the other RCs in that it does not have a bound cytochrome subunit (Falkowski and Raven 2007). PSI contains three iron-sulfur clusters and therefore uses non-heme irons are on the acceptor side, but do not replace the donor side cytochromes as in other RC's (Blankenship 2014). In some cases, depending on copper and iron content, Photosystem I receives electrons from the cytochrome b_6f complex via the blue copper protein plastocyanin and ultimately reduces ferredoxin to make NADPH (Blankenship 2010). However, most cyanobacteria and some algae contain a cyt c_6, which will be discussed below, that can substitute for plastocyanin, with the expression dependent on copper availability (Hill and Merchant 1992).

2. Photosystem II

The Reaction Center with the most subunits and variety of cofactors is Photosystem II (Williamson et al. 2011). PSII is found in cyanobacteria, algae and plants. PSII houses a Mn-containing Oxygen Evolving Complex (OEC) within the protein (Pokhrel and Brudvig 2014). The OEC was a critical development in Earth's evolution providing the oxygen that allowed higher forms of life to evolve (Blankenship 2010). The OEC, as the electron donor, aides PSII to participate in both cyclic and linear electron flow. The cytochrome subunits of PSII also help the complex function in both of these scenarios (Fig. 3.2c).

Cytochrome b_{559} in PSII is a heterodimeric monoheme subunit with alpha and beta subunits, each contributing one heme coordinating histidine (Shinopoulos and Brudvig 2012). A single heme being coordinated across two small subunits is a unique structural feature of the cyt b_{559} compared to other known cytochromes. Cyt b_{559} is a required subunit for the assembly of PSII (see Müh and Zouni 2015), but it does not appear to function in the electron transport chain and its precise functional role has long been elusive. It has been proposed to have a role in secondary electron transfer pathways such as putative cyclic electron flow around PSII, photoinhibition, or reactive oxygen species protection (Pospíšil 2011; Shinopoulos and Brudvig 2012). Recent work has investigated the enzymatic function of cyt b_{559} as an oxidoreductase because it was observed that four distinct forms of cyt b_{559} with different redox potentials appear in vivo under different conditions (Feyziyev et al. 2013; Kaminskaya and Shuvalov 2013; Guerrero et al. 2014). The range of redox potentials for a monoheme cytochrome (see Müh and Zouni 2015 for discussion of functional significance of these potentials) and the bridging of the heme between two proteins make cytochrome b_{559} a unique cytochrome in photosynthetic electron transport chains.

PSII from some organisms, has two other known cytochromes, PsbV and PsbV2. PsbV and PsbV2 are low redox potential cytochromes, but so far their function is not understood (Fig. 3.2c). These two cytochromes have been found in cyanobacteria and red algae, but not in plants (Kerfeld et al. 2003). The PSII subunit PsbV encodes for a cytochrome c_{550} and is an extrinsic protein on the lumenal side of the cyanobacterial thylakoid membrane. The subunit binds PSII in close proximity

to the Mn cluster of the OEC (Roncel et al. 2012) (see also Chap. 8, Muh and Zouni, this volume). PsbV is one among several cyt's of unknown function in oxygenic phototrophs (Katoh et al. 2001; Roszak et al. 2012). Since PsbV is implicated in PSII's affinity for calcium and chloride ions in the OEC, the presence of the subunit is required for the function and stability of the complex (Kirilovsky et al. 2004). The role of the heme, specifically, in PsbV is still unknown since the heme is not thought to act in the ion affinity function. The heme of PsbV is also not thought to have a redox function in the OEC due to the low redox potential of the heme. Recent data suggest that it protects the Mn atom in the OEC from reductants (Roncel et al. 2012). Additional new mass spectrometry techniques have shown that the disruption of PsbQ affects the stability of PsbV and decreases the overall function of PSII (Liu et al. 2013, 2014).

An alternate protein to PsbV is PsbV2. PsbV2 was found in the genome of thermophilic cyanbacteria immediately following PsbV. The amino acid sequences of PsbV and PsbV2 are highly similar (Kerfeld et al. 2003). PsbV2 has been isolated and purified. Biochemically, it appears to be similar to PsbV and can partially replace PsbV in the event of disruption of PsbV expression. Both proteins have sequence similarity to PetJ or cyt c_6, formerly referred to as cytochrome c_{553} (Katoh et al. 2001). The crystal structure of PsbV2 was recently solved revealing a new His/Cys axial heme ligation, but an overall structure like that of cyt c_6 and cyt c_{550} (Suga et al. 2013). The novel ligation adds a further question to an enzymatic role for the heme of PsbV and PsbV2. The cytochrome subunits of PSII are diverse members of the cytochrome family in their unique heme coordination, ligation and putative functional roles in the RC complex.

B. Cytochromes in b_6f Complexes

In oxygenic phototrophs, including cyanobacteria, algae and plants, the functional role of the cytochrome bc complex is filled by the cytochrome b_6f complex (Kallas 2012). Unlike anoxygenic photosynthetic organisms, which only have cyclic electron transport, the b_6f complex is involved in LEF and CEF (Hasan et al. 2013). Their role in CEF mirrors that of the cyt bc_1 complex sending cycles electrons around the RC, PSI. Under conditions of CEF only, b_6f becomes the sole protein responsible for the proton gradient that drives ATP production. LEF is a feature of oxygenic photosynthetic electron transport chains and involves both photosystems to split water and reduce $NADP^+$ with b_6f in between (Blankenship 2014). Whether in CEF or LEF, cyt b_6f undergoes a Q cycle that analogous to the mechanism in bc_1. The mechanism is bifurcated electron transfer mediated by the same classes of cofactors, b and c hemes and iron sulfur clusters (Fig. 3.3d). Recent work on the evolution of bc complexes has pointed to a common ancestor for bc_1 and b_6f complexes that is closer to the bc complex from Heliobacteria (Dibrova et al. 2013). After their appearance, b_6f complexes have remained largely unchanged and are highly similar among species that are separated by hundreds of millions of years of evolution (Cramer et al. 2004).

The cytochrome b subunit is much like cyt b of the bc_1 complex with two b type hemes interacting with the quinone pool. The differences lie in the addition of pigments within the complex, the length of the b subunit, and the presence of an addition c-type heme. The anoxygenic and/or prokaryotic cyt b has a longer amino acid sequence than its oxygenic counterpart (Cramer et al. 2004). The function of the carotenoid and chlorophyll pigments in the b_6f complex is unknown since the complex is not known to do photochemistry. Reports have suggested a protective mechanism of these pigments against ROS species by capturing the triplet energy of ROS (Stroebel et al. 2003; Cramer et al. 2005). Heme c_i was a surprise discovery when the crystal structure of the cytochrome b_6f complex was solved (Fig. 3.3d) (Stroebel et al. 2003). Heme c_i is located near heme b_L in the Q_i site of the complex. It has

at midpoint potential of $+100$ mV, but no axial ligands and may be accessed by an exogenous ligand (Alric et al. 2005). Mutation to the c_i resulted in a slowly of b heme reoxidation (de Lacroix de Lavalette et al. 2009). The role of c_i in the complex is still being investigated (Hasan et al. 2013). The oxygenic cyt b subunit of the b_6f complex, while being similar to its anoxygenic counterpart, is a diverse cytochrome because of the presence of pigments in the subunit.

The most unusual cytochrome protein in photosynthesis is cytochrome f (Fig. 3.3e). Cytochrome f takes the place of cytochrome c_1 as a c-type heme being the last step of electron transfer within the b_6f complex (Whitmarsh and Cramer 1979; Stroebel et al. 2003). Cyt f, however, is primarily a beta-sheet protein that does not resemble the typical alpha helical cytochrome c fold. Additionally, the axial coordination of the heme involves an unusual histidine and tyrosine combination (Prince and George 1995). Cyt f belongs not to a cytochrome family of proteins but rather to the barrel sandwich hybrid superfamily (Prince and George 1995). Within the crystal structure, it was observed that heme-binding residues are located on alpha helices of only a few residues in length (Fig. 3.3e) (Martinez et al. 1994). Fundamental work by Hill and coworkers in the early 1950's first identified and then isolated cytochrome f (Davenport and Hill 1952). By the late 1970s, cytochrome f was known, and its role in the cell was being clarified (Whitmarsh and Cramer 1979). Today, even with hundreds of cyt f genes sequences and high-resolution crystal structures, the origin and evolution of the cytochrome f remains a mystery. It is speculated to be an important link in the transition from anoxygenic to oxygenic photosynthesis (Blankenship 2014).

C. Soluble Cytochromes in Oxygenic Cyclic Electron Transport

Soluble cytochromes in oxygenic cyclic electron transport are not as widely utilized as is the case in their anoxygenic photosynthetic counterparts. Oxygenic organisms have several alternative soluble electron carriers. A small blue copper protein, plastocyanin, fills the major role of shuttling electrons between the cyt b_6f complex and PSI in plant and algal chloroplasts and in many cyanobacteria (Gupta et al. 2002). Likewise, electrons arrive to the special pair in PSII from water splitting in the OEC. Small molecules such as ferredoxin or NADPH also serve as electron carriers in oxygenic electron transport chains (Nelson and Ben-Shem 2004). Soluble cytochromes are still valuable oxygenic photosynthesis. In many cyanobacteria, cyt c_6 is the sole known electron donor to PSI (Kerfeld and Krogmann 1998). Cytochromes can also have new functions during stress conditions providing flexibility for the organisms (Rutherford et al. 2012; Simon and Klotz 2013).

One family of soluble oxygenic photosynthetic cytochromes is cyt c_6 and cyt c_{6a}. They are lumenal electron carriers found in cyanobacteria and eukaryotic algae and plants (Figs. 3.1b and 3.4c). Cyt c_6 has been shown to replace plastocyanin donation to PSI when the medium is copper limited (Hill and Merchant 1992). Cyt c_6 is a class I c-type cytochrome of about 10 kDa with standard histidine and methionine axial ligands (Kerfeld and Krogmann 1998). The flash-induced kinetics of cyt c_6 donation to PSI were compared in many organisms and situations (Hill et al. 1991; Hervas et al. 1995). The cyt c_6 of the chlorphyll-d containing cyanobacterium *Acaryochoris marina* has also been purified and characterized. It was found to weigh 8.9 kDa, have alpha helical structure and a midpoint potential of $+327$ mV (Bell et al. 2009). The work by Bell et al. also suggested that cyt c_6 was the electron acceptor from cyt b_6f rather than plastocyanin. Characterization of the cyt c_6 family has continued with new techniques and new species. Recent work has characterized the rapid electron transfer between cytochrome f and cyt c_6, shedding light on the efficiency of this process (Díaz-Moreno et al. 2014) (see also Bialek et al. 2015).

The cyt c_{6a} designation emerged amid evidence that higher plants had by evolution eliminated cyt c_6 from their chloroplasts (Gupta et al. 2002). Oxygenic organisms have a very high degree of conservation in their photosynthetic machinery within their families. Cyanobacteria predominantly use cyt c_6 to interact with cyt b_6f. Algae use both cyt c_6 and plastocyanin and plants only express and use plastocyanin (Hippler 1999). The discovery of cyt c_{6a} in *Arabidopsis* challenged this notion. Compared to the sequence of cyt c_6, cyt c_{6a} has an insertion of 12 amino acids with two conserved cysteine residues. It also has a particularly low potential heme that is 200 mV lower than cyt c_6 (Gupta et al. 2002). This indicates that cyt c_{6a} could not effectively donate electrons to PSI. This low potential was surprising because the axial heme coordination is the same as cyt c_6 (Worrall et al. 2008). The exact functional role of low potential cyt c_{6a} is not clear (Howe et al. 2006, 2015).

Besides cyt c_{6a}, there are other known alternatives to cyt c_6. In cyanobacteria, two other forms of cyt c_6 have been identified as c_{6b} and c_{6c}. These cytochromes were identified from the *petJ1* gene and a *petJ1*-like gene, *petJ2*, in *Synechococcus* sp. PCC 7002. The *petJ1* gene encodes a typical cyt c_6, which is the only known electron carrier between the cyt b_6f complex and PSI in *Synechococcus* 7002 and many other cyanobacteria. PetJ2 had a much lower midpoint potential $+148$ mV, compared to $+319$ of PetJ1, and was designated as the cyt c_{6c} class of cytochromes, which are widely distributed among cyanobacteria (Bialek et al. 2008). Cytochrome cyt c_M from cyanobacteria is another possible c_6 substitute and is an example of a protein that may be used in both CEF and LEF. Cyt c_M was shown to donate electrons to cytochrome c oxidase cyanobacterial respiration during in vitro assays as rapidly as cyt c_6 and faster than plastocyanin (Bernroitner et al. 2009). However, there is no available evidence to show that cyt c_M is expressed at sufficient levels in vivo to support such functions (Mullineaux 2014). The varied soluble oxygenic cytochromes allow organisms to adapt to stress conditions or different living environments.

IV. Conclusions

From large membrane protein complexes to small soluble proteins, cytochromes play crucial roles in electron transport chains, accomplishing the major processes of energy conversion and oxygen production on the planet. Most that have been studied are b or c-type cytochromes, but significant variations exist despite overall similarity in functions. Most cytochromes share evolutionary origins, with the notable exception of cytochrome f, whose origin remains obscure. Photosynthetic organisms take advantage of the diversity of cytochromes to adapt to different environments and metabolic needs.

Acknowledgments

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Chapter 4

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Evolution of Photosynthetic NDH-1: Structure and Physiological Function

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Summary

Chloroplast and cyanobacterial NADH dehydrogenase-like complex (photosynthetic NDH-1) is structurally related to the multi-subunit-type NADH dehydrogenase present in bacteria and mitochondria (respiratory NDH-1). They both have a common ancestor, namely the group 4 membrane-bound hydrogenases, including energy-converting hydrogenase (Ech). During its evolution, respiratory NDH-1 (complex 1) acquired the electron input N module that functions in NADH oxidation. Homologs of the N module have not been identified in photosynthetic NDH-1. The electron donor system for photosynthetic NDH-1 has apparently remained similar to that in the common ancestor and accepts electrons directly from ferredoxin (Fd). Indeed, the discovery of the NdhS protein required for high-affinity binding of Fd to NDH-1 in chloroplasts and cyanobacteria supports the idea that photosynthetic NDH-1 accepts electrons from Fd. Through modification of its subunit composition NDH-1 in cyanobacteria is involved in divergent functions, including the concentration of CO_2

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by NDH-1MS complexes. Nonetheless, chloroplast NDH-1 originated from cyanobacterial NDH-1L that in the light mediates cyclic electron transport around photosystem I (PSI) and in darkness regulates respiratory electron transport. Consistent with this, chloroplast NDH-1 also mediates PSI cyclic electron transport as well as chlororespiratory reduction of the plastoquinone pool in order to maintain redox homeostasis in chloroplasts. During the evolution of land plants, the number of subunits of chloroplast NDH-1 increased and it became associated with PSI to form a supercomplex in flowering plants, the process which is necessary to stabilize NDH-1. Despite conservation of the core skeleton forming the L-shape, photosynthetic NDH-1 is likely to have undergone evolution distinct from that of respiratory NDH-1. Consequently, photosynthetic NDH-1 has gained different physiological functions and operates, at least partially, by molecular mechanisms different from those of respiratory NDH-1.

I. Introduction

Multi-subunit-type NADH dehydrogenase (complex I) couples electron transfer from NADH to quinone, with translocation of protons (or cations) across the membrane. The complex consists of a peripheral arm mediating electron transfer and a membrane arm consisting of the proton-transfer machinery, forming an L-shaped skeleton (Baradaran et al. 2013). NADH-dependent quinone reduction is also mediated by a single-subunit-type enzyme (NDH-2), but this reaction is not coupled with proton translocation (Melo et al. 2004). Multi-subunit proton/cation-transferring enzymes are referred to as NDH-1 to distinguish them from NDH-2. A prototype of NDH-1 is a prokaryotic enzyme consisting of a minimum set of 14 subunits (Friedrich and Scheide 2000) in three distinct modules (N, Q, and P). The peripheral arm contains all of the redox cofactors and is subdivided into the electron-input NADH oxidizing (N) module (composed of NuoE, F, and G in *Escherichia coli* NDH-1) and the Q module mediating electron transfer to the quinone-binding site (composed of NuoB, C, D, and I in *E. coli* NDH-1). The membrane arm forms

the proton-translocating P module composed of 6 subunits (NuoA, J, K, L, M, and N in *E. coli* NDH-1). NuoH does not belong to any module and connects the Q and P modules (Efremov and Sazanov 2012).

Complete sequencing of chloroplast genomes has revealed 11 *ndh* genes in tobacco (*Nicotiana tabacum*) and liverwort (*Marchantia polymorpha*) (Ohyama et al. 1986; Shinozaki et al. 1986). This set of genes encodes 4 subunits of the Q module, 6 subunits of the P module, and NdhA, corresponding to NuoH (Matsubayashi et al. 1987). Why do the chloroplast genomes encode subunits of the respiratory complex? Chloroplasts originated via endosymbiotic events from cyanobacteria, in which photosynthetic and respiratory electron transport share a common quinone pool in the thylakoid membrane (Mullineaux 2014). Reflecting this evolutionary scenario, some electron transport routes in the thylakoid membrane resemble respiratory electron transport in mitochondria. Both NDH-1 and NDH-2 reduce the plastoquinone (PQ) pool, which in turn is oxidized by plastid terminal oxidase (PTOX), which is related to alternative oxidase (AOX) in mitochondria (Rumeau et al. 2007). Chlororespiration is electron transport mediated by these components; particularly high chlororespiration activity has been reported in *Chlamydomonas reinhardtii* (Peltier and Schmidt 1991; Peltier and Cournac 2002). However, the green algae,

Abbreviations: BN – Blue native; Fd – Ferredoxin; Lhc – Light harvesting complex; NPQ – Nonphotochemical quenching; pmf – Proton motive force; PQ – Plastoquinone; PSI/II – Photosystem I/II; PTOX – Plastid terminal oxidase

including *Chlamydomonas*, have lost their chloroplast NDH-1, and their chlororespiration depends on NDH-2 (Desplats et al. 2009). Even though chloroplast NDH-1 is structurally closely related to respiratory NDH-1, the enzymes have experienced differential evolution and are likely to have acquired different functions from the common ancestor. On the basis of its distinct difference from "respiratory NDH-1" in bacteria and mitochondria, here we call the NDH-1 in chloroplasts and cyanobacteria "photosynthetic NDH-1." In fact, photosynthetic NDH-1 is unlikely to be a true NADH dehydrogenase and the term "NDH" has been proposed to represent the NADH dehydrogenase-like complex in cyanobacteria and chloroplasts (Ifuku et al. 2011; Yamamoto et al. 2011). Here, we summarize our knowledge about the evolution of cyanobacterial NDH-1 from the common ancestor with respiratory NDH-1. This prototype of photosynthetic NDH-1 further experienced evolution in land plant chloroplasts. We do not yet understand the reasons for this evolution, but we summarize the current progress in research toward elucidation of the mysterious features of the photosynthetic NDH complex. To avoid overlap with recent reviews (Suorsa et al. 2009; Battchikova et al. 2011a; Peng et al. 2011b), we specifically focus on the evolution of photosynthetic NDH-1.

II. Function and Structure of Cyanobacterial NDH-1

To review the research history of photosynthetic NDH-1, it is natural to start with the story of the M55 mutant of *Synechocystis* sp. PCC 6803 (Ogawa 1991). The M55 mutant was isolated on the basis of its requirement for a high CO_2 concentration for growth; the mutation was identified in the *ndhB* gene encoding an NDH-1 subunit. The discovery was followed by extensive physiological research, the results of which supported the idea that NDH-1 mediates cyclic electron transport around photosystem I (PSI) (Mi et al. 1992). However, the original idea that NDH-1 operates in PSI cyclic electron transport in order to energize the CO_2-concentrating machinery was soon found to be overly simplified and was actually an erroneous assumption. Most of the cyanobacterial *ndh* genes are present as a single copy per genome; *ndhD* and *ndhF* are the only exceptions (Table 4.1). The *Synechocystis* genome has 6 *ndhD* genes (*ndhD1* to *ndhD6*) and 3 *ndhF* genes (*ndhF1*, *ndhF3*, and *ndhF4*) (Kaneko et al. 1996). Selective knockout of specific *ndhD* genes has elucidated the distinct functions of NDH-1 (Ohkawa et al. 2000). NdhD1 and NdhD2 (but not Ndh5 and -Ndh6), which are related to chloroplast NdhD, and their double knockout results in a low rate of respiration (Bernát et al. 2011) and impaired photoheterotrophic growth. The NDH-1 complex containing NdhD1 or NdhD2 mediates electron transport from the electron donor to the PQ pool in respiratory electron transport in the dark and in PSI cyclic electron transport in the light. These conclusions made from mutant phenotypes have been supported by proteomics analysis of thylakoid membrane complexes separated by blue native (BN) gels (Herranen et al. 2004; Zhang et al. 2004). In the BN gel, two bands represent distinct forms of NDH-1, namely the NDH-1L (~490 KDa) and the NDH-1M (~350 KDa) complexes (Battchikova et al. 2005). NDH-1L and NDH-1M share all other subunits except for NdhD1/D2 and NdhF1, which are specifically present only in NDH-1L (Prommeenate et al. 2004; Battchikova et al. 2005). Nevertheless, the original idea based on the mutant phenotypes (low rate of respiration and impaired photo-heterotrophic growth) may require modification, because succinate dehydrogenase, at least in *Synechocystis* sp. PCC 6803, has been shown to mainly donate electrons to the PQ pool in darkness (Cooley and Vermaas 2001); thus the role of NDH-1 in respiration may be related to light conditions. Moreover, cyanobacterial NDH-1 includes NdhS (see the discussion on the electron donor for chloroplast NDH-1) and

Table 4.1. A list of genes encoding NDH-1 subunits.

SC[a]	Subunit	Other name	At[b]	Mp[c]	Syn[d]	E. coli	motif
M	NdhA		*ndhA*	*ndhA*	slr0851	NuoH	TM[e]
	NdhB		*ndhB*	*ndhB*	sll0223	NuoN	TM, H$^+$ translocation
	NdhC		*ndhC*	*ndhC*	slr1279	NuoA	TM
	NdhD		*ndhD*	*ndhD*	slr0331 (D1) slr1291 (D2)	NuoM	TM, H$^+$ translocation
	NdhD'				sll1733 (D3) sll0027 (D4)		TM
					slr2007 (D5) slr2009 (D6)		TM
	NdhE		*ndhE*	*ndhE*	sll0522	NuoK	TM
	NdhF		*ndhF*	*ndhF*	slr0844 (F1)	NuoL	TM, H$^+$ translocation
	NdhF'				sll1732 (F3) sll0026 (F4)		TM
	NdhG		*ndhG*	*ndhG*	sll0521	NuoJ	TM
	NdhH		*ndhH*	*ndhH*	slr0261	NuoD	
	NdhI		*ndhI*	*ndhI*	sll0520	NuoI	
	NdhJ		*ndhJ*	*ndhJ*	slr1281	NuoC	
	NdhK		*ndhK*	*ndhK*	slr1280	NuoB	
A	NdhL	CRR23	At1g70760	HE855888	ssr1386		TM
	NdhM		At4g37925	HE855889	sll1623		
	NdhN		At5g58260	HE855890	sll1262		
	NdhO		At1g74880	HE855891	ssl1690		
N[f]	NuoE					NuoE	
	NuoF					NuoF	NADH binding
	NuoG					NuoG	

B	PnsB1	NDH48/NDF1	At1g15980	HE855895	
	PnsB2	NDH45/NDF2	At1g64770	HE855896	
	PnsB3	NDF4	At3g16250	HE855897	
	PnsB4	NDF6	At1g18730	HE855898	sml0013 (NdhP)	TM
	PnsB5	NDH18	At5g43750	HE855899		TM
	NdhQ				slr1311	TM
L	PnsL1	PPL2	At2g39470			PsbP-like
	PnsL2	PQL	At1g14150			PsbQ-like
	PnsL3	PQL	At3g01440			PsbQ-like
	PnsL4	FKBP16-2	At4g39710	HE855903		FKBP– type PPIase
	PnsL5	AtCYP20-2	At5g13120	HE855903		PPIase
ED	NdhS	CRR31	At4g23890	HE855892	ssl0352	SH3-like fold
	NdhT	CRRJ	At4g09350	HE855893		J domainTM
	NdhU	CRRL	At5g21430	HE855894		J-like domainTM
	NdhV		At2g04039	CUFF.9031	sll0272	DUF2996
1S[g]	CupA				sll1734	
	CupB				slr1302	
	CupS				sll1735	
LK[h]	Lhca5		At1g45474			LhcI
	Lhca6		At1g19150			LhcI

The bolditalics indicate chloroplast-encoded genes
[a]Subcomplex
[b]*Arabidopsis thaliana*
[c]*Marchantia polymorpha*
[d]*Synechocystis* sp. PCC 6803
[e]*trans*-membrane domain
[f]N module
[g]NDH-1S
[h]Linkers

probably accepts electrons from ferredoxin (Fd) rather than from NAD(P)H (Battchikova et al. 2011b). It is highly conceivable that our original concept of photosynthetic NDH-1 was influenced too much by the homology with respiratory NDH-1.

In contrast to the *Synechocystis* △ndhD1/ △ndhD2 mutant, the △ndhD3/△ndhD4 mutant is defective in CO_2 uptake, in addition to PSI cyclic electron transfer, and requires increased levels of CO_2 for optimal growth (Ohkawa et al. 2000; Shibata et al. 2001). *ndhD3* is co-transcribed with *ndhF3* and *cupA*/*chpY* from an operon induced at low CO_2 concentrations (Maeda et al. 2002; Wang et al. 2004; Zhang et al. 2004). On the other hand, *ndhD4* is constitutively expressed with *ndhF4* and *cupB*/*chpX*. These subunits, which are specific for the CO_2 concentrating machinery, form NDH-1S complexes, which associate with NDH-1M to form active NDH-1MS complexes (Zhang et al. 2004, 2005). NDH-1MS complexes are involved in CO_2 concentration. The M55 mutant is defective in NdhB, which is present in both NDH-1L and NDH-1MS complexes; this results in disruption of both functions. This research history has been extensively reviewed in other articles (Battchikova and Aro 2007; Ogawa and Mi 2007).

Like the *Thermus thermophilus* and *E. coli* NDH-1 complexes, which consist of a minimal set of 14 subunits (Sazanov et al. 2013) and have been characterized as the prototypes of mitochondrial NDH-1, cyanobacterial NDH-1 can be regarded as a model for chloroplast NDH-1. As discussed below in greater detail, photosynthetic NDH-1 lacks the N module composed of three subunits in *E. coli* NDH-1, indicating that 11 subunits form a core conserved both in the respiratory and photosynthetic NDH-1 complexes (Table 4.1). In addition to these 11 subunits, the cyanobacterial NDH-1 contains the NdhL, M, N, and O subunits (Ogawa 1992; Prommeenate et al. 2004; Battchikova et al. 2005). These 15 subunits (NdhA to NdhO), at a minimum, form the core of photosynthetic NDH-1. The crystal structure of NDH-1 has been determined only in the prototypical bacterial enzymes (Efremov et al. 2010; Efremov and Sazanov 2011; Baradaran et al. 2013). Nevertheless, single-particle electron microscopy has provided structural information about cyanobacterial NDH-1 (Arteni et al. 2006). In *Thermosynechococcus elongatus* BP-1, NDH-1 forms an L-shaped structure with a relatively short peripheral hydrophilic arm, which probably reflects the lack of the N module (Fig. 4.1). However, a minor population of the complex contains a longer peripheral arm, implying the presence of an unknown electron input module. Another low-abundance type of the complex forms a U-shaped structure, probably corresponding to the NDH-1MS complex (Arteni et al. 2006; Folea et al. 2008). By adding a YFP-tag to the subunits, the positions of NdhL, M, N, and O have been determined by single-particle
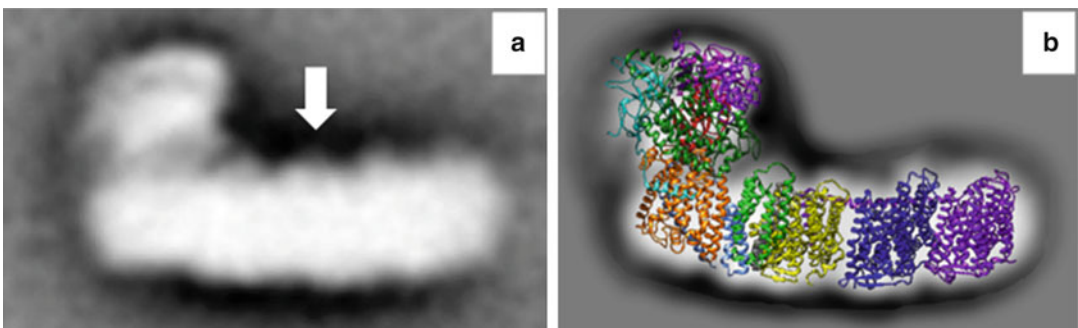
Fig. 4.1. Single-particle images of cyanobacterial NDH-1L (*side view*). An *arrow* in (**a**) indicates the position of NdhL, M, N, and O. (**b**) Overlap with the crystal structure of respiratory NDH-1, from which the N module is omitted (Single-particle images were kindly provided by E. J. Boekema).

analysis (Birungi et al. 2010). Unexpectedly, the NdhL, M and N subunits were localized to the central part of the membrane arm, which is inconsistent with the *Arabidopsis* model based on genetic and biochemical evidence (Peng et al. 2009, 2012) (see below).

III. Chloroplast NDH-1 and Cyclic Electron Transport Around PSI

A breakthrough in cyanobacterial study made it possible to seriously reconsider the enigmatic occurrence of NDH-1 in chloroplasts. Meanwhile, a technique for plastid transformation was established (Svab and Maliga 1993), enabling knockouts of the chloroplast *ndh* genes (Burrows et al. 1998; Kofer et al. 1998; Shikanai et al. 1998; Horváth et al. 2000). The initial physiological characterization of the NDH knockout tobacco has been a topic of another review (Shikanai 2007) and is only briefly discussed here. Chloroplast NDH-1 mediates electron transport from the stromal electron pool (see information below on the electron donor) to the PQ pool. However, knockout of the NDH-1 complex has no influence on photosynthetic electron transport under greenhouse conditions (Burrows et al. 1998; Shikanai et al. 1998). This is also true for *Arabidopsis* mutants defective in chloroplast NDH-1 (Hashimoto et al. 2003; Munekage et al. 2004). Chlororespiratory reduction of the PQ pool shares a route with cyclic electron transport around PSI in the light. However, the contribution of chloroplast NDH-1 to the total proton motive force (pmf; $\triangle$pH plus membrane potential) is small, at least in tobacco and *Arabidopsis*. The phenotypes of the NDH-1 mutants have been detected from the post-illumination changes in chlorophyll fluorescence and from $P700^+$ levels (Burrows et al. 1998; Shikanai et al. 1998; Hashimoto et al. 2003; Munekage et al. 2004). Importantly, virtually no phenotype has been able to be monitored during steady-state photosynthesis. For example, it has not been possible to monitor the reduction in the extent of non-photochemical quenching (NPQ) of chlorophyll fluorescence, which in leaves reflects the size of $\triangle$pH. This is also true for attempted measurements of the activity of Fd-dependent PQ reduction in ruptured chloroplasts, which is smaller than the main, antimycin A-sensitive, PGR5-dependent pathway (Okegawa et al. 2008). Although there is evidence that NDH-1-deficient tobacco is sensitive to various environmental stresses (Endo et al. 1999; Horváth et al. 2000), the exact molecular mechanism of the resistance provided by photosynthetic NDH-1 remains obscure.

PSI cyclic electron transport mediates the recycling of electrons from Fd to the PQ pool, contributing to the formation of pmf without accumulation of NADPH (Shikanai 2007). The *Arabidopsis pgr5* (*proton gradient regulation 5*) mutant is defective in the main pathway of PSI cyclic electron transport (Munekage et al. 2002) and this pathway corresponds to the antimycin A-sensitive route of electrons, initially discovered by the group of Arnon (Tagawa et al. 1963; Joët et al. 2001; Munekage et al. 2002; Hertle et al. 2013; Sugimoto et al. 2013). *Arabidopsis* double mutants defective in both the NDH-1- and PGR5-dependent pathways of PSI cyclic electron transport exhibit very severe phenotypes of photosynthesis and plant growth (Munekage et al. 2004), providing genetic evidence that PGR5-dependent and NDH-1-dependent pathways partly redundantly contribute to the formation of pmf. On the basis of the *pgr5* mutant phenotype in *Arabidopsis* (Munekage et al. 2002, 2004) and rice (Nishikawa et al. 2012), the physiological function of PSI cyclic electron transport can be summarized as (Shikanai 2014): (1) Acidification of the thylakoid lumen to induce NPQ (Munekage et al. 2002) and downregulation of the activity of the cytochrome b_6f complex (Suorsa et al. 2012), both of which are required to protect PSI from photodamage (Munekage et al. 2002; Suorsa et al. 2012). This process is especially important for growth under fluctuating light conditions (Suorsa et al. 2012; Kono et al. 2014). (2)

The pmf formed by PSI cyclic electron transport, as well as that formed by linear electron transport, contributes to the ATP supply for CO_2 fixation (Munekage et al. 2002, 2008; Nishikawa et al. 2012). The defect in chloroplast NDH-1 accelerates the *pgr5* phenotype (Munekage et al. 2004), suggesting that NDH-1 also contributes to the formation of pmf in chloroplasts by mediating PSI cyclic electron transport. This was also supported by the direct analysis of the size of pmf in the double mutants (Wang et al. 2015).

Although the contribution of chloroplast NDH-1 to pmf formation is minor during steady-state photosynthesis, it may regulate the redox state of the PQ pool under certain conditions in combination with PTOX (Joët et al. 2002; Trouillard et al. 2012). The variegated phenotype of the PTOX mutant (*immutans*) is alleviated by the absence of chloroplast NDH-1, suggesting that chlororespiration has a regulatory function in maintaining the proper redox state of the PQ pool during early chloroplast development (Okegawa et al. 2010). PGR5-dependent PQ reduction also contributes substantially to redox homeostasis at this stage (Okegawa et al. 2010). Nevertheless, the question remains regarding the physiological function of NDH-dependent PSI cyclic electron transport in mature chloroplasts. This topic is discussed further below, on the basis of the different phenotypes of NDH-1-deficient mutants between flowering plants and *Marchantia*.

IV. Structure of Chloroplast NDH-1

Respiratory NDH-1 in *E. coli* is composed of 3 modules, whereas photosynthetic NDH-1 in chloroplasts consists of 5 subcomplexes assigned as subcomplexes A, B, ED, L, and M (Ifuku et al. 2011). Eleven core subunits, which are homologous to those forming the Q and P modules in *E. coli*, are encoded by the chloroplast genome (Table 4.1). NdhH, I, J, and K form the core of subcomplex A, which corresponds to the Q module in *E. coli*,

whereas NdhB, C, D, E, F, and G are core components of subcomplex M (membrane), corresponding to the P module. NdhA corresponds to NuoH in *E. coli* NDH-1, which connects the Q and P modules. In addition to the 4 plastid-encoded subunits, the subcomplex A of chloroplast NDH-1 contains NdhL, M, N, and O, which are encoded in the nuclear genomes in eukaryotes (Rumeau et al. 2005; Shimizu et al. 2008). Although single-particle analysis has detected NdhL, M and N in the center of the stromal surface of subcomplex M in cyanobacteria (Fig. 4.1), genetic evidence suggests that they are components of subcomplex A in chloroplasts (Peng et al. 2011b). This is in accordance with a recent report, which re-localizes the NdhO subunit to the peripheral hydrophilic arm of cyanobacterial NDH-1 (Zhao et al. 2014). In-depth research on the assembly of subcomplex A in the stroma also provide support for this localization in *Arabidopsis* (Peng et al. 2012): (1) In the assembly intermediates of subcomplex A in the stroma, the stability of NdhK depends on NdhM, implying that there is an interaction between the two proteins. (2) NdhO forms the scaffold for further assembly with NdhH and the assembly factor CRR41, implying that NdhO also directly interacts with NdhH. (3) Finally, NdhL and NdhN are probably needed for incorporation of the peripheral arm into the proper position of the membrane arm. Thus, NdhL and NdhN should be located close to the peripheral arm. More study is needed to determine the exact positions of these subunits specific to photosynthetic NDH-1.

Although the exact positions of NdhL, M, N, and O are still unclear, 8 subunits in total form subcomplex A in chloroplast NDH-1 (Table 4.1). NdhL has 2 trans-membrane domains and was first discovered in cyanobacteria (Ogawa 1992); subsequently, its homolog with an additional trans-membrane domain was identified in *Arabidopsis* (Shimizu et al. 2008). NdhL likely interacts with NdhA, which interconnects subcomplexes A and M. NdhL is essential for NDH activity but is not required for stabilizing NDH-1 subunits in

cyanobacteria (Ogawa 1992). In contrast, the *ndhL* defect destabilizes subcomplex A in *Arabidopsis* (Shimizu et al. 2008). In *Arabidopsis*, subcomplex A is assembled in the stroma (Peng et al. 2012) as is the Q module in mitochondria (Mimaki et al. 2012). NdhL may be required for placing subcomplex A in the proper position on subcomplex M to build the L-shape. It is likely that the assembly factor CRR7 is required for this process (Peng et al. 2010) and that NdhN is incorporated into the NDH-1 complex in this step, because NdhN has not been detected—even in the final assembly intermediate—in the stroma (Peng et al. 2012). In the absence of NdhL, subcomplex A may be placed in a wrong position on subcomplex M, thereby rendering the subcomplex A subunits more susceptible to proteolytic attack in chloroplasts than in cyanobacteria. Probably reflecting this assembly step, the individual proteins of the M, L, and B subcomplexes are stable in the absence of subcomplex A and remain associated with PSI (Peng et al. 2008, 2009).

Compared with cyanobacterial NDH-1, a striking structural difference in chloroplast NDH-1 is the presence of additional B and L (lumen) subcomplexes (Table 4.1). Subcomplex B, composed of 5 protein subunits, was first believed to be specific to chloroplast NDH-1, but recently a novel NDH-1 subunit, NdhP was discovered in cyanobacteria; it shows weak similarity to PnsB4/NDF6 in the region including the trans-membrane domain (Nowaczyk et al. 2011; Ishikawa et al. 2008). Chloroplast NDH-1 may have required more accessory subunits that interact with PnsB4.

In *Arabidopsis* mutants defective in subcomplex L, the stability of subcomplex A is drastically affected, suggesting that subcomplex L interacts with NdhL, a sole subcomplex A subunit with trans-membrane domains, thereby stabilizing the entire subcomplex A (Peng et al. 2009). Unexpectedly, 3 subunits of subcomplex L are homologs of the oxygen-evolving complex of PSII. PnsL1 is a PsbP-like protein 2 (PPL2), whereas PnsL2 and PnsL3 are PsbQ-like proteins (PQLs) (Ishihara et al. 2007; Suorsa et al. 2010; Yabuta et al. 2010). On the basis of the amino acid sequence, we categorized PnsL3 into subcomplex L, but its stability is more closely related to that of subcomplex B (Yabuta et al. 2010). Most likely, PnsL3 attaches to the lumenal side of subcomplex B, possibly via interaction with PnsB4 or PnsB5, or both (Ifuku et al. 2011).

Subcomplex ED (electron-donor binding) was finally identified in a proteomics study of the NDH-1 complex in *Arabidopsis* and is composed of NdhS, T, and U (Yamamoto et al. 2011). Subcomplex ED most likely interacts with subcomplex A, but the subunit stability is largely independent of the other subcomplexes, distinguishing this subcomplex from others. Recently, NdhV was shown to be required for the stability of subcomplexes A and ED (Fan et al. 2015). NdhV is likely to be a member of subcomplex ED. Subcomplex ED forms the binding site of NDH-1 to the electron donor (Fd); this topic is discussed below in detail.

In *Arabidopsis*, chloroplast NDH-1 forms a supercomplex with PSI (Peng et al. 2008). Lhca5 and Lhca6 are minor LhcI proteins that mediate this interaction (Peng et al. 2009). Chloroplast NDH-1 has been proposed to interact with at least two copies of PSI (Peng et al. 2009), as determined by the mobility in BN gels of the supercomplexes isolated from *lhca5* and *lhca6* single mutants and an *lhca5 lhca6* double mutant. Consistent with this, single-particle electron microscopy has revealed that NDH-1 is sandwiched between two copies of the PSI-LhcI supercomplex, in which the LhcI side faces NDH-1 (Fig. 4.2; Kouřil et al. 2014). The interaction of NDH-1 with one of the two PSI copies is probably weak, because often a partial supercomplex with only one PSI copy is detected. An unassigned protein density has been observed between NDH-1 and another copy of PSI adjacent to Lhca2 and Lhca3; this area has been proposed to represent both Lhca5 and Lhca6 (Kouřil et al. 2014). However, this model cannot explain the phenotype of *lhca5* and *lhca6* single mutants simply; each mutant lacks a
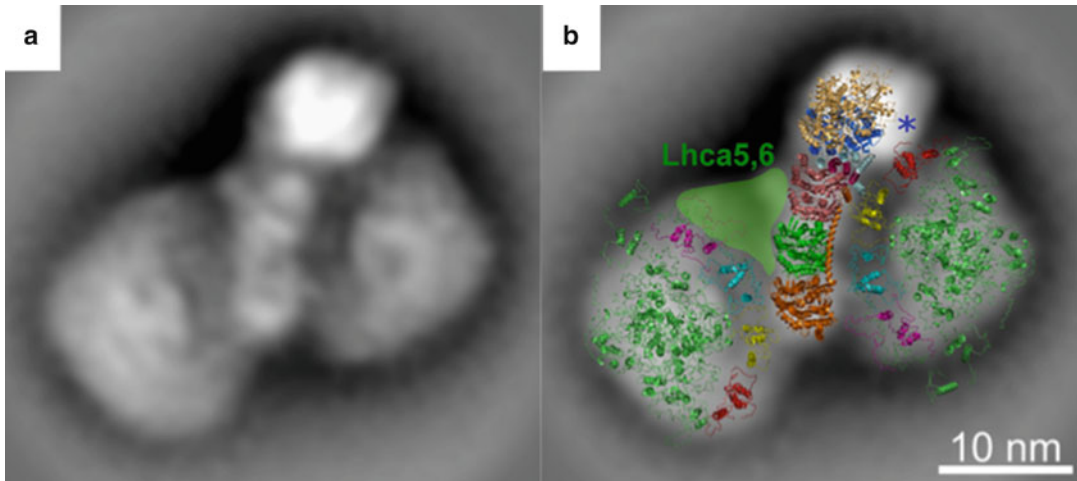
Fig. 4.2. Single-particle images of the NDH-1-PSI supercomplex (*top view*). An averaged projection map (**a**) and overlaps with the crystal structures of the PSI complex (*both sides*) and the respiratory NDH-1 (*center*) (**b**). The N module, which is missing in photosynthetic NDH-1, is omitted from the structure of respiratory NDH-1. Each PSI complex interacts with NDH-1 via the surface, composed of 4 LhcI molecules (Lhca1 to 4). The *blue asterisk* indicates an assigned density in the hydrophilic arm. The *green area* was assigned to Lhca5 and 6 in the original manuscript (Kouřil et al. 2014), but further research is needed for this to be conclusive (see text) (Pictures were kindly provided by R. Kouřil).

single copy of PSI, suggesting that Lhca5 and Lhca6 independently interact with PSI (Peng et al. 2009). The unassigned space may be for subcomplex B, which is absent in cyanobacterial NDH-1 except for PnsB4 (Ifuku et al. 2011). Because the sequence of Lhca6 is similar to that of Lhca2, one copy of Lhca2 (possibly in the weakly-associated PSI) may be substituted by Lhca6 to form the supercomplex with NDH-1. However, it is unlikely that both copies of Lhca2 are substituted by Lhca6, because Lhca2 has been detected in the NDH-PSI supercomplex (Peng et al. 2009). Because Lhca5 interacts with Lhca2 and Lhca3 in the PSI supercomplex (Lucinski et al. 2006), it is conceivable that the unassigned space includes Lhca5, as well as the subcomplex B.

V. Evolution of Chloroplast NDH-1 in Land Plants

Liverwort (*M. polymorpha*) is highlighted as an emerging model plant because of its earliest divergence in the evolution of land plants (Qiu et al. 2006). Because transformation techniques for both nuclear and plastid genomes have been established, *Marchantia* is an ideal model plant for organelle research (Chiyoda et al. 2007; Ishizaki et al. 2008). We took advantage of this available genome information to survey the nuclear-encoded genes for NDH-1 subunits (Table 4.1). In flowering plants, NDH-1 interacts with PSI to form a supercomplex (Peng et al. 2008, 2009), but the *Marchantia* genome does not encode Lhca6. Consistent with this, the NDH-1-PSI supercomplex has not been detected in BN gels (Ueda et al. 2012). The high levels of sequence identity between Lhca2 and Lhca6 suggest that Lhca6 originated from Lhca2 after gene duplication, but the origin of Lhca5 is unclear. In fact, a gene has been annotated as Lhca5 in *Physcomitrella patens* (Alboresi et al. 2010), and small amounts of NDH-1 subunits have been detected in high-molecular weight positions on BN gels (Armbruster et al. 2013). Functional analysis is necessary to conclude whether *Physcomitrella* has Lhca5. In Arabidopsis, Lhca6 plays a key role in stabilizing the NDH-1 complex (Peng et al. 2009), and
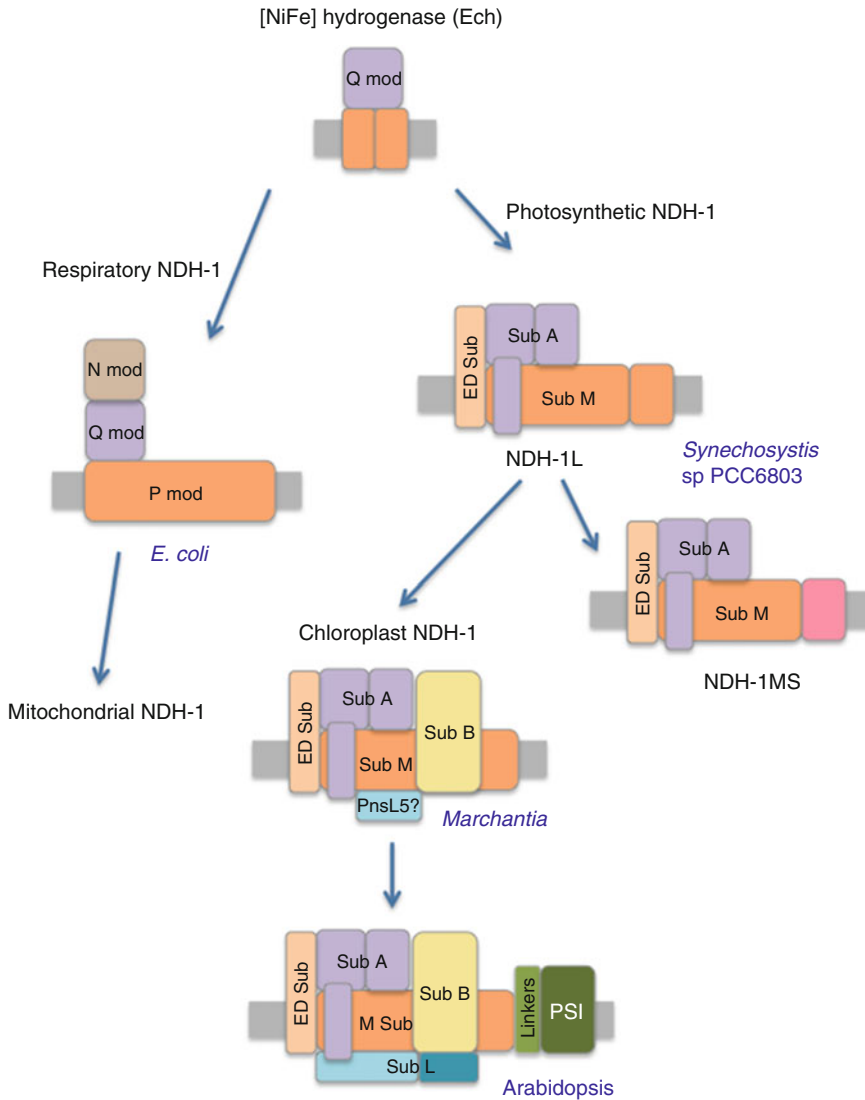
Fig. 4.3. Model for the evolution of NDH-1 complexes. Both respiratory and photosynthetic NDH-1 complexes are considered to have originated from energy-converting hydrogenase (Ech). During the evolution of respiratory NDH-1, the enzyme acquired an N module to accept electrons from NADH. Chloroplast NDH-1 originated from cyanobacterial NDH-1L and increased its number of subunits during the evolution of land plants. In flowering plants, chloroplast NDH-1 associates with PSI.

Physcomitrella is unlikely to have completed evolution in regard to flowering plant–type supercomplex formation (Fig. 4.3).

What is the physiological significance of NDH-1-PSI supercomplex formation in flowering plants? In the *Arabidopsis lhca6* mutant, NDH-1 is destabilized in mature leaves and Lhca5 has a similar function, especially at high light intensity (Peng and Shikanai 2011). Because NDH-1 activity is detected in immature leaves of the *lhca5 lhca6* double mutant, supercomplex formation is not necessary for enzyme activity (Peng et al. 2009). The phenotype of *lhca5 lhca6* is probably explained by the reduced stability of NDH-1 in chloroplasts. The question is: How is the NDH-1 complex stabilized in *Marchantia* and cyanobacteria,

in which NDH-1 does not interact with PSI? High light intensity decreases the stability of NDH-1 in the *lhca5 lhca6* double mutant (Peng et al. 2009). In flowering plants that are adapted to adverse light environments, the protein complexes in the thylakoid membrane may require an additional device for stability. The exact reason for the evolution of the supercomplex is still unclear.

The *Marchantia* genome does not encode the genes for subcomplex L, with the exception of that for PnsL5 (Ueda et al. 2012). This is also true for *Physcomitrella* (Armbruster et al. 2013). PnsL5 is an unusual subunit of chloroplast NDH-1 in *Arabidopsis* and may possess peptidyl-prolyl isomerase (PPIase) activity (Sirpiö et al. 2009). This is not specific to PnsL5. PnsL4 is FKBP (FK506-binding protein) 16–2, which has been annotated as a chloroplast-lumen targeting immunophilin (Peng et al. 2009). NdhT and U form subcomplex ED with NdhS and are J and J-like proteins, respectively (Yamamoto et al. 2011). It is unclear why chloroplast NDH-1 incorporated these proteins, which may be involved in protein folding, as subunits. These subunits may retain the original enzyme activity in the complex and may be necessary for the assembly of NDH-1. In *Marchantia*, the PnsL5 homolog may be conserved as PPIase and may not be a genuine NDH-1 subunit. This is likely, because the stability of PnsL5 in *Arabidopsis* depends on other subunits of the subcomplex L (Peng et al. 2009).

Although it is unclear whether PnsL5 is an NDH-1 subunit in *Marchantia*, the entire subcomplex L was likely acquired during the evolution of land plants. In *Arabidopsis*, the mutant phenotype suggests that subcomplex L stabilizes subcomplex A (Peng et al. 2009). However, NDH-1 lacks subcomplex L in *Marchantia* and cyanobacteria, suggesting that this straightforward hypothesis based simply on the mutant phenotype is unlikely to explain the function of subcomplex L in the wild type. The occurrence of the subcomplex L coincides with supercomplex formation

with PSI (Fig. 4.3), and this may be the reason for the evolution of subcomplex L.

The exact reason for the structural difference in chloroplast NDH-1 between *Marchantia* and flowering plants is unclear, but we can extend our speculation a little further. Both the full subcomplex L and supercomplex formation with PSI may be required for stabilizing NDH-1 in the chloroplasts of flowering plants. To test this hypothesis, a straightforward strategy would be the introduction of *Marchantia*-type or cyanobacterial-type NDH-1 into chloroplasts of flowering plants. However, our technology is still premature for handling such huge multi-subunit complexes in transgenic research. Chloroplasts are the sites of reactive oxygen generation, and the thylakoid lumen is acidified to less than pH 6.0 to induce NPQ and downregulation of the cytochrome b_6f complex. Assembly and stabilization of the protein complexes in the thylakoid membrane may have required additional machineries in the course of land plant evolution. As described above, a defect in *ndhL* has a different impact on the stability of subcomplex A in cyanobacteria and flowering plants (Ogawa 1992; Shimizu et al. 2008). This structural adaptation might have been particularly important because of the concomitant disappearance of some photoprotective pathways in the course of evolution. One example is the disappearance of flavodiiron proteins in angiosperms, suggesting that switching of the systems regulating photosynthetic electron transport occurred during the evolution of land plants (Allahverdiyeva et al. 2013; Zhang et al. 2012). On the other hand, more assembly factors were recruited to photosynthetic NDH-1 in the course of land plant evolution. In *Arabidopsis*, the assembly of subcomplex A requires the non-subunit factors CRR41 and CRR42, the functions of which are not conserved in cyanobacteria (Peng et al. 2012). In *Marchantia* chloroplasts, NdhH is folded properly with the assistance of the Cpn60 complex. In *Arabidopsis*, however, the evolution of an unusual β subunit (Cpn60β4) was necessary for the folding

of NdhH, although the NdhH sequence is highly conserved between *Marchantia* and *Arabidopsis* (Peng et al. 2011a). We speculate that adaptation to the severe light conditions of terrestrial environments required the more robust assembly, or a stabilizing system, of protein complexes in the thylakoid membrane.

VI. Physiological Reason for Evolutionary Increase in Complexity of Chloroplast NDH-1

How does the structural difference in chloroplast NDH-1 between flowering plants and *Marchantia* reflect differences in physiological function? As in flowering plants, knockout of *ndhB* does not affect the growth of thalli in *Marchantia* (Ueda et al. 2012). Consistently, photosynthetic electron transport is not drastically affected in the mutants. Similar to the case in flowering plants, chloroplast NDH-1 is unlikely to mediate the main route of electrons that contributes substantially to pmf formation in *Marchantia*. At low light intensity, however, the PQ pool is slightly more reduced by electrons in the *ndhB* mutant, suggesting that chloroplast NDH-1 is required for redox homeostasis in *Marchantia* chloroplasts. A similar phenotype has been observed in rice NDH-1-deficient mutants (Yamori et al. 2011). The contribution of NDH-1 to pmf is not negligible, at least in these plants at low light intensity. Actually, we observed a minor but statistically significant decline in pmf even in the Arabidopsis NDH-less mutants (Wang et al. 2015). The difference in mutant phenotypes among plant materials may depend on the activity of other, alternative, electron transport pathways, including the water-water cycle. The physiological function of NDH-1 has been analyzed by focusing on stress tolerance (Endo et al. 1999; Horváth et al. 2000), and it is conceivable that chloroplast NDH-1 is involved in redox homeostasis in chloroplasts under various stress conditions and may even

be involved in the plant immune response (García-Andrade et al. 2013). Fine-tuning of the chloroplast redox state may be necessary for plant fitness. Despite the obscure physiological function of chloroplast NDH-1, its molecular function is clear: Chloroplast NDH-1 couples electron transport from Fd to PQ with proton/cation translocation across the thylakoid membrane. The direct consequence of an NDH-1 defect should be reduced pmf, as in the *Arabidopsis pgr5* mutant. In flowering plants, PGR5-dependent PSI cyclic electron transport is essential for survival under field conditions, through ATP synthesis and also through regulatory processes via lumenal acidification (Suorsa et al. 2012; Shikanai 2014). Indeed, both *Marchantia* and flowering plants possess at least two pathways of PSI cyclic electron transport, which differ substantially in terms of rates of electron transport. A central question is how plants selectively use the two pathways of PSI cyclic electron transport.

C_4 photosynthesis requires additional ATP for recycling phosphoenolpyruvate, and this ATP is believed to be synthesized by PSI cyclic electron transport (Nakamura et al. 2013). Bundle sheath cells of maize contain higher levels of NDH-1 than do mesophyll cells, suggesting that some C_4 plants utilize NDH-1-dependent PSI cyclic electron transport to energize the CO_2-concentrating machinery (Takabayashi et al. 2005). Chloroplast NDH-1 is likely to have proton-pumping activity; this machinery seems to be superior to that of the PGR5/PGRL1 complex for pmf formation. In C_3 plants, however, $\triangle pH$ formation is required not only for ATP synthesis but also for regulation of photosynthesis, NPQ induction (Munekage et al. 2002), and downregulation of the cytochrome b_6f complex (Suorsa et al. 2012). It is likely that, for these purposes, the PGR5-dependent pathway was selected over chloroplast NDH-1. Bundle sheath cells of maize contain only small amounts of PSII, and regulation of photosynthesis in these plants via low lumen pH is probably not necessary. In *Marchantia*, NDH-1—rather than the main machinery

for pmf formation—may have a regulatory function; this is probably true also for C_3 plants.

VII. The Origin of Photosynthetic NDH-1

Among the protein complexes related to NDH-1, cyanobacterial and chloroplast (photosynthetic) NDH-1 is unique because of the absence of the N module. In *E. coli* NDH-1, the N module consists of NuoE, F, and G and functions in oxidizing NADH (Friedrich and Scheide 2000). Because, in cyanobacteria and higher plants, despite complete genome sequencing of nearly 100 different oxygenic photosynthetic organisms, no orthologs of *E. coli* N module subunits have been identified, a completely different electron input module for photosynthetic NDH-1 has started to emerge. Indeed, for decades the nature of the electron donors for photosynthetic NDH-1 has raised confusion, and conflicting results have been reported from physiological and biochemical studies. In *Arabidopsis*, NDH-1-dependent PQ reduction is strictly dependent on Fd (Munekage et al. 2004) when ruptured chloroplasts are used as an assay system, but NAD(P)H has also been shown to donate electrons to the same complex in spinach (Endo et al. 1997), potato (Corneille et al. 1998), and barley (Bernhard Teicher and Vibe Scheller 1998). A purified NDH complex from pea chloroplasts also showed NADH dehydrogenase activity (Sazanov et al. 1998), although the purified complex apparently lacked many subunits. The dilemma regarding identification of the various functional electron donors for photosynthetic NDH-1 is at least partly due to the fragile nature of the complex. It is also possible that soluble or membrane-bound FNR has contributed to NAD(P)H dehydrogenase activity (Guedeney et al. 1996). More problematically, there has been no consensus among researchers on how to measure photosynthetic NDH-1 activity: The conclusions drawn are fully dependent on the methods used (Johnson 2005; Leister and Shikanai 2013).

Sequence identity between NDH-1 and [NiFe] hydrogenase was discovered more than two decades ago (Böhm et al. 1990) and led to the idea that the two enzymes share a common ancestor (Friedrich and Weiss 1997; Friedrich and Scheide 2000). Photosynthetic NDH-1 especially shares structural similarity with group 4 membrane-bound [NiFe] hydrogenase (Fig. 4.3; Efremov and Sazanov 2012; Marreiros et al. 2013). In this group of hydrogenases, the Q module directly accepts electrons from Fd to reduce protons (Marreiros et al. 2013). Independent of conflicting physiological and biochemical analyses, this sequence identity suggests that photosynthetic NDH-1 accepts electrons from Fd.

NdhS/CRR31 is a subunit of chloroplast NDH-1 discovered in the NDH-1-PSI supercomplex of *Arabidopsis* (Yamamoto et al. 2011). The Src homology 3 domain-like fold of NdhS is structurally similar to that of PsaE, which forms the Fd-binding site in PSI, as well as to that of PsaC (Sétif et al. 2002; Ben-Shem et al. 2003). In ruptured chloroplasts, NdhS is required for high-affinity binding of Fd to chloroplast NDH-1 in order to reduce PQ; NdhS is also required for NDH-1 activity *in vivo* (Yamamoto et al. 2011). As in the case of other Fd-interacting proteins, binding of Fd to NdhS also depends on electrostatic interaction. Arginine 193 of NdhS is required for the formation of a positively-charged pocket in the domain; this pocket is required for high-affinity Fd binding (Yamamoto and Shikanai 2013). This function of NdhS is conserved in cyanobacterial NDH-1 (Battchikova et al. 2011b), suggesting that NDH-1 accepts electrons from Fd also in cyanobacteria.

VIII. Concluding Remarks

Since the discovery of 11 *ndh* genes in chloroplast genomes (Matsubayashi et al. 1987), our knowledge of this enigmatic enzyme has progressively improved. However,

several fundamental questions related to its activity and physiological function still remain. Some of these questions are common to all photosynthetic NDH-1 complexes, from cyanobacteria to flowering plants, whereas some are related to changes that have occurred in the course of evolution of the land plants. If photosynthetic NDH-1 accepts electrons directly from Fd, how are electrons transferred from Fd to the iron-sulfur cluster present in NdhI? Are some subunits still missing from our models? Photosynthetic NDH-1 is considered to possess proton-pumping activity, but the experimental evidence is totally missing. It has become clear that regulation of pmf components ($\triangle$pH and membrane potential) is important, because both $\triangle$pH and membrane potential contribute to pmf but only $\triangle$pH triggers the regulation of photosynthesis (by induction of NPQ, at least in chloroplasts, and down-regulation of the cytochrome b_6f complex) (Carraretto et al. 2013). Is it possible that photosynthetic NDH-1 pumps cations rather than protons? Hopefully the answers will be found at the level of the crystal structure. What is the function of cyanobacterial NDH-1 in the light and in darkness? Does the function in light of photosynthetic NDH-1 in cyanobacteria differ from that in chloroplasts of flowering plants? What is the exact reason for the supercomplex formation with PSI in flowering plants and for the appearance of the B and L subcomplexes during the evolution of land plants? Despite the low levels of NDH-1 in chloroplasts, genetic approaches have identified many assembly factors specifically required for chloroplast NDH-1 (Peng et al. 2010, 2011a, b, 2012; Armbruster et al. 2013). Chloroplast NDH-1 can be an excellent model for the assembly of huge protein complexes, the subunits of which are encoded by both nuclear and chloroplast genomes. The exact physiological function of chloroplast NDH-1 is also unclear. We have to bridge the gap between the slow rate of NDH-1-dependent electron transport observed *in vivo* and the drastic phenotypes of *Arabidopsis* double mutants defective in both NDH-1 and PGR5

(Munekage et al. 2004). The central question is the physiological function of chloroplast NDH-1 in the wild type.

Acknowledgements

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Part II

Theoretical Aspects of Electron Transfer

Fundamentals of Electron Transfer
in Proteins

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Summary

An outline of the Marcus–Levich–Dogonadze theory of charge transfer reactions in polar media is given with an emphasis on the physical sense of its concepts rather than on mathematical details. The reorganization energy has been considered as well as the dependence of the activation energy on this parameter and on the reaction energy. The quantum—mechanical treatment of the pre-exponential factor is described, and the regularities of the non-adiabatic and adiabatic electron transfer are qualitatively explained. Regarding intraprotein electron transfer, the electrostatic interaction of a moving charge with its surroundings, primarily with protein and water, is the major factor determining the thermodynamics and kinetics involved. A specific feature of protein structure is the presence of a high concentration of strongly polar groups, mainly of peptide groups. Tight packing of these dipoles inside certain structures substantially restricts their ability to change orientation in an external electric field. This has two consequences: (i) a low static dielectric permittivity (i.e., dielectric constant) of proteins and (ii) the presence of a substantial permanent intraprotein electric field pre-existent before charging of the active site, e.g., redox center. Therefore, the internal environment of proteins can be defined as a pre-organized highly-polar, low-dielectric medium. These specific protein

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features are important for acceleration of electron transfer reactions. The semi-continuum electrostatics approach, viz. description of a protein as a set of point atomic charges in a continuum dielectric is presented. The specificity of quantitative calculations in the framework of this approach of all the parameters determining the activation energy has been considered. It is shown that the intraprotein pre-existing field should be calculated employing the protein's optical permittivity, while for the dielectric response energy one should use the static quantity (in both cases, the static dielectric constant of water). The reorganization energy of a protein does not depend on the pre-existing field. The reorganization energies calculated by microscopic simulations and by semi-continuum electrostatics (including quantum—chemical data) are in reasonable agreement with each other and with experiment. The mechanism of long-range electron transfer is discussed.

I. Introduction

A great many biochemical processes are essentially electron transfer reactions, i.e., the processes involving transfer of charged particles. The purpose of this chapter is to provide a brief summary of the general theory of charge transfer in condensed polar media, and to discuss the consequences of this theory applied to biochemical processes, namely, charge transfer reactions in the specific polar medium of a protein. Accordingly, the chapter consists of two main parts. The first aims at presenting the physical sense of the theory with an emphasis on the qualitative understanding of its main concepts rather than on the rigorous deduction of its equations, (including the most important formulae). The second part considers the charge transfer reactions in proteins. Its main goal is to show how the general architectural principles of protein structure are reflected in their behavior as media of charge transfer.

There exists a vast literature on the topic that is impossible to cover completely. Therefore, in many cases, I will cite reviews, the original papers in which some specific problem was first considered, and/or some more recent publications where the reader can find a more detailed bibliography.

II. The General Theory of Charge Transfer Reactions in Polar Media: An Outline

The foundations of the theory in the framework of the semi-classical approach have been laid by Marcus (1956) (Nobel Prize, 1992), and the rigorous quantum—mechanical theory was first developed by Levich and Dogonadze (1959). The theory has been reviewed many times, and for a more detailed description the reader is referred to representative manuscripts and books (Dogonadze and Kuznetsov 1975, 1983; Ulstrup 1979; Marcus and Sutin 1985; Krishtalik 1986a; Kuznetsov et al. 1988; Marcus 1996; Kuznetsov 1995, 1999; Kuznetsov and Ulstrup 1999).

A. Electron Transfer and Charge: Medium Interaction, Activation and Reorganization Energies

Let us consider electron transfer from A to B

$$A^- + B = A + B^-$$

Abbreviations: bpy – Bipyridine; D – Debye the unit of dipole moment; DMF – Dimethylformamide; ESP – Electrostatic potential; HOMO – Highest occupied molecular orbital; im – Imidazole; LUMO – Lowest unoccupied molecular orbital; MD – Molecular dynamics; MM – Molecular mechanics; py – Pyridine; SHE – Standard hydrogen electrode; QM – Quantum mechanics; WT – Wild type
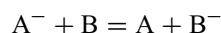
Any charge in a polar medium causes orientation of its dipoles. The orientation of many dipoles results in medium polarization. In subsequent figures, this polarization is described by the coordinate q. The energy of the charge-polarization interaction is an important constituent of the system's free energy, G. The optimum dipole orientation corresponds to the equilibrium state having a minimal energy. Any deviation from this initial optimum polarization q_o caused by thermal fluctuations of dipole orientations results in an increase in energy. As the dielectric polarization responds linearly to the electric field in the absence of dielectric saturation, the energy depends quadradically on polarization, i.e., the energy as a function of q, is a parabola (the polarization q serves as

the reaction coordinate in the framework of the continuum electrostatic description.

In the left panel of Fig. 5.1, curve 1 represents the dependence of the system free energy on the medium polarization for the initial state (electron residing on A). The corresponding equilibrium polarization is q_o^i. When charge is transferred from one reactant to another (from A to B), a new final equilibrium polarization (q_o^f) and a corresponding new equilibrium energy appears, which is described by another energy curve (curve 2, Fig. 5.1, left panel). The energy difference at the minima of these two curves is equal to the reaction free energy, ΔG_0.

It should be mentioned that the quadratic dependence of energy on the reaction coordinate was also obtained by molecular
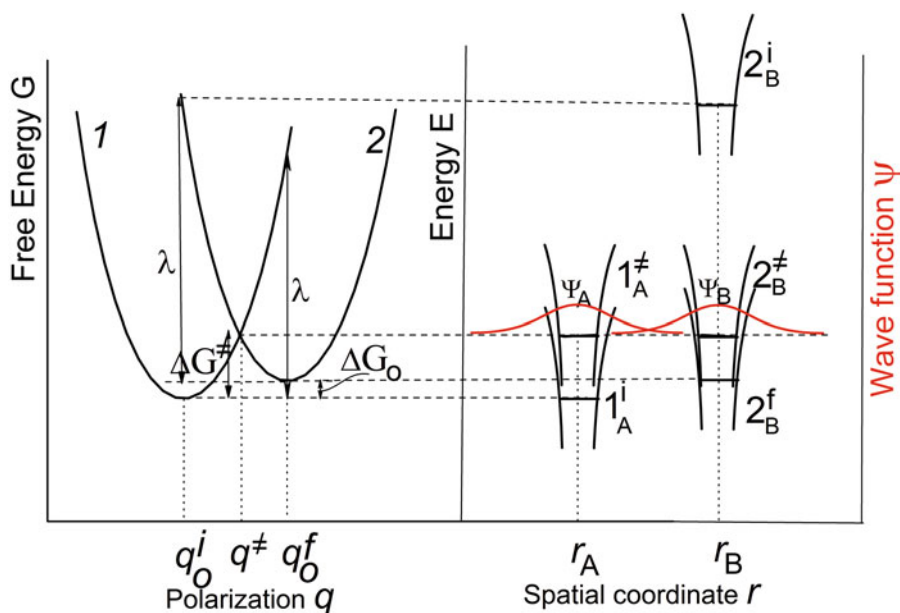
Fig. 5.1. Energy diagrams for the electron transfer process. *Left panel*: System free energy (G) dependence on the medium polarization q. Curve 1 corresponds to the initial state (electron residing on A), curve 2 to the final one (electron on B). The polarizations q_o^i and q_o^f are the equilibrium values for initial and final states, polarization $q^{\neq}$ relates to the intersection point of curves 1 and 2, i.e., to transition state. ΔG_0 is the equilibrium reaction free energy, and $\Delta G^{\neq}$ the activation energy. *Right panel*: The intra-atom electron potential energy dependence on its spatial coordinate r (*black curves*). Curves 1_A correspond to the electron residing on A, curves 2_B—on B. Superscript i means that the polarization coordinate equals to q_o^i, superscript f relates to polarization q_o^f, superscript $\neq$ — to $q^{\neq}$. *Horizontal bars* inside the electronic wells show the electron energy levels, occupied for A, and empty for B. The states with equal energies in the *left* and *right* panels are connected by *thin horizontal dashed lines*. The wave functions of electron on A or B (ψ_A and ψ_B) are represented as *red curves*.

dynamics simulations for some concrete systems (e.g., Yadav et al. 1991; Simonson 2002; see also Sect. V). Hence, the microscopic simulations support the continuum dielectric description.

The potential energy curves for electron as a function of the spatial coordinate r are represented (Fig. 5.1, right panel). The potential wells for the electron in the atom or an atomic group are approximately hyperbolic. Curve $1_A{}^i$ relates to the initial state (electron residing on A); curve $2_B{}^f$—to the final state (electron on B). The electron energy levels shown here (horizontal bars in the potential wells described by curves $1_A{}^i$ and $2_B{}^f$) relate to an equilibrium, i.e., they are at the same energy as the minima of the curves 1 and 2 at the left panel (the equivalent states in the left and right panels are connected by dashed horizontal lines).

Let us consider now the process of the electron transfer from A to B. At the dipole coordinates corresponding to the equilibrium initial state $(q_o{}^i)$, the electron energy level is shown at the curve 1_A^i (right panel) when the electron is on A, and at curve 2_B^i when it is on B. The energy of the system with the electron on B at dipole coordinates $q_o{}^i$ is described by curve 2, left panel. Due to the great difference in masses, the rate of the electron movement is many orders of magnitude faster than that of the heavy particles (dipoles) movement. Therefore, the electron transfer should proceed at practically constant coordinates of heavy particles (nuclei), and the latter can be considered as practically immobile in the course of this process. This very important statement is called the Franck-Condon principle. We see that, at the constant coordinate $q_o{}^i$, the electron levels in the initial and final states are quite different, and hence the electron cannot jump from one reactant to the other without an input of energy from an external source. In this system, there are no other energy sources except thermal motion of the molecules, but molecular motion is too slow in comparison to electron movement. Therefore, the electron cannot borrow energy from this source. Thus, electron transfer at the equilibrium

polarization $q_o i$ is impossible as it contradicts the law of energy conservation.

The situation changes when, as a result of relatively slow thermal fluctuation of dipole orientation, the system comes to the intersection point of the potential curves of the initial and final states (Fig. 5.1, left panel, $q^{\neq}$). At this coordinate, the energy levels of the electron at A and B become equal (curves $1_A^{\neq}$ and $2_B^{\neq}$, right panel) and electron transfer becomes possible. Thereby, this electron transition should be preceded by a thermal activation bringing the system to the top of the barrier. This barrier is called the Franck-Condon barrier. The probability of the electron transition from A to B at the top of the barrier will be considered in the next Sect. (II.B).

The height of the barrier (activation energy $\Delta G^{\neq}$) is determined by the equilibrium energy gap between two states, ΔG_o, and the shape of the energy curves. In the case of dielectric polarization of the medium, only the positions of the charge in the initial and final states are different, but not its interaction with the medium; therefore, the two parabolic curves 1 and 2 have the same curvature. Their shape can be characterized by the quantity called the reorganization energy, λ. As shown in Fig. 5.1, λ is the energy necessary to bring the system from the initial to final equilibrium polarization (coordinates, $q_o{}^i$ and $q_o{}^f$) under the condition that electron remains in the initial configuration, i.e., resides on A; this means that the system reaches the final polarization $q_o{}^f$ remaining on curve 1. As shown in Fig. 5.1, the same quantity, λ, corresponds to charge transfer in the opposite direction, from $q_o{}^f$ to $q_o{}^i$. The activation energy, $\Delta G^{\neq}$ is the difference between the initial equilibrium energy and the energy at the intersection point of the two parabolae. The corresponding formula (the Marcus equation; Marcus 1956) is

$$\Delta G^{\neq} = \frac{(\lambda_s + \Delta G_o)^2}{4\lambda_S} \qquad (5.1)$$

The subscript "s" here means that λ relates to reorganization of the medium (solvent).

In principle, reorganization of coordinates of other heavy particles is possible, e.g., change of the covalent bonds lengths inside a complex ion (inner—sphere reorganization, λ_i, as opposed to the outer—sphere reorganization, λ_s). In that case, Eq. 5.1 should involve the sum of all reorganization energies (at the strongly asymmetric potential curves of the inner coordinates, λ_i's for the two reaction directions are non-equivalent; in this case, Eq. 5.1 takes a more complex form; however, when these two λ_i's are not too different (e.g., by more than a factor of two), and markedly smaller than λ_S, one can use their arithmetic mean.

Figure 5.1 presents a one-dimensional scheme of the potential energy curves. The polarization coordinate q involves coordinates (degrees of freedom) of a great many dipoles, and hence the real picture is a multi-dimensional energy surface. Averaging statistically over all degrees of freedom gives the free energies that enter Eq. 5.1.

As defined above, the reorganization energy, λ_s, is the energy necessary to change the polarization from $q_o{}^i$ to $q_o{}^f$ with the electron remaining at its initial position. This polarization is due to the orientation of dipoles, i.e., of heavy particles moving slowly relative to electron movement. This kind of polarization is called 'inertial polarization' as opposed to the inertia-less electronic polarization of the medium (the electrons of the medium follow instantaneously the movement of the electron being transferred, and hence electronic polarization does not present any obstacle for electron transfer).

To clarify the physical sense of the reorganization energy, we will calculate the value of λ_s following the Marcus scheme. At the first step, we will find the equilibrium energy of a slow charge transfer from A to B in some medium with the static dielectric constant (dielectric permittivity), ε_s. This static constant reflects the total dielectric response of inertial and inertia-less polarization. The second step is a very fast charge transfer backwards from B to A. This transfer is so fast that only electronic polarization (described by the optical dielectric constant ε_o) has time to adapt itself to the new charge position. Therefore, the inertial polarization remains the same as after the first step. Therefore, as the result of this two-step process, we have the system with the initial charge distribution and with the inertial polarization corresponding to the final charge position. The total energy of this process is nothing else than the reorganization energy. Let us perform these calculations using the simple model of spherical conducting spheres with radii a_A and a_B.

The electron transfer from A to B $(A + B \rightarrow A^+ + B^-)$ can be described as the charging of A by the positive charge $+e$ and, simultaneously, charging of B by $-e$ (e is the unit electron charge). At some moment in the course of the charging process, a charge $+q$ resides on A $(0 \leq q \leq e)$ and $-q$ resides on B; then, we add $+dq$ to A and $-dq$ to B. The additional charge $+dq$ is distributed on the surface of A, and is subject to the potential

$$\varphi_A^s = +q/\varepsilon_s a_A - q/\varepsilon_s R \qquad (5.2)$$

The first term is due to the charge of A itself, and the second is the potential set up by the charge of B (R is the distance between centers of A and B). A similar expression describes the potential at B.

$$\varphi_B^s = -q/\varepsilon_s a_B + q/\varepsilon_s R \qquad (5.3)$$

The work of bringing $+dq$ to A is $+\varphi_A dq$, and the corresponding quantity for $-dq$ is $-\varphi_B dq$, and the total work of the first step of the charging process is obtained by integration over q from 0 to **e**

$$W_I = W_s = \int_0^e \left(\frac{1}{a_A} + \frac{1}{a_B} - \frac{2}{R} \right) \frac{1}{\varepsilon_s} q dq$$

$$= \frac{e^2}{\varepsilon_s} \left(\frac{1}{2a_A} + \frac{1}{2a_B} - \frac{1}{R} \right) \qquad (5.4)$$

The designation W_s stresses that the process considered is charging in a "static" medium characterized by dielectric constant ε_s.

Now we shall calculate the charging energy for the second step, i.e., the charging of A by $-e$, and charging of B by $+e$ leaving both reactants uncharged. The situation differs from the first step because the potentials on A and B are not zero before the new charging process, as in the first step, but have some constant values corresponding to the final state after the first charging:

$$\varphi_A^{sf} = +e/\varepsilon_s a_A - e/\varepsilon_s R \qquad (5.5)$$

$$\varphi_B^{sf} = -e/\varepsilon_s a_B + e/\varepsilon_s R \qquad (5.6)$$

The contribution of this field to the charging energy is:

$$W_{sf} = \int_0^e \left(\varphi_B^{sf} dq - \varphi_A^{sf} dq \right)$$

$$= -\frac{e^2}{\varepsilon_s} \left(\frac{1}{a_A} + \frac{1}{a_B} - \frac{2}{R} \right) \qquad (5.7)$$

Note that $W_{sf} = -2\, W_s$. Now we calculate the contribution to the charging energy due to polarization of the "optical" medium (with ε_o) by the charge added. The calculation is quite analogous to the calculation of W_s (in Eqs. 5.2, 5.3 and 5.4; one should exchange lower indices A and B, and substitute ε_o for ε_s):

$$W_o = \int_0^e \left(\frac{1}{a_A} + \frac{1}{a_B} - \frac{2}{R} \right) \frac{1}{\varepsilon_o} q dq$$

$$= \frac{e^2}{\varepsilon_o} \left(\frac{1}{2a_A} + \frac{1}{2a_B} - \frac{1}{R} \right) \qquad (5.8)$$

The total work of the second step is

$$W_{II} = W_{sf} + W_o \qquad (5.9)$$

Correspondingly, the reorganization energy is

$$\lambda_s = W_I + W_{II}$$

$$= e^2 \left(\frac{1}{\varepsilon_o} - \frac{1}{\varepsilon_s} \right) \left(\frac{1}{2a_A} + \frac{1}{2a_B} - \frac{1}{R} \right)$$

$$(5.10)$$

For the sake of simplicity, Eq. 5.10 was derived for the initially uncharged reactants. It can be shown that it is valid for any initial charges of reactants because the contribution due to these initial fields cancels out.

Equation 5.10 defines the medium reorganization energy as function of properties both of the reaction complex and of the medium. So, it cannot be ascribed to some reaction in any medium or to some medium for any reaction, but only to a definite reaction in a definite medium. The reaction complex is described by the geometric factor (second parentheses), and the medium properties are represented by the difference of reciprocals of the optical and static dielectric constants. The last quantity is called the coupling constant (it determines coupling of charge movement with the inertial polarization) or the Pekar parameter (it was first introduced by Pekar in the polaron theory; Pekar 1954).

The optical permittivity ε_o corresponds to the medium polarization that follows inertialess the electron transfer. First, it is a purely electronic polarization, and it can be calculated as $\varepsilon_o = n^2$, where n is the refraction index for visible light. However, in some media, other vibrational modes can be present that are fast enough to follow the movement of the electron to be transferred. This effect is expressed especially in water due, for instance, to the high–frequency vibrations of O-H bonds. This results in a frequency dependence of the permittivity up to the frequency of the "quantum limit" kT/h (on the "quantum limit", see Sect. II.B). This effectively causes an increase in ε_o and a corresponding decrease in reorganization and activation energies. However, the influence of this effect on the reaction rate is smaller than one can expect on the basis of the decrease in activation energy: the shift of atoms at frequencies higher than kT/h proceeds via tunneling, and the rate decreases by factor $\exp(-\sigma)$ where σ is the medium tunneling factor. On the whole, the effect of the medium quantum vibrations is difficult to estimate quantitatively (Kuznetsov 1955, 1999).

Inspection of Eq. 5.10 shows that it can be rewritten as

$$\lambda_s = W_o - W_s \qquad (5.11)$$

Equation 5.10 was derived for a simple model of two spherical reactants in a homogeneous medium. It can be shown that Eq. 5.9 has a quite general character and is valid for any geometry of reactants, their internal charge density distribution, and an inhomogeneous medium. Its application demands a proper calculation of charging energies, i.e., for a solution of the corresponding electrostatic problem; in the case of complex geometries, e.g., redox centers inside a protein globule, this solution can be achieved by numerical methods.

Let us turn again to Eq. 5.1. The reaction activation energy $\Delta G^{\neq}$ depends on the reaction free energy, ΔG_o. With increasing driving force (i.e., ΔG_o more negative), the activation energy decreases. However, this decrease takes place only until ΔG_o becomes equal to $-\lambda$. At this point, the activation energy becomes zero (activationless process; see scheme in Fig. 5.2, intersection of curves 1 and 2), and the reaction rate achieves its maximal possible value. At more negative values of ΔG, in the so-called "inverted region," the activation energy increases with the increasing driving force (intersection of curves 1 and 3, Fig. 5.1. Curve 3 corresponds to an equilibrium energy $\Delta G_1 < \Delta G_o$, and the activation energy $\Delta G_1^{\neq} > 0$). Due to the quadratic form of Eq. 5.1, the dependence of the activation energy $\Delta G^{\neq}$ on ΔG_o has a symmetric parabolic form with a minimum ($\Delta G^{\neq} = 0$) at $\Delta G_o = -\lambda$ (the corresponding dependence for the rate constant has a maximum). However, the experimental dependencies are asymmetric—the decrease in the rate constant at high driving forces is markedly slower than its increase at lower absolute values of ΔG_o. The explanation of such behavior lies in the involvement in the process of the vibrationally excited states of the products. Eq. 5.1 relates to both initial reactants and final products being in their
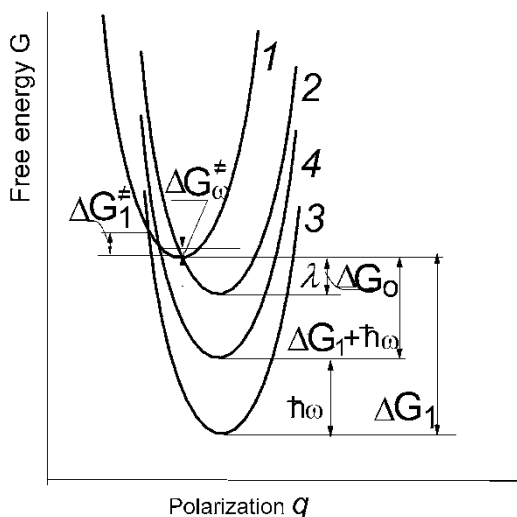
Fig. 5.2. Free energy diagrams for activationless and inverted regions. System free energy dependence on the medium polarization q. Curve 1—initial state, curves 2–4—different final states, Intersection of curves 1 and 2 at the minimum of curve 1 ($\lambda = -\Delta G_0$) results in activation energy equal to zero (activationless process). At more negative free energy ($-\Delta G_1 > \lambda$, curve 3) activation energy ($\Delta G_1^{\neq}$) increases. When in the final state, a vibrationally–excited level of the product is involved (curve 4), its energy becomes higher by the value of the vibrational quantum $\hbar\omega$ (the reaction free energy ($\Delta G_1 + \hbar\omega$) becomes less negative). Correspondingly, the activation energy $\Delta G_\omega^{\neq} < \Delta G_1^{\neq}$. The situation described by curves 3 and 4 is called the "inverted region".

ground states. This is the most favorable pathway before the reaction rate maximum. However, at very negative ΔG's, formation of a vibrationally-excited product decreases the absolute value of ΔG by the energy of the vibration quantum, $\hbar\omega$ ($\Delta G_1 + \hbar\omega$) decreasing the activation energy $\Delta G_\omega^{\neq}$ (intersection of curves 1 and 4 in Fig. 5.2). For some products, more than one vibrational state, and even an electronically excited state, may be involved.

Equation 5.1 describes the activation free energy inside a reaction complex having some optimal configuration of reactants. Often, some work (W_i) associated with approach of reactants must be expended to achieve this optimum configuration (inter-reactant distances, and orientation). In this case,

$$\Delta G^{\neq} = W_i + \frac{(\lambda_s + \Delta G)^2}{4\lambda_s} \qquad (5.12)$$

Here ΔG is the free energy of the elementary act of reaction inside the reaction complex; it differs from the reaction free energy for the separated reactants ΔG_o by the difference of the energies of approach for initial reactants and final products

$$\Delta G = \Delta G_o + W_f - W_i \qquad (5.13)$$

The quantity ΔG_o in Eqs. 5.1 and 5.13 refers to the process inside the reaction complex, According to its physical sense, it is independent of the presence of the reactants outside the complex, and of their concentration in the bulk solution. This concentration—independent quantity is called the configurational free energy ΔG_c which depends on the configuration of the reactants but not on their concentration. It differs from the total free energy of reaction by the difference of the free energies of mixing of initial and final reactants with the solvent:

$$\Delta G_c = \Delta G_o + RT \sum_1^n \ln X_{oi} - RT \sum_1^m \ln X_{of} \qquad (5.14)$$

Here ΔG_o is the standard free energy of the reaction in solution, and X_{oi} and X_{of} are the molar fractions of n initial and m final reactants in solution in their standard states. The origin of the last two terms in this equation is purely entropic: a decrease in the fraction of some substance increases the number of transpositions of its molecules with other molecular species (normalized to one mole of this substance), and hence increases the probability of the state, i.e., increases the molar entropy.

Equation 5.12 allows one to calculate ΔG_c, which should enter Eqs. 5.1 and 5.13 as ΔG_o, when the standard free energy is known. If the standard states of all reactants are the same (e.g., 1 M), and the number of molecules does not change upon the reaction

($n = m$), the last two terms in Eq. 5.11 cancel out, and $\Delta G_c = \Delta G_o$. That means that the free energy expenditure necessary to take the dissolved reactants from the bulk and gather them into the reaction complex (the demixing process that is accompanied by a decrease in entropy) is exactly compensated for by the free energy of mixing of the products with solution. In other, also frequently occurring cases, the correction according to Eq. 5.12 is large, and thereby substantially influences the reaction equilibrium and rate (Krishtalik 1986b).

B. Underbarrier Transfer (Quantum—Mechanical Tunneling): Adiabatic and Non-adiabatic Transitions

The electron transfer from the initial to the final position is described as an underbarrier transition (quantum—mechanical tunneling). For any particle, there are two possible pathways of transition—classical 'overbarrier' and quantum—mechanical 'underbarrier'. In the case that the distance between two neighboring energy levels (for harmonic oscillator $\hbar\omega$) is smaller than the average energy of thermal fluctuations, kT, the predominant pathway is the classical overbarrier transition. In the opposite case ($\hbar\omega > kT$) the particle transfer proceeds through a quantum—mechanical underbarrier pathway. Let us qualitatively describe how this underbarrier transition proceeds.

For the particle behaving quantum–mechanically, its state is described by the wave function ψ, and this function is not confined in the space inside the well, but protrudes outside it; in the "outer space" it decays approximately exponentially with distance. The probability of finding the particle at some position is determined by its wave function, so it is not zero outside the well. With two wells not too far from each other, the wave functions corresponding to an electron in wells A and B (ψ_A and ψ_B, red curves in Fig. 5.1) overlap. In the region of this overlap, the electron belongs simultaneously to both atoms—it has a common wave function and the same energy.

Therefore, it can transfer from one atom to the other; the probability of this transition is larger, the greater the overlap of the wave functions (more precisely, the stronger is the interaction between the initial and final states, see below). It decays exponentially with increasing distance.

Not only electrons, but any particle can in principle, behave quantum-mechanically. In special cases, the proton also undergoes an underbarrier transition, and the process is accompanied by a substantial kinetic isotope effect (Krishtalik 2000).

In the preceding considerations, we have used potential curves (more strictly, free energy surfaces) corresponding to the initial and final states separately. These energy curves, unaffected by the interaction of the reactants are called "diabatic curves". However, the interaction of reactants can influence the form of potential curves and the position of the corresponding energy levels. This interaction is substantial when the electron has time to rearrange its wave function, i.e., zero time for a transition from one potential well to the other (from the initial i to the final f). Let us designate this time, τ_{if}. According to the Franck-Condon principle, this transition can take place only near the top of the barrier along the q coordinate. On the other hand, the coordinate q changes continuously because of thermal fluctuations, and the slow classical subsystem remains near the barrier top only for some restricted time τ_c.

When $\tau_c \ll \tau_{if}$, the interaction between initial and final states does not have sufficient time to develop, the system remains in the diabatic state, and the barrier height is determined by intersection of the diabatic curves. In the course of its thermal fluctuations, the classical subsystem will pass the crossing point many times before the particle jumps to its final position. Therefore, at the top of the classical barrier, the transfer probability is much less than unity and is close, within an order of magnitude, to the ratio τ_c/τ_{if}. Such reactions are called "non-adiabatic."

In contrast, when $\tau_c \gg \tau_{if}$, the electron can exchange many times between the re-

actants before the system leaves the region of the barrier top. Therefore, they can interact effectively and form a unified state (this is possible near the barrier top where the energies of the two reactants are similar). More exactly, from two initially existing states (i and f) two new states appear, one which is lower than the intersection point of the diabatic curves by the interaction energy (coupling matrix element) V_{if}, and the other lying higher by the same energy difference. These curves (free energy surfaces) are called "adiabatic" (Fig. 5.3, left panel). The intersection region of diabatic curves are shown Fig. 5.3 by the dashed segments of curves 1 and 2, and the adiabatic curves are presented as solid lines. Two adiabatic curves correspond to two different electronic states and, instead of one energy level in each potential well (dashed curves in the right panel of Fig. 5.3), two levels appear (solid lines, Fig. 5.3, right). In the course of a slow movement of the classical subsystem, at any moment the electron has enough time to adjust adiabatically to the new coordinates, and hence when the system goes over the q-barrier, the electron can transit readily to the final state. Such a transition called adiabatic, and it has a probability of 1.

As is explained below the rate constant k can be written as

$$k = \frac{\omega_{eff}}{2\pi} P \, \exp\left(-\frac{\Delta G^{\neq}}{kT}\right) \qquad (5.15)$$

In addition to the purely electrostatic treatment, the medium can be described also as a set of harmonic oscillators, or one oscillator with an effective circular frequency ω_{eff}. In Eq. 5.15, $\omega_{eff}/2\pi$ is the number of the classical subsystem's oscillations, and $\exp(-\Delta G^{\neq}/kT)$ determines the fraction of these oscillations having an energy sufficient to reach the barrier top. P is the probability of the electron transition to the final state when the system has reached the barrier top, where the electron levels in the initial and final states become equal. For a non-adiabatic process, the quantum mechanical Levich—Dogonadze theory gives
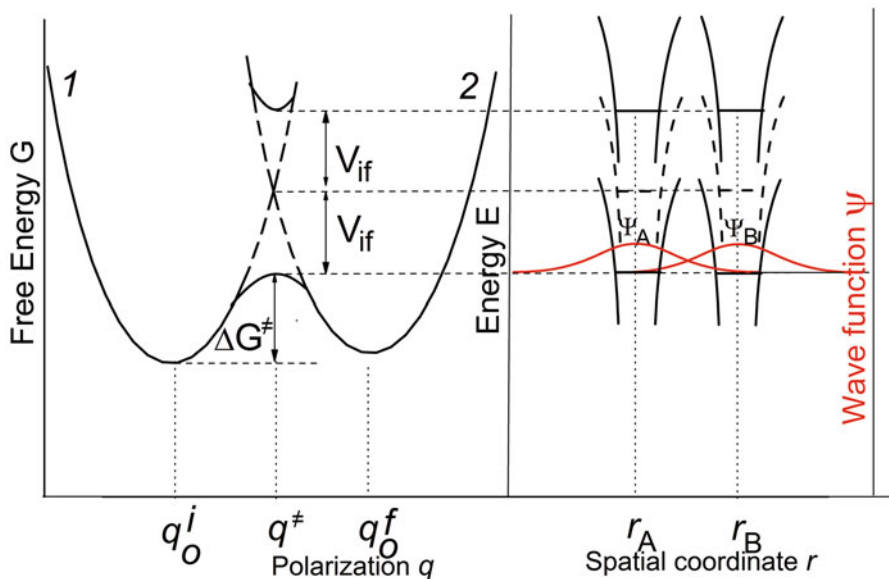
Fig. 5.3. Energy diagrams for the adiabatic electron transfer process. Plotted similarly to Fig. 5.1, the designations are also similar. On the *left panel*, two continuous *solid lines* relates to two adiabatic curves with the upper and lower energies at the transition polarization $q^{\neq}$. V_{if} is the interaction energy. The *dashed* segments represent the diabatic curves for the initial and final states. On the *right panel*, the *dashed* potential wells correspond to the diabatic state, i.e., the situation where the interaction of initial and final states is absent (the point of intersection of diabatic curves on the *left panel*). The *solid* potential wells relate to two electronic (adiabatic) states at the transition point $q^{\neq}$; their energy levels are separated by $2V_{if}$.

the following expression for the rate constant:

$$k = 2\pi \frac{V_{if}^2}{h} \left(\frac{\pi}{kT\lambda_s} \right)^{1/2} \exp\left(-\frac{\Delta G^{\neq}}{kT} \right) \quad (5.16)$$

Let us explain qualitatively some features of this formula. As to its strict description, we refer the reader to the references cited at the beginning of Sect. II, especially to Kuznetsov (1995, 1999).

We have seen that the system behavior depends substantially on the ratio τ_c/τ_{if}. The value of τ_c depends on two factors. The first is the width of the transition region that should be crossed by the slow subsystem; it increases with increasing V_{if}, and decreases with the increasing curvature of the potential curves in the vicinity of their intersection that depends on λ_s. The second factor is the velocity of crossing the transition region which is proportional to the frequency of the classical oscillator (medium oscillator) ω_{eff}.

As a result, τ_c is inversely proportional to ω_{eff}. Therefore, the frequencies in the numerator and denominator cancel out, and the rate constant is independent of ω_{eff}. The physical basis is that an increasing ω_{eff} implies a higher frequency of barrier crossings, but simultaneously a larger velocity of this crossing, and hence a compensating decrease in transition probability at each crossing event.

On the other hand, the crossing time τ_{if} is inversely proportional to V_{if} (it equals h/V_{if} by approximately an order of magnitude). The stronger the interaction, the faster the transformation of the electronic cloud. This explains why k is proportional to V_{if}^2.

For the non-adiabatic reaction, the small value of V_{if} corresponds usually to a weak overlap of the wave functions ψ_A and ψ_B (see Fig. 5.1, right panel). In this case, the activation energy $\Delta G^{\neq}$ is determined by the intersection of two diabatic curves. The activation energy is expressed through the reorganization energy λ_s and the free

energy of the elementary act, ΔG_o, by the usual quadratic expression of the Marcusian type.

For adiabatic reactions, the activation barrier is lower than the crossing point of two diabatic curves by the value of the interaction energy V_{if} (see Fig. 5.3, left panel; in this figure, the large V_{if} corresponds to the strong overlapping of the wave functions). At the same time, the probability P for these reactions equals one. Hence, the medium vibration frequency enters the rate equation explicitly (Eq. 5.13).

III. Proteins as Specific Structured Polar Media

The properties of proteins as polar media are determined by the distinctive features of their structure (for review, see Krishtalik 2012). The main principle underlying protein 'architectronics' is the formation of a linear sequence of amino acid residues linked together by strong peptide bonds and rather tightly packed into secondary and tertiary structures. The main elements of this structure, peptide groups, are characterized by a large dipole moment. For example, the dipole moments of many free amides are close to 4D; there is evidence that the formation of certain secondary structures, such as α-helices, leads to mutual polarization of peptide bonds and enhancement of their dipole moment up to ∼6D. Tight packing of peptide sequences and polar-side chains inside certain structures substantially restricts the ability of protein dipoles to change orientation (to reorient) under the action of an external electric field. Of course, the dipoles retain some mobility but their orientation is constrained to narrow limits. This has two important consequences:

First, the dielectric response of a protein to the external electric field is very weak (the extent of their reorientation is small), and hence its static dielectric permittivity is low. For certain dry proteins, the dielectric permittivity was found to be ∼3.5–4.0 (Pethig 1979); similar values were obtained with different variants of microscopic simulations for regions inside protein molecules (e.g., Gilson and Honig 1986; Simonson 1998). At the same time, liquid low-molecular weight amides with practically the same or a lower concentration of polar amide groups are characterized by high dielectric permittivity (e.g., 37 for dimethylformamide). Second, an important difference between proteins and low-molecular weight polar solvents lies in the existence of a permanent intraprotein electric field. The electric potential created by dipoles at any point of a low-molecular weight liquid at an arbitrary time can differ from zero. However, random fluctuations of dipole orientation make the time averaged potential equal to zero. The averaged dipole orientation ceases to be random only after an ion is introduced into the liquid; then, the potential created by the dipoles assumes a definite value. Unlike dipole orientation in liquids, that in proteins is determined by their structure. Certainly, dipole orientation in proteins undergoes fluctuations but the permissible variations are very limited. This accounts for the existence of a permanent time-averaged electric field at any point in the protein molecule. The field is present prior to the introduction of a new free charge; therefore, it may be called a 'pre-existent field'. Dipoles in proteins are intrinsically ordered, whereas in liquids their ordering develops only after the introduction of a charge. The pre-existent field is organized in such a way to facilitate charge transfer (see below, Sects. IV.A and V). For this reason, proteins can be described as 'pre-organized polar media' (Krishtalik 1974).

The proteins as pre-organized media possess one more feature different from that of liquid dielectrics. In liquid solvents, the main contribution to the solvation energy is brought about by the interaction of the ion with the nearest molecules; the more distant dipoles are less oriented because the field of the ion at larger distance is weaker. In proteins, the orientation of dipoles is determined by the entire protein structure, and therefore one can observe in some cases that

the dominant effect is the interaction not only with the nearest ion's neighbors but with the larger number of more distant dipoles.

It follows from the foregoing that proteins are highly-polar, low-dielectric media, i.e., they exhibit a combination of properties that can never be found in low-molecular weight liquids showing practically unlimited dipole mobility. Therefore, there is no sense, in the case of proteins, to consider a high polarity and a low dielectric permittivity to be antithetical properties.

In real biological systems, proteins practically never function as the sole dielectric phase; they are usually in contact with other dielectrics, e.g., water or lipids. The long-range action of Coulomb forces accounts for the substantial interaction of charges inside the protein with a non-protein phase that must be necessarily taken into consideration in the quantitative description of charge transfer processes. The introduction of a certain effective dielectric permittivity, sometimes proposed to describe an averaged description of the response of the two phases, is a rough approximation having no clear physical sense.

The aqueous environment influences both the energy of interaction between the charge and dipoles created by polarization and the strength of the pre-existent field. The latter is due to the shielding of the charge field in a protein by polarization of its aqueous environment, characterized by high dielectric permittivity. This effect was first reported, in quantitative terms in the classic work by Tanford and Kirkwood (1957) for the field of charged groups in the model of a homogeneous dielectric sphere. For dipole fields, the effect of aqueous surroundings was first considered in the microscopic model by Warshel and Levitt (1976). For the field of free charges and dipoles in the protein of a real geometry, the first accurate computations in the framework of a semi-continuum model, were performed by Warwicker and Watson (1982). At present, a few programs, e.g., DelPhi (Nicholls and Honig 1991) and MEAD (Bashford and Karplus 1990) are available for the electrostatic calculation

of arbitrarily shaped macromolecules in an electrolyte solution. The point in question is the numerical solution of the Poisson–Boltzmann equation (solution of the Poisson equation taking account of the Boltzmann distribution of the surrounding electrolyte ions in the Debye-Hückel approximation).

Structural constraints on the mobility of dipoles affect not only the permissible degree of their orientation, but also their rotation rate. A wide range of different-scale movements are known to occur in proteins, with different activation barriers and, therefore, with different characteristic times (see, e.g., Brooks et al. 1988). Dipole rotation associated with different types of protein movements correspond to different dielectric response rates reflected in the broad spectrum of dielectric relaxation times (from hundreds of femtoseconds to a few seconds). This situation can be phenomenologically described as a set of effective dielectric permittivities, each corresponding to a certain time interval (Krishtalik 1996).

To summarize, the consequences of common principles of the protein architecture are low dielectric permittivity, the presence of a permanent intraprotein electric field, and a wide range of dielectric relaxation times. How these specific features of proteins as dielectric media manifest themselves in the rate of electron transfer will be discussed in the following sections.

An exhaustive theoretical analysis of the charge transfer reactions in proteins demands a quantum-mechanical treatment of all parts of the system—reactants, protein and its surroundings. However, it is an extremely difficult task even for modern powerful computers, and therefore more tractable multi-scale models were developed (Karplus, Levitt, Warshel, Nobel Prize in Chemistry, 2013). In these models, the reactants are treated quantum-mechanically (QM), and the protein and its surroundings by various classical mechanics methods—molecular mechanics (MM), molecular dynamics (MD) (reviewed by Shurki and Warshel 2003).

For the processes involving bond breaking—bond formation, and hence

the mutual movement of reacting groups, MM/QM or MD/QM are the computational methods of choice. However, for reactions of the pure electronic transfer, another approach can be used; the redox centers are treated quantum-chemically (QC) or (more often) by a combination of QC with the experimental data, and the protein and surroundings—by classical electrostatics. For the protein, the semi-continuum model is used: a set of point charges (full and partial) imbedded in a continuum dielectric (the latter can be inhomogeneous one). A comparison of these two approaches has been discussed in a review by Simonson (2008), and the conclusion was that for redox reactions both these methods give reasonably similar results. In practical applications, both microscopic and semi-continuum electrostatics models have to resort to various approximations and simplifications. For these approaches, the approximations used are different, and therefore it is instructive to compare their results for the same concrete system. Some examples of such a comparison will be given below (Sect. V).

In the following, we will employ the semi-continuum protein description. First, in our opinion, it gives a more transparent general physical picture. Second, the calculations in this model consume, by orders of magnitude, less computer time than any microscopic simulation. We will analyze the problems in the framework of the Marcus–Levich–Dogonadze formalism, described above in Sect. II (also, see Chap. 6 by G. Matthias Ullmann et al. 2015).

IV. Equilibrium Energies of Electron Transfer

The free energy of the elementary act of charge transfer is the sum of the energy determined by the inherent properties of reactants (e.g., ionization potential) and the energy of interaction between the charge and its environment. The latter energy, in turn, comprises two components. One is the energy of polarization, ΔG_{pol}, of the dielectric medium by the charge. The polarization energy generated by the dielectric response of the medium is manifested in both low-molecular weight liquids and proteins, where it differs only quantitatively in agreement with the difference of their static dielectric permittivities.

The usual polarization of the medium by the charge is supplemented in protein media by a qualitatively new effect absent in liquid dielectrics; namely, the pre-existing field effect mentioned in Sect. III. A charge introduced into a protein (or transferred from one of its sites to another) interacts not only with the polarization it induces but also with the pre-existing intraprotein electric field, giving a contribution, $\Delta G_{p.f.}$.

A. The Pre-existent Field

The effect of the pre-existent field of protein dipoles, and not only of superficial charged groups, described in the early 1970s (Johannin and Kellersohn 1972; Krishtalik 1974; Warshel and Levitt 1976) was later extensively investigated. However, the analysis of electrostatic effects in proteins was for a long time inconsistent due to the ambiguity of the notion of dielectric permittivity used in these calculations (see, e.g., Schutz and Warshel 2001). In many calculations, the same static permittivity ε_s was used for evaluation of both the pre-existing field and the polarization energy. Warshel and coworkers (King et al. 1991) noticed a contradiction inherent in such an approach. Indeed, it suggests that permanent dipoles not only create an intraprotein field but also screen it; in other words, the permanent dipole field is shielded by the dipoles themselves which is physically unreasonable. This contradiction can be resolved by the following considerations (Krishtalik et al. 1997).

In the framework of the semi-continuum approximation, we consider a protein as some set of charges imbedded in a continuum dielectric medium. Hence, we substitute the true electron density

distribution over all atoms of the protein by certain effective partial atomic charges obtained in quantum chemical calculations of small molecules or their fragments, which describe the field outside these molecules. The field of each fragment distorts the electron density distribution of all other fragments by inducing respective dipole moments. In the framework of the semi-continuum approximation, mutual electronic polarization of protein molecule fragments is averaged and described by a certain mean electronic (optical) dielectric permittivity ε_o. The optical permittivity is used to calculate the pre-existent field, because the coordinates of all the atoms are specified and their shift under the action of the pre-existing field should not be taken into consideration The appearance in the protein of a new free charge (e.g., attachment of electrons or protons to a group) causes both displacement of the medium (protein) heavy atoms (and/or dipole rotation, an analog of atomic and orientational polarization of ordinary dielectrics) and polarization of all electron shells. Therefore, all types of polarizability are responding, and the effect of the interaction of the new charge with the polarization it induces is determined by the total, i.e., static, dielectric permittivity, ε_s. This suggests an algorithm for the ΔG calculation in which $\Delta G_{p.f.}$ and ΔG_{pol} are calculated using the protein dielectric constants, ε_o and ε_s, respectively.

The optical dielectric permittivity of liquid amides is close to 2. However, at the practically identical polarizability of an amide molecule and each link of the polypeptide chain, the protein density is approximately 30 % higher. According to the Clausius-Mosotti equation (Atkins 1978), that elevates ε_o to 2.5. Extrapolation of refraction indices of α-chymotrypsin aqueous solutions yields a similar value (Mertz and Krishtalik 1999).

Let us consider a typical case when a certain group, e.g., a center for oxidation-reduction, has some definite charge (including zero) under conditions in which the protein structure is determined. The additional charging of this group would cause a change in protein dipole orientation. Therefore, the potential created by its charge at other points must be calculated with the use of static permittivity, ε_s. This potential is added to that created by all the remaining protein's charges (full and partial) giving the pre-existent field and computed based on the optical permittivity, ε_o.

The choice of atomic partial charges is important. These values are inaccessible to any direct experiment, and hence are determined as effective values that describe the extramolecular field corresponding to the electronic density distribution obtained from quantum chemical calculations (so called electrostatic potentials ESP). Various systems of parameterized partial charges have been proposed. We deem it necessary to choose, in the first place, values of partial charges that correspond to the experimentally measurable characteristic of a given molecule that is directly related to its charge density distribution, i.e., the dipole moment. Quantum chemical calculations can provide the electric field of a molecule in a vacuum with the ε inside the molecule set to be equal to one. Because we describe proteins as continuous media with ε_o when calculating the pre-existent field, the same optical permittivity is automatically attributed to the inner region of each molecular fragment. Such 'filling' of a fragment with a medium having $\varepsilon_o > 1$ weakens its field. In order to retain the correct field value outside the fragment, the 'vacuum' partial charges should be multiplied by a coefficient close to $(\varepsilon_o + 2)/3$ (the field of a dipole placed in a sphere with ε_o is $3/(\varepsilon_o + 2)$ times weaker than the field of the same dipole in a vacuum). This coefficient relates only to the partial charges of dipoles or other electro-neutral groups. Free charges must not be corrected (the field outside the charged sphere being independent of dielectric permittivity inside the sphere).

It should be noted that most of the modern systems (such as CHARMM, AMBER etc.) that employ partial charges increase them over the values that correspond

to the experimental dipole moments of small molecules, thereby achieving better agreement of MD simulations. In particular, the PARSE set of charges (Sitkoff et al. 1994) generates intraprotein fields close to results obtained with charges that are corrected as described above, for the effect of filling of the fragment "body" by ε_o (Ptushenko et al. 2008).

In real biological systems, a protein is surrounded by different media, water, and lipids for intra-membrane proteins. Therefore, calculations must be performed for a heterogeneous system. Unlike atomic coordinates in proteins, the coordinates of the surrounding water molecules are not fixed, and its dielectric response includes all types of polarizability; this means that the pre-existing field must be calculated using ε_o for the protein and at the same time the static permittivity (dielectric constants) ε_{sw} for water and ε_{si} for lipids.

As shown above, the energy of polarization of the dielectric medium, ΔG_{pol}, is computed using the static permittivity of both the protein and the surrounding water. Since the ε_s of protein is much smaller than that of water, the absolute value of the energy, ΔG_{pol}, in the protein-water system is significantly lower than in the purely aqueous environment. This makes charge transfer from water into a low-dielectric medium energetically disadvantageous, as is well known from the Born formula (discussed below). An ion can exist in the protein environment only when the loss of the solvation energy is compensated by the action of the intraprotein pre-existing field. Such more or less full compensation has been observed many times (e.g., Gunner et al 2000; reviewed in Krishtalik 2012). A drawback of most of these studies is that the pre-existing field was calculated with the use of protein static permittivity; the use of optical permittivity enhances the field and makes compensation for the low solvation energy a more reliably established fact. This compensation is especially substantial in the case of multi-charged ions; here, the difference between the more accurate computations using ε_o and

the traditional approach where only one permittivity is employed, namely the static one, becomes very large. For example, the corresponding error in calculation of the redox potentials of multi-charged Fe_4S_4 clusters (in the Photosystem I and in ferredoxins) reaches 0.8–1.0 V (Ptushenko et al. 2008).

Concluding this section, let us mention that besides the well-known structural specificity of enzymatic catalysis, which is due to the structure of active sites, an additional structural factor exists. This is the spatial configuration of the intraprotein field that is needed to ensure a favorable energy of charge transfer. As was described in Sect. III, this field depends not only on the amino acid composition of the active site, but also on a large number of dipoles of peptide groups and surrounding side chains.

B. The Medium Polarization Energy

The notion of the ion solvation energy as due to the interaction of the ion's charge with the dielectric's polarization induced by it dates back to the famous paper by Born (1920). Born described the solvation energy of a spherical ion of radius 'a' as a difference of its charging energies by charge e in vacuum and sample dielectric, ε_s.

$$\Delta G = \frac{e^2}{2a} \left(1 - \frac{1}{\varepsilon_s} \right) \qquad (5.17)$$

Notice the similarity of this formula with the Marcus equation for the reorganization energy (Eq. 5.10)—both are based on continuum electrostatics.

It is well known that the Born equation, although it provides a clear physical picture, does not provide quantitative results. This is due to the discreteness of solvents. Most common inorganic ions are small compared to solvent molecules and water clusters. Their electric field at small distances, less than the size of solvent molecules, changes rapidly, and inside the solvent molecule it cannot be described as a field in a continuum medium.

The foregoing suggests that for ions larger than solvent molecules the Born equation

may be sufficiently accurate. However, the "chemical solvation energy", i.e., the energy only due to interaction with the solvent, cannot be determined experimentally by any thermodynamically correct experiment; only the so-called "real solvation energy" that includes, in addition to the chemical energy, the energy of ion transfer across the solvent-dependent surface potential difference at the liquid/air boundary is experimentally accessible. The surface potential components cancel out when we determine the sum of solvation energies for cation and anion or difference of solvation energies for two ions of the same sign. Such a determination was performed for the cationic and anionic forms of cobaltocene $CoCp_2$ (bis-cyclopentadienylcobalt $Co(C_5H_5)_2$) that have an effective radius 3.7 Å as well as for two anionic forms of bis-o-dicarbollylnickel $NiCb_2$ ($Ni(C_2B_9H_{11})_2$), which has an effective radius of 4.17 Å (Krishtalik et al. 1991; this and subsequent studies summarized in Krishtalik 2008). The data presented in Fig. 5.4 show that for $CoCp_2$ the Born equation is fulfilled practically quantitatively in a wide interval of static dielectric constants from 65 to 7, the latter a permittivity close to those of proteins, especially at their active sites. Similar data were obtained for $NiCb_2$ (but in a narrower range of permittivities, 65–21).

In proteins, the size of redox centers (e.g., hemes, flavins) is much larger than the size of the peptide group or the polar ends of side chains. Therefore, the results described above may serve as some substantiation of the continuum electrostatics approximation in these systems. The shape of redox centers is far from spherical, and hence the Born equation in its simple form cannot be applied. However, the existing programs for numerical solution of the Poisson equation allow calculations of the dielectric response energy for a reactant of any shape.

For computation of redox potentials one employs two methods—the first based on quantum—chemical calculations, and
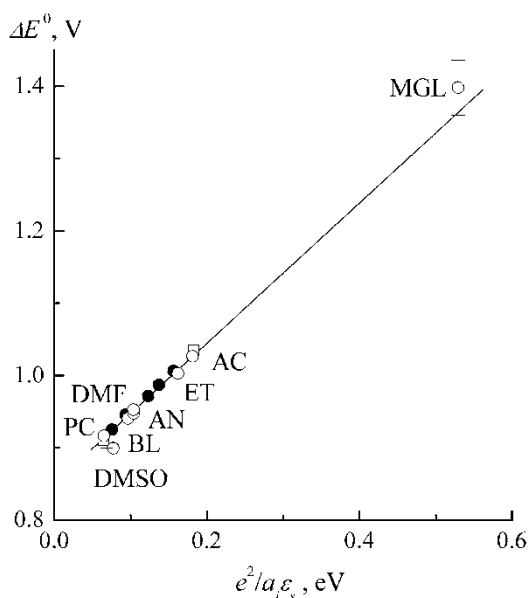
Fig. 5.4. Dependence of the redox potential difference of the $CoCp_2{}^+/CoCp_2$ and $CoCp_2/CoCp_2{}^-$ couples on the sum of charging energies of cation and anion, calculated according to Born equation. Designations: *circles* are the results obtained with a mercury electrode; *squares* are the results obtained with a platinum electrode. These charging energies are calculated assuming the same electronic density distribution for each of the components in all solvents. However, DMSO is more polarizable than the other solvents studied (electronic dielectric permittivity 2.2 compared to 1.8), and therefore interacts stronger with solute; as a result, in this solvent the covalent bond in $CoCp_2{}^-$ is more polar, making the charging energy somewhat lower. The cross presents the point for DMSO with the charging energy recalculated on the basis of quantum-chemical data (An. Kuznetsov et al. 2009); open points correspond to propylene-carbonate (PC), butyrolactone (BL), dimethylsulfoxide (DMSO), dimethylfomamide (DMF), acetonitrile (AN), ethanol (ET), acetone (AC), monoglyme (dimethoxyethane) (MGL); *black circles* correspond to mixtures of PC and AC.

the second—on the experimental data and quantum—chemical results.

In the first method, one computes the ionization energy of the corresponding group in a gas phase, and then calculates the difference of the electrostatic transfer energies from gas to protein for the reduced and oxidized forms (including both the dielectric response energies and the energies in the pre-existing intraprotein electric field). These transfer energies are the chemical ones.

The electron formed in the gas phase should be used for reduction of the other oxidized species, for instance H^+ with the final formation of $\frac{1}{2} H_2$. Their transfer energies (with an account for half of the H_2 dissociation energy) give the potential of the standard hydrogen electrode (SHE). The difference of the corresponding energies of the redox couple under study and the potential of the hydrogen electrode gives the redox potential relative to the standard hydrogen electrode– an experimentally measurable quantity.

In these calculations, very frequently one employs the so-called absolute SHE potential defined by Reiss and Heller (1985). This potential is actually nothing else than the potential suggested for the first time by Kanevskii (1952). This 'absolute' potential can be strictly computed thermodynamically from experimental data, as the sum of the metal work function and the potential difference at the metal-solution interface at a given electrode potential, specifically the SHE potential. However, the calculations described above employ the chemical solvation energies, and hence should use the hydrogen electrode potential based on the 'chemical' hydration energy (An. Kuznetsov et al. 2008). Unlike this potential, Kanevskii's potential is determined by the 'real' hydration energy, including the water surface potential, χ

$$\Delta G_{real} = \Delta G_{chem} + \chi q \qquad (5.18)$$

The 'chemical' hydration energy enters the so-called Trasatti potential E_T (1974), differing from the Kanevskii potential E_K by the water surface potential, χ.

$$E_K = E_T + \chi \qquad (5.19)$$

The Trasatti potential should be employed in calculations of redox potentials.

Determination of the water-gas potential jump implies the use of extra-thermodynamic assumptions. Different assumptions were applied by Trasatti (1974) and Krishtalik (2008), but they give similar

values close to 0.14 V. In conjunction with the known experimental value of $E_K = 4.44$ V, it yields $E_T = 4.30 \pm 0.02$ V.

Calculations of this type were performed for the Rieske iron-sulfur protein that functions in respiratory and photosynthetic electron transport, using E_T and the optical dielectric permittivity for the calculation of the intraprotein field. Their agreement with the experimental data is reasonably close. It is interesting that the distortion of the Fe_2S_2 cluster due to the steric restrictions imposed by the protein matrix affects the redox potential substantially (An. Kuznetsov et al. 2010).

The second type of computation uses quantum-chemical data for the charge distribution in the reactants that are necessary for electrostatic calculations, and experimental data for the redox potential in some, usually aprotic, solvents. One calculates the electrostatic transfer energies from the solvent into the protein for both reduced and oxidized forms, and adds this component to the experimental redox potential. However, there is a pitfall similar to that in the first method—the unknown liquid junction potential between solution in the nonaqueous solvent and the aqueous solution of the reference electrode, e.g., SHE. This potential is caused by a nonequivalent distribution of cations and anions of the background electrolyte between water and the organic solvent due to the difference of their transfer energies. This quantity is inaccessible to direct measurement and hence, for its evaluation, one needs to resort to some extra-thermodynamic assumptions. The correction is minimal if one has measured the redox potential relative to a proper internal standard in the same solvent, e.g., the $FeCp_2^+/FeCp_2$ couple; for this couple, there are good estimates of its redox potential in aprotic solvents relative to an aqueous reference electrode. This approach combined with quantum—chemical data, data on the distribution of static dielectric permittivity, and the employment of the optical permittivity for the intraprotein field evaluation, resulted in a reasonable agreement with the experiment for ten

cofactors of the Photosystem I and two soluble ferredoxins (Ptushenko et al. 2008).

V. Reorganization Energy and Activation Energy

Let us start by considering the simple case of charge transfer in a homogeneous medium. According to the Marcus equation (5.10), the medium reorganization energy is determined by its optical and static dielectric permittivities, ε_o and ε_s, as it is expressed via the so-called polaron coupling constant (Pekar constant) $C = 1/\varepsilon_o - 1/\varepsilon_s$. (as discussed in Sect. II.A). For the usual polar solvents, 'C' depends weakly on static permittivity. For example, for water, $\varepsilon_o = 1.8$, $\varepsilon_s = 78$ at 25 °C, and $C = 0.54$; for DMF, $\varepsilon_o = 2.04$, $\varepsilon_s = 36.7$, $C = 0.46$. However, the low static dielectric permittivity of proteins has a dramatic effect on their reorganization energy. At typical values of the optical and static permittivity of proteins (roughly 2.5 and 4) the C value is as low as 0.15. Hence, the protein reorganization energy can be expected to be several times lower than for water and other polar solvents. The low reorganization energy of charge transfer in proteins (together with the pre-existing field favoring the electron transfer; see below) is one of the main physical causes of low activation energy and, therefore, of high catalytic activity of enzymes.

As was discussed in Sect. III, a specific property of proteins is the presence of a pre-existing electric field. This field is due to the full and partial charges of the amino acid residues, but is not dependent on the charge that is transferred to the reacting group. Therefore, it is the same during the charging and discharging processes used for determination of the reorganization energy as described in Sect. II.A. In the charging process, this field contributes the energy

$$\sum_i \varphi_i q,$$

where φ_i designates the field set up by the protein atom i on the reactant (but not the po-

tential created by the reacting group), and q is the charge transferred to the reactant (usually distributed over the reactant's atoms). This energy is a part of the total reaction energy, ΔG, and hence has an important role in charge transfer in proteins.

In the second step of the reorganization energy computation, viz. discharge in an optical medium, the potential of the pre-existing field is the same, and the charge transferred is $-q$. Hence, the corresponding energy is

$$-\sum_i \varphi_i q$$

The sum of these two expressions is zero, and hence the pre-existing field does not affect the reorganization energy. The latter is the same as in the structureless medium with the same spatial distribution of dielectric permittivities. Therefore, the reorganization energy calculation in the framework of the semi-continuum approximation is insensitive to the choice of a parameter such as the partial charge of protein atoms.

In real systems, one deals with an inhomogeneous medium, i.e., proteins (that can have some spatial distribution of permittivities), reactants, and the surrounding aqueous electrolyte solution. In a general case, one can employ a numerical solution of the Poisson–Boltzmann equation to find the dielectric response energies in optical and static media, W_o and W_s (Eq. 5.9) using a program that accounts for the presence of several dielectric regions. A more detailed description of the algorithm is given in Krishtalik (2011, 2012).

The reorganization energy semi-continuum calculation is independent on the partial charges of the protein atoms while the molecular-dynamics calculations employ this parameter explicitly. It is interesting to compare the results of these quite different methods. Below, some examples related to cytochromes and similar systems are given (Krishtalik 2011).

The experimental evaluation of the reorganization energy of horse heart cytochrome c, in electrochemical reaction on the electrode, modified by a covalently tethered long-chain

aliphatic alcohol gives a value of 0.58 eV (Terrettaz et al. 1996). These experimental data are related to electron transfer into metal for a distance determined by the thickness of the adsorbed layer, while the calculations were performed for the electron transfer to an infinite distance. The corresponding extrapolation results in a value of 0.64 eV (Krishtalik 2011).

The calculated reorganization energy of the medium turned out to be 0.45–0.48 eV, about half of it due to the reorganization of surrounding water. To obtain the full reorganization energy, one should add other terms. First, there is the inner-sphere reorganization energy of a liganded heme equaling about 0.04 eV. There is one more component of the medium reorganization energy associated with a marked displacement upon the heme charging of water molecule 156, as well as of Asn-152, Tyr-57, and Thr-78 adjoining the heme (this is the standard numbering of residues for the horse cytochrome c). This shift cannot be described in the usual dielectric approximation that suggests a linear response, which is feasible in practice only for small displacements of atoms. Our quantum chemical calculations yielded 0.28 eV for this 'non-linear dielectric reorganization' of the environment. The total reorganization energy is 0.77–0.80 eV.

Microscopic simulations of cytochrome c yielded a reorganization energy of 0.4–0.65 eV (Muegge et al. 1997) and 0.77 eV (Simonson 2002; the latter study employed a much longer molecular dynamic simulation), in reasonable agreement with the results of experimental measurements and semi-continuum calculations.

Gray and coworkers have studied the electron transfer between the heme of cytochrome c and a ruthenium complex covalently tethered to $N\varepsilon2$ of His33 of the protein. For a series of Ru-ammonia-pyridine modified Zn-cytochromes $c,$ the reorganization energy was estimated to be 1.20 ± 0.05 eV (Gray and Winkler 1996, 2003; Meade et al. 1989).

The calculation for the $Ru(NH_3)_5$ derivative gives $\lambda_s = 1.12$–1.18 eV, for the more typical members of the series studied, viz. the $Ru(NH_3)_4$-pyridine derivative, the medium reorganization energy equals to 0.95–1.01 eV. It is somewhat smaller than the experimental value due to the aforementioned contributions of the inner-sphere reorganization and, especially, the shift of some residues in the vicinity of the heme (the data for the Zn-substituted cytochrome are unknown, but one can expect an effect of the same order of magnitude).

Blumberger and coworkers (Tipmanee et al. 2010) have studied the reorganization energy for three amine-ruthenated cytochromes using a quantum mechanical–molecular mechanical approach. For Ru(NH3)5—substituted cytochrome c, a total reorganization energy was found to be 1.34 eV, which is close to the experimental data and to our estimates. An important finding is the good agreement with experiment obtained using only the 'polarizable force field' model (Tipmanee et al. 2010). Without accounting for the molecular electronic polarizability, the calculated reorganization energies are substantially overestimated by 40 %. This is in line with the idea of the dielectric continuum theory where optical (electronic) dielectric permittivity plays an important role (Sect. II.A, this Sect. V, above).

For the Ru $(bpy)_2(im)$ derivative (bpy = bipyridine, im = imidazole), a value $\lambda = 1.26$ eV was calculated (Tipmanee et al. 2010). In our calculations, λ_s for this system equals to 0.70–0.74 eV. The approximate experimental estimate for the total λ is 0.75 eV (Mines et al. 1996).

The reorganization energy for the electron transfer between heme a and heme a_3 in cytochrome c oxidase was roughly estimated from the kinetic data by Jasaitis et al. (2005) as lying between 0.05 and 0.20 eV (the last figure seems to be more probable). A semi-continuum calculation gives the value of 0.2 eV; the same result was obtained by molecular dynamics simulations (Kailaa et al. 2010).

Concluding, one can state that in the computation of reorganization energies, the microscopic modeling and semi-continuum

electrostatics (plus quantum chemistry) approach are generally in good correspondence, and similarly agree with experiment.

The activation energy is determined by two quantities—the reaction free energy and the reorganization energy. Therefore, one should consider the protein's influence on both of these quantities together (Krishtalik 1974, 1985, 2012). The contribution of the aqueous surroundings to the reorganization energy depends on its accessibility to the active site of the protein. In the examples given above, the heme of cytochrome c lies close to the protein surface, and hence the effect of water reorganization is substantial. It plays an even greater role for amine-ruthenated cytochromes because the ruthenium complex is surrounded by water. However, for interheme electron transfer in cytochrome c oxidase, it is only of minor importance, as the reactants are situated deep in the membrane protein. In any case, the presence of aqueous surroundings somewhat increases the reorganization energy.

For the reaction free energy, the effect of the aqueous phase depends on the direction of charge transfer. When the charge is transferred from the interior of the protein towards its surface, the charge interaction with the high-dielectric phase makes ΔG more negative, and therefore decreases the activation energy. The opposite direction of charge transfer makes ΔG more positive. Therefore, for several enzymes with two-step processes, the step that would be less favorable in a homogeneous medium proceeds with the charge transfer from inside to outside, compensating in this way for energy consumption.

In globular proteins, the active site is usually situated close to the globule surface to ensure effective interaction with the reaction partners. An increase in the globule radius results in better screening of the opposite side of the active site from interaction with water. This screening results in both the reorganization energy and the activation energy of the slow step of the charge transfer decreasing with increasing globule radius; for small values of the radius, this decrease is initially relatively rapid. This suggests the existence of a range of optimal radii within which the activation energy is almost minimal, while the consumption of protein for the globule formation is also not too high. The initially fast decrease in the activation energy with increasing protein radius accounts for the experimentally observed fact that the size of enzyme globules is, as a rule, substantially greater than the size of the active site proper—an additional volume of protein insures better screening of the charge in the active site from the interaction with water.

As discussed in Sect. III, the main principle of protein structure results in two consequences: a low static dielectric permittivity (dielectric constant) and the presence of a permanent intraprotein electric field. On the one hand, the low static permittivity of proteins results in a small reorganization energy. On the other, the low dielectric permittivity accounts for the disadvantage of the presence of charged groups in a protein that may, in principle, cause an increase in the reaction activation energy compared with its value in an aqueous medium. A consistent comparison of these two media implies taking account of the energy of charge transfer, ΔG_{tr}, from one medium to the other. The effective activation energy is expressed as

$$\Delta G_{eff}^{\neq} = \Delta G^{\neq} + \Delta G_{tr} \qquad (5.20)$$

The activation energy for certain typical reactions (charge separation, neutralization, transport of charges from an initially charged reactant to an uncharged one, etc.) has been analyzed (Krishtalik 1985, 2012). The activation energy of the elementary act proper in a medium with low dielectric permittivity $\Delta G^{\neq}$ can be in some cases lower than in water, and greater in the other cases. However, the effective activation energy in the low-dielectric medium is invariably higher than in the high-dielectric one. In other words, the additional energy expenditure necessary to transfer a charge into a low-dielectric permittivity medium (or generate a charge in such a

medium) always outweighs the gain due to a decrease in reorganization energy.

This inference holds for a structureless dielectric for which the polarization interaction between the charge and its environment (Born solvation energy) is the sole contribution to the electrostatic energy of the system. However, proteins are highly structured systems possessing a permanent electric field, and the energy of the reactant charge in this field is an essential constituent of the system's electrostatic energy; usually, it compensates for the loss of the Born solvation energy given a proper potential in the region of the reactant localization. The existence of a strong intraprotein electric field confers on proteins one of their evolutionary preferences over many other possible biopolymers (Krishtalik 1985).

In conclusion, it should be stressed that only the joint action of two factors, the low reorganization energy and the intraprotein electric field, ensures a substantial reduction in the activation energy, i.e., the high catalytic activity of enzymes. Both effects directly follow from the general principles and architecture of protein structural organization.

It is relevant to add some additional comments.

The compensation effect of the intraprotein field may be either slightly smaller or greater than the loss of the Born solvation energy; it is important that the total ΔG value must be reduced to a mechanistically feasible level. Excessive stabilization of the charge is sometimes unfavorable because it may hamper realization of a subsequent electron transfer, or release of a free ion.

Often, one sees the statement that the intraprotein field stabilizes the transition state. This is literally correct as far as heavy particle transfer reactions are concerned when the transition state has a definite charge. However, the notion of transition state charge makes no sense for the non-adiabatic electron transfer that is widespread in biochemical reactions. In these processes, the charge is wholly concentrated at one of the reactants, either in the initial or final states of the reaction. In such a situation, one may speak

of electrostatic stabilization of the reaction product relative to the initial reactants, which lowers the activation barrier. In other words, a decrease in the transition state energy is an indirect result of a decreased ΔG of the reaction rather than the consequence of a direct action of the field on the transition state charge. Let us notice that the transition state includes not only the reactive groups proper, but also the surrounding medium in a non-equilibrium configuration. The behavior of this medium is governed by its reorganization energy, which does not depend on the intraprotein electric field; its low value is due to the protein's weak dielectric response as described in this section.

VI. Long-Range Electron Transfer

In the great majority of biological systems, two redox centers are not in the direct contact that assures the adiabatic electron transfer, but are situated at a longer distance resulting in non-adiabatic transfer. So, there is a "tunneling barrier" formed by the protein matrix. In the last two—three decades, this problem has been the subject of intensive studies, both experimental and theoretical.

For non-adiabatic electron transfer its probability decreases strongly with increasing distance. A useful empirical formula describing the distance dependence has been proposed by the Dutton's group (Moser et al. 1992; review, Moser et al. 2010). It has the form

$$\log_{10} k = 13 - 0.8 \, (1.2 - \rho) \, (R - 3.6)$$
$$- 3.1 \frac{(\Delta G + \lambda)^2}{\lambda} \qquad (5.21)$$

In this formula, the constant '13' is the logarithm of kT/h, i.e., the pre-exponential in Eyring's absolute rate theory. The quantity ρ is the packing factor (calculated for some pathway connecting the donor and acceptor); it accounts for the difference in the tunneling probabilities through the protein atoms and through the empty space (the former term substantially higher); the generic value

of $\rho \approx 0.76$. R (in Å) is the edge–to–edge distance between the redox partners (for the molecules with conjugated bonds, like heme, the molecular orbitals encompass the entire molecule, and decay exponentially outside the molecule); 3.6 is the approximate sum of the donor-acceptor van der Waals radii, so (R−3.6) presents the distance at which the electron transfer becomes non-adiabatic. The last term has a usual Marcusian form but the empirical coefficient equals to 3.1 instead of the value of 4.2 in the Marcus formula (energies expressed in eV) and is supposed to account for a decrease in activation energy due to the involvement of quantum modes as described in Sect. II.A above. In the derivation of this formula, several approximations are made. For example, λ has been assigned the same value for all reactions and reactions at all distances, which seems to be an oversimplification. Notice, however, that the described R—dependence was observed for the activationless processes where the last term of Eq. 5.17 equals zero. In spite of all approximations, this formula works well in many cases (Moser et al. 2010).

Often one considers the exponential dependence on the distance as due to the electron tunneling through a rectangular barrier according to the Gamow equation (Gamow 1928), and estimates the proper barrier height. However, these estimates give values too low compared to the barrier height expected from physical considerations. The most probable mechanism of the long–range electron transfer in proteins as well as the transfer through synthetic spacers is the so called "super-exchange" via some "pathway" (Beratan and Onuchic 1989; for review see Gray and Winkler 2010). Let us describe qualitatively the latter mechanism using a simple scheme presented in Fig. 5.5.

As shown above (Fig. 5.1), when the classical subsystem reaches the top of the barrier, the electronic levels in initial and final states become equal; in (Fig. 5.5) these are the levels of donor D and acceptor A. However, direct electron transfer from D to A is impractical because the donor-acceptor distance is too large, and hence the overlapping of their wave functions is negligible. At the same time, the wave function of D overlaps markedly with the wave function of the molecular orbital (LUMO) of the intermediate protein group P1 (Fig. 5.5); the same takes place for A and P4. The simple elec-
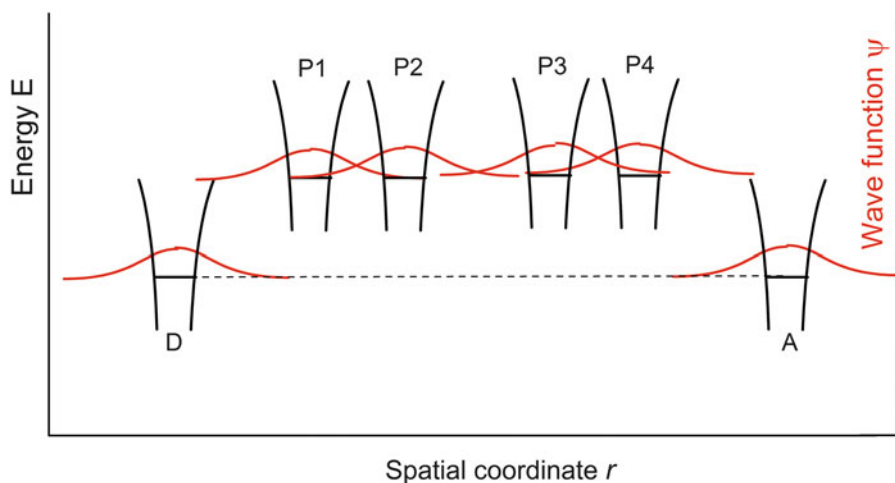
Fig. 5.5. Scheme of the super-exchange electron transfer through intervening protein. Plotted similarly to the right panel of Fig. 5.1. The black curves represent the electron potential energy E versus its spatial coordinate r, the horizontal bars inside the potential wells mark the electron energy levels. Designations of potential wells: D—donor, A—acceptor, P1–P4—groups of the protein that form the pathway. Horizontal dashed line shows that the energies of electronic levels in D and A are equal. Red curves represent the corresponding wave functions.

tron transfer from D to P1 is impossible because they have different energies. However, the substantial overlapping of wave functions from P1 to P4 makes possible the overlap between D and A via the chain P1–P4. This chain is called "the pathway". One should not understand this term as some trajectory of a real electron movement (the electron cannot jump from D to P1 or from P4 to A). It rather means "the combination of protein orbitals which dominates the coupling between donor and acceptor" (Beratan and Onuchic 1989). A similar picture holds true for the hole transfer from A to D; in this case, the HOMO's of the intervening protein are employed.

In Fig. 5.5, the distances between P1 and P2 as well as between P3 and P4, are drawn shorter than the P2–P3 distance. The former correspond to covalent bonds, the latter to the space between unboundatoms. As seen from the shape of the wave functions, the electronic coupling through the covalent bond is substantially stronger than through-space. The extent of these couplings can be estimated on the basis of quantum—chemical analysis. The various calculations give somewhat different results. For through-bond coupling, the decrease in $\log_{10}k$ is   0.45–0.6 per covalent σ-bond. The through-space coupling is strongly distance– dependent, with the rate constant, k, changing by $\Delta \log_{10}k$ -1.5 R_{space} where R_{space} is the length of this interval (Jones et al. 2002). The distance between donor and acceptor R is approximately proportional to the number of bonds connecting them (n). For the synthetic compounds with D and A linked by some regular covalent chain; R and n are strictly proportional. Therefore, both the density model (Eq. 5.18) and the pathway model usually yield qualitatively similar results. A detailed comparison of these two models was done by Jones et al. (2002). For 33 of 38 donor–acceptor pairs in 28 proteins a linear correlation of coupling parameters was found. The slope of the best—fit linear function = 0.7, which is due primarily to the difference in the through–space parameters used in these models. The correlation observed is understandable

because both approaches have a similar physical basis, viz. the difference between the transfer probabilities, through-bond and through-space.

For five systems, a substantial deviation from the common fit/linear function was observed. It was caused by a non-coincidence of the optimal pathway with the shortest Donor–Acceptor distance or by a large difference in the estimation of the role of through–space coupling.

In some model systems, a strong discrepancy between the pathway approach and distance dependence was observed. For example, Niki et al. (2003) studied the electron transfer to cytochrome c from a metal coated with a covalently tethered layer of long-chain carboxylic acid. Several lysines, surrounding the heme edge are attracted to caboxylates. Using the WT and various mutants, it was shown that only one of the mutations (eliminating Lys 13) results in a drastic, approximately six orders of magnitude, decrease in the reaction rate, and it is precisely that residue which is connected to the heme by the shortest chain of covalent bonds. The distance to the electrode for the WT and different mutants is similar, but the pathways from the different Lys's to the heme are quite different.

Another example is the electrochemical reduction of hemin through the physically adsorbed (and hence more flexible) carboxylic acid monolayer (Khanova and Krishtalik 2005). An alteration in the orientation of hydrocarbon chains of the carboxylic acid upon a change in pH of the solution results in a twofold decrease in the layer thickness. However, the reaction rate remains practically unaltered because the number of covalent bonds between the electrode and hemin does not change.

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Chapter 6

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Theoretical Analysis of Electron Transfer in Proteins: From Simple Proteins to Complex Machineries

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Summary

Electron transfer plays a central role in many biological processes such as, for instance, photosynthesis or oxidative phosphorylation, but also in other bioenergetic processes such as denitrification or sulfate and sulfite reduction. Moreover, electron transfer is a key step in

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many enzymatic reactions. The framework of Marcus theory provides the theoretical basis to describe the kinetics of these reactions. The parameters to calculate rate constants can be estimated using protein crystal structures. Namely, the electronic coupling is related to the edge-to-edge distance between the redox-active sites. The reaction free energy and the reorganization energy can be obtained, for instance, from continuum electrostatic calculations. However, to perform complicated tasks, proteins often combine many redox cofactors and couple the redox reactions to protonation reactions or conformational changes. Moreover, electron transfer proteins are often embedded in membranes, and thus membrane potential and concentration gradients influence the reactions. One approach to describe such complex systems is the so-called microstate model, in which each state of a system is represented by a vector in which each component defines the status of each site (for instance oxidized or reduced, protonated or deprotonated). On the basis of this microstate description, it is possible to calculate the thermodynamics and kinetics of a complex protein system. In this article, we will review the principle features of the microstate model and explain how the parameters of the microstate model can be calculated using continuum electrostatics. The microstate model provides the theoretical framework to go from molecular structures to the mechanism of complex protein machines.

I. Introduction

Electron transfer reactions are central in many biological processes such as photo-synthesis and oxidative phosphorylation, to name just the most prominent biochemical pathways. Additionally, electron transfer also plays a central role in biochemical pathways such as the nitrogen cycle and the sulfur cycle that are of geochemical relevance. In recent years, the biochemical electron transfer processes also raised an increased interest because of its use in microbial fuel cells (Logan 2008; Zhou et al. 2013). Electron transfer reactions are even possible candidates to be processes at the origin of life in hydrothermal vents where electron transfer reactions are driven by pH gradients (Martin et al. 2008). These reactions are in some respect just the reverse of the chemi-osmotic theory of Mitchell (1961) and may thus help to explain the origin of the non-equilibrium state that is characteristic for living organisms.

Considering the central role of redox reactions in biochemical systems, it is not surprising that the redox potential is tightly controlled in the cell (Banerjee 2008). In fact, there are several redox buffering systems in the cell that are interconnected through various complex enzymatic systems. One of the most prominent biological redox buffers is glutathione, a tri-peptide which forms dimers through its thiol group upon oxidation (Deponte 2013). Glutathione functions as an antioxidant in plants, animals, fungi, and some bacteria and archaea and prevents harmful reactions which can be caused by reactive oxygen species such as free radicals and peroxides. Oxidized glutathione is reduced by nicotinamide adenine dinucleotide phosphate (NADP) through a reaction that is catalyzed by glutathione reductase. Glutathione reductase links the redox buffer glutathione to the anabolic redox pool of NADPH, which is mainly generated in the pentose phosphate pathway and, in the case of plants and cyanobacteria, in the light phase of photosynthesis. The major catabolic redox coenzyme is nicotinamide adenine dinucleotide (NAD) which lacks one phosphate group in comparison to NADP and thus has different binding properties, enabling enzymes to differentiate between NAD and NADP. The redox pools of NAD/NADH and NADP/NADPH are connected through the enzyme nicotine amide nucleotide transhydrogenase, a multi-domain membrane protein (Pedersen et al. 2008). Quinones, especially ubiquinone,

plastoquinone, menaquinone and related molecules play a central role in many bioenergetic reactions. Quinones take up electrons from various metabolic reactions and can channel their redox-energy into the Q-cycle that is catalyzed by *bc*-type cytochromes (Mitchell 1976; Crofts 2004). These membrane-linked redox buffers are in contact with the redox buffers in the aqueous medium in the cell through several enzymes. A description of the mechanism of *bc*-type cytochromes are discussed in articles by Xia et al. Trumpower and Hasan et al. in this volume. Although this short discussion of the different redox reactions in the cell shows only the tip of the iceberg, it underlines the prevalence and importance of complex biological redox reactions for biochemical processes. Many more different redox reactions play a central role in the metabolism and we are only at the beginning of understanding what is happening in the cells and how complex enzymes direct and use the electron flow in the cell.

There are two groups of proteins that can undergo redox changes: (i) the electron transport proteins that simply take up electrons from a protein complex and transport them further to other complexes (e.g. monomeric cytochromes, ferredoxins, flavodoxins, thioredoxins, and cupredoxins); (ii) the large group of oxido-reductase enzymes which catalyze redox reactions often with the help of metal ions or other prosthetic groups. The first group played an important role in the theoretical analysis of electron transport in proteins since they are relatively simple and therefore allow a detailed study of the electron transfer reactions. Much of our understanding of electron transfer reactions in proteins originates from studies of such small proteins such as cytochrome *c* or azurin in connection with non-natural electron transfer partners (Gray and Winkler 1996). Complex oxido-reductase enzymes are instead a challenge, since they often involve many redox-active sites and couple the redox reactions for instance to protonation

reactions or conformational changes. The theoretical understanding of these complex reactions is still in its infancy, however much of the theoretical framework developed from simple electron transfer reactions can be employed.

Although electron transfer proteins often contain cofactors, there are also a few proteinogenic amino acids that are redox-active. In particular, pairs of cysteine residues, which can form disulfide bridges, are common redox-regulated motives, for instance in thioredoxins and also in many other redox sensitive proteins (Woycechowsky and Raines 2000). Furthermore, also tyrosine and tryptophan residues are known to undergo redox state changes in proteins (Warren et al. 2012). Another amino acid that can undergo redox changes in proteins is glycine, which can form radicals (Stubbe and van der Donk 1998). These glycine radicals in conjunction with a cysteine residue can catalyze complex chemical reactions in so-called glycine radical enzymes (Stubbe and van der Donk 1998). In the resting form of the enzyme, a radical is localized at a glycine residue. Upon substrate binding, the glycyl radical abstracts a hydrogen atom from an adjacent cysteine residue, which then performs the actual catalytic reaction. Another redox-active amino acid that is sometimes found in proteins is seleno-cysteine in which the thiol group is replaced by a selenol group. The pK_a value of the selenol is 5.2, much lower than that of a thiol (8.3), and its redox properties are also considerably shifted (Wessjohann et al. 2007). Besides these redox-active aminoacids, there are also protein-derived cofactors that are post-translationally formed by chemical modifications of amino acids (Davidson 2007). Examples of such groups are trihydroxyphenyl-alanine quinone, lysine tyrosylquinone, cysteine tryptophylquinone or tryptophan tryptophylquinone. Other cofactors are generated by cross-linking aminoacids such as, for instance, tyrosine and histidine in the active site of cytochrome c oxidase.

However, the large majority of electron transfer proteins bind redox cofactors as prosthetic groups. Such cofactors can be organic cofactors such as flavins or quinones; pure metal clusters such as copper centers, iron sulfur centers, non-heme iron centers, nickel-iron or molybdenum-iron centers, or manganese centers; or they can be of mixed organic/inorganic origin such as hemes or molybdopterin-centers (da Silva and Williams 2001). A special role is played by chlorophyll molecules, which are tetrapyrrole cofactors, similar to hemes, with a non-redox-active magnesium ion in the center of the ring. Pheophytins have an identical organic scaffold but lack any central ion. These later two cofactors are normally involved in light harvesting and participate in electron transfer only in photosynthetic reaction centers.

Many of the redox enzymes that perform complex reactions contain more than one redox center. Famous examples are the photosynthetic reaction center, which couples photoexcitation to charge separation, and the cytochrome bc_1 and cytochrome b_6f complexes, which generate a trans-membrane proton electrochemical gradient through bifurcation reactions, and cytochrome c oxidase, which couples the exergonic reduction of oxygen to an endergonic proton transfer across the membrane. Some examples are shown in Fig. 6.1. A crucial point for the understanding of the mechanism of these enzymes is the coupling of the electron transfer reactions to proton transfer reactions, to conformational changes and to other electron transfer reactions. A theoretical analysis of electron transfer reactions can help to reveal the mechanism of complex enzymes. In order to properly model complex electron transfer reactions that are coupled to conformational changes, to proton transfer reactions, or to other chemical reactions, different theoretical methods need to be combined. In this article, we will review the most important methods and explain the theoretical framework for analyzing complex reactions.

II. Theoretical Description of Electron Transfer Reactions

A. Marcus Theory of Electron Transfer

Even if biological redox reactions can involve association and dissociation reactions and can thus be bimolecular (Eq. 6.1), the actual electron transfer reaction is monomolecular:

$$D^- + A \leftrightharpoons D^- \cdots A \overset{ET}{\leftrightharpoons} D \cdots A^- \leftrightharpoons D + A^-$$
(6.1)
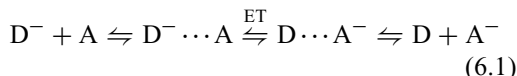
This system consists of two redox active sites, the donor (D) and the acceptor (A). In the reactant state ($D^- \cdots A$), the electron is localized at the donor and in the product state ($D \cdots A^-$), the electron is localized at the acceptor. The actual electron transfer step can be described by transition state theory, i.e. to obtain the following rate constants for the forward (k_{for}) and for the backward (k_{back}) reaction. One can write:

$$k_{for} = \frac{\zeta}{\beta h} \exp\left(-\beta G^{\neq}\right)$$
(6.2)

$$k_{back} = \frac{\zeta}{\beta h} \exp\left(-\beta(G^{\neq} + \Delta G^{\circ})\right)$$
(6.3)

where $G^{\neq}$ is the free activation energy, ΔG° is the reaction free energy, ζ is the transmission coefficient which accounts for the probability of being reactive once the transition state is reached, β is $1/k_B T$ (k_B is the Boltzmann constant, T the absolute temperature), and h the Planck constant (see Fig. 6.2).

For long range electron transfer reactions, the donor and the acceptor are only weakly electronically coupled. Thus, the reaction is non-adiabatic and its rate can be obtained from Fermi's golden rule (Marcus and Sutin 1985).

$$k_{ET} = \frac{2\pi}{\hbar} H_{DA}^2 (FC)$$
(6.4)

where H_{DA}^2 is the electronic coupling between the reactant state and the product state (sometimes called donor state and acceptor state, respectively) and (FC) is the
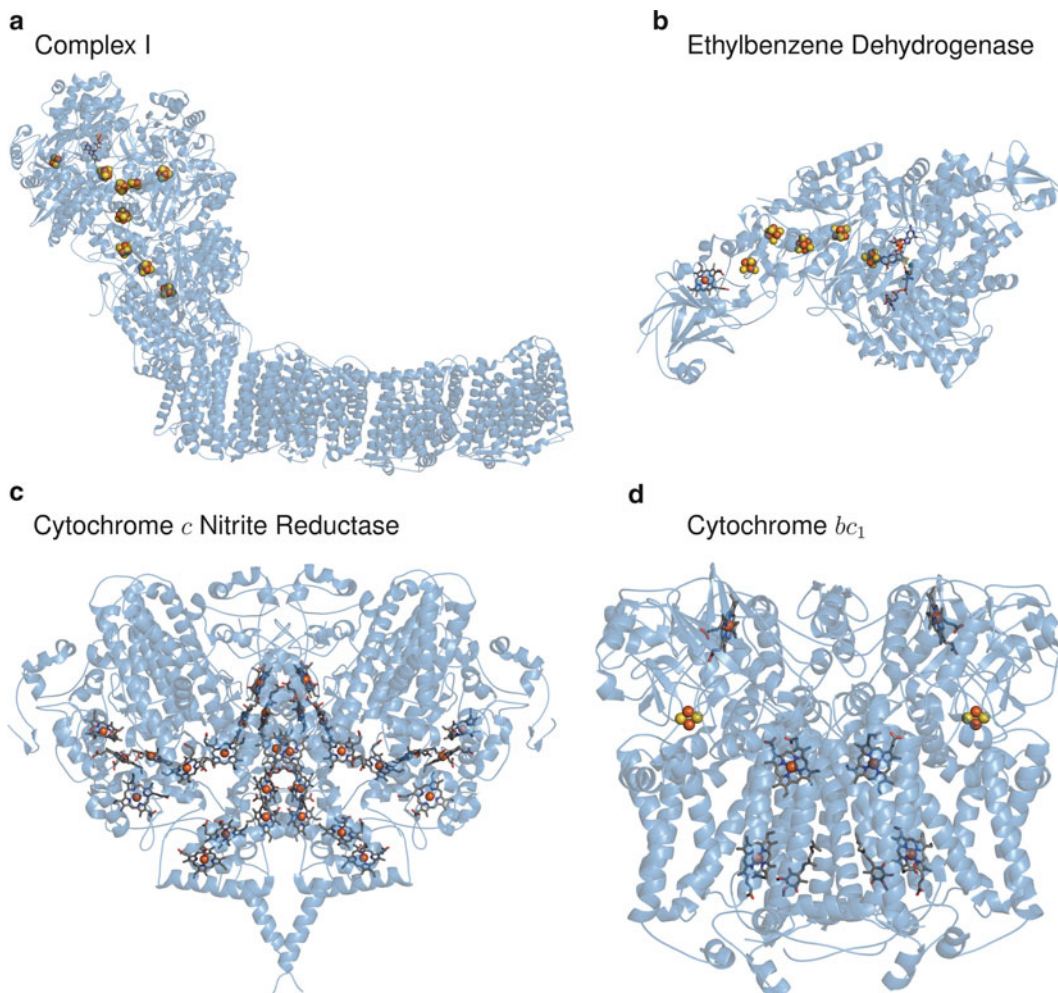
Fig. 6.1. Multicenter redox proteins. (**a**) Complex I catalyzes the transfer of electrons from NADH to ubiquinone. The electron transfer via several iron sulfur centers drives a proton transfer across the membrane in a not understood way. (**b**) Ethylbenzene Dehydrogenase is a molyptopterin enzyme. The electron transfer to the active site occurs via one heme and several iron-sulfur centers. (**c**) Cytochrome *c* Nitrite Reductase catalyzes the six-electron reduction of nitrite to ammonia. The electrons are provided by several heme centers in the protein. This enzyme couples the reduction of ammonia to the quinone/quinol pool in the membrane. (**d**) Cytochrome *bc*₁ uses the driving force of the electron transfer from ubiquinol to cytochrome *c* to generate a proton gradient. This proton pumping is achieved by a complex redox loop reaction called the Q-cycle.

Franck-Condon weighted density of states, i.e. the Franck-Condon factor. Equation 6.4 separates the electronic factors (represented by the electronic coupling) from the nuclear factors (represented by the Franck-Condon factor which accounts for the structural adaptation of the donor site, the acceptor site, and their surroundings) (Marcus and Sutin 1985). A classical form of the Franck-Condon factor is given by the following expression:

$$(FC) = \frac{1}{\sqrt{4\pi\beta^{-1}\lambda}} \exp\left(-\beta\frac{(\Delta G^\circ + \lambda)^2}{4\lambda}\right)$$

(6.5)

where λ is the reorganization energy and ΔG° is the reaction free energy. The reor-
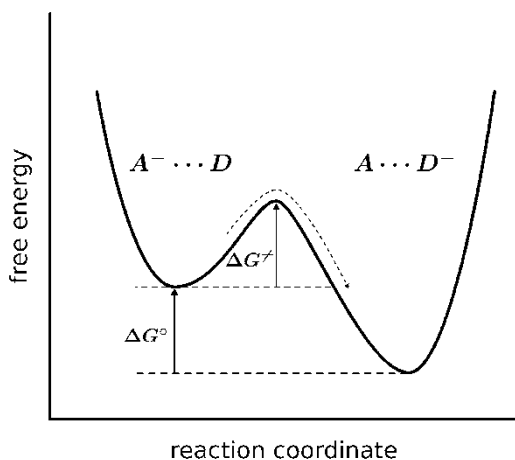
Fig. 6.2. The transition from the reactant state (or donor state) to the product state (or acceptor state) occurs over a barrier $\Delta G^{\neq}$. The free energy difference between the two states is ΔG°. Thus, the back reaction has a barrier that is the sum of the two energies $\Delta G^{\neq} + \Delta G^{\circ}$.

ganization energy accounts for the structural reorganization of the reactant state to the product state upon the reaction. Comparing Eq. 6.2 with Eqs. 6.4 and 6.5, one can recognize that the term $\frac{(\Delta G^{\circ} + \lambda)^2}{4\lambda}$ corresponds to the activation free energy. Instead, the electronic coupling together with the preexponential factor of Eq. 6.5 are related to the transmission coefficient ζ in Eq. 6.2.

In summary, electron transfer reactions are influenced by three energetic parameters: the electronic coupling of the donor and the acceptor, the reaction free energy, and the reorganization energy. Each of these parameters can be estimated from molecular structures using various theoretical models as discussed below.

B. Electronic Coupling

The electronic coupling quantifies how easily an electron can move from the donor to the acceptor. The closer the donor site and the acceptor site the more rapid the transfer. If the distance between the redox centers in a protein becomes larger than 20 Å, the electron transfer rate between them is so slow

that it is basically inconsequential. The actual electron transfer is a tunneling event, in which the electronic states of the protein are used as a bridge. Several theoretical methods have been developed to calculate the tunneling efficiency.

In the simplest model, an electron with the mass m tunnels between two narrow wells that are separated by a uniform energy barrier of the height V. The electronic coupling in such a system decreases exponentially with the distance R between the wells (Gamow 1928; Gray and Winkler 2005).

$$\begin{aligned} {H_{DA}}^2 &= {H_{DA}^{\circ}}^2 \exp\left(-\frac{2\sqrt{2mV}}{\hbar}(R - R_{\circ})\right) \\ &= {H_{DA}^{\circ}}^2 \exp\left(-b(R - R_{\circ})\right) \end{aligned} \qquad (6.6)$$

This model describes reasonably well the distance and the barrier height dependence of electron transfer between two cofactors embedded in a protein matrix. This picture captures the view that the electronic coupling between redox cofactors in a protein medium is mainly dependent on the distance between the cofactors. The intervening medium represents a barrier to the electron. The protein medium lowers the barrier compared to the vacuum and provides an electronic bridge. To estimate electronic coupling between cofactors in a protein, different models have been developed ranging from methods based on quantum chemical calculations (Stuchebrukhov 2003), to electron transfer pathways (Gray and Winkler 1996), or a simple, experimentally parametrized distance dependence (Moser et al. 1992; Page et al. 1999) just to name some of the models [also see Krishtalik in this volume]. In the later model, the specific nature of the intervening protein medium such as secondary or tertiary structure is not taken into consideration. Surprisingly, the different methods gave similar results leading to an exponential decay of the electronic coupling as a function of the distance between the donor site and the acceptor site. This finding indicates that the

excited reactant state r*

excited product state p*

reactant state r
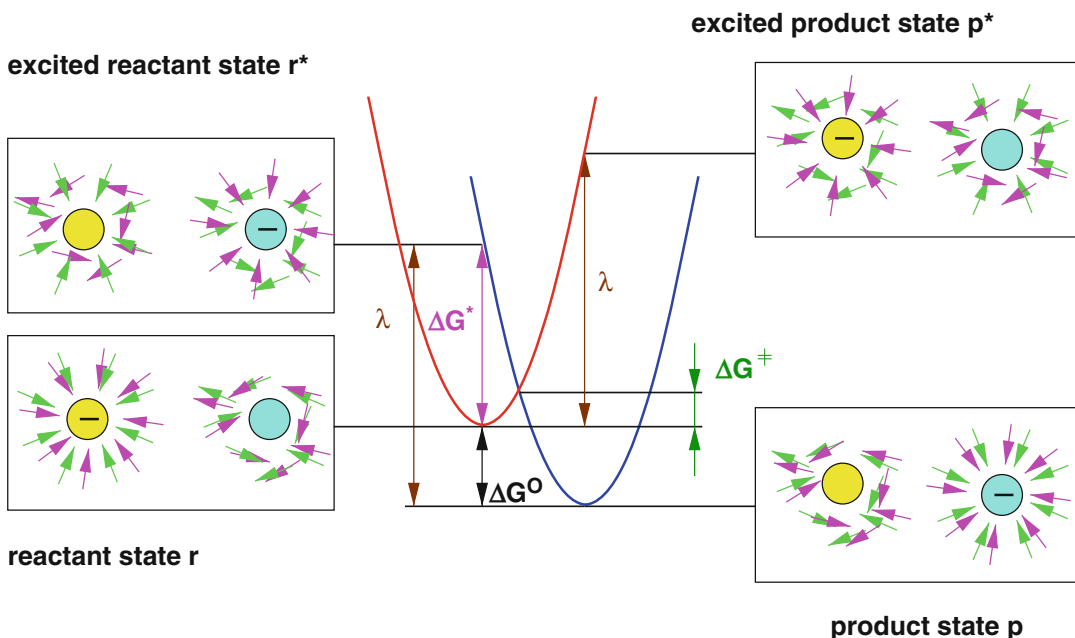
product state p

Fig. 6.3. The Marcus model of electron transfer. The reactant and the product states are described as *red* and *blue* harmonic potentials, respectively. The energy difference between the two states, i.e. the reaction free energy, is given by ΔG°. The energy ΔG^* represents the vertical excitation energy leading to the Franck-Condon state. The reorganization energy λ is the energy required to adopt the nuclear configuration of the product state without leaving the potential energy curve of the reactant state. The same reorganization energy can be defined for the backward reaction. As can be seen, $\lambda = \Delta G^\circ + \Delta G^*$. The activation free energy $\Delta G^{\neq}$ can be obtained from the reorganization energy and the reaction free energy. The idea of Marcus was to separate the fast relaxing electronic polarization (represented by *magenta arrows*) from the slowly relaxing solvent polarization (represented by *green arrows*). In the equilibrium states, both polarizations adapt fully to the given charge distribution, while in the vertically excited Franck-Condon state only the electronic polarization is adapted to the charge distribution.

electronic coupling is not much influenced by the specific nature of the protein medium.

C. Reorganization Energy

The reorganization energy λ accounts for the structural reorganization of the reactant and the product state including the surrounding medium. Figure 6.3 shows the meaning of the reorganization energy. Assuming a harmonic potential, the reorganization energy is the energy required to adapt the geometry of the product state in the reactant state and vice versa. The potential energy of the two states can then be expressed as

$$V_r(\vec{x}) = G_r + \frac{1}{2}k(\vec{x} - \vec{x}_r)^2 \qquad (6.7)$$

$$V_p(\vec{x}) = G_p + \frac{1}{2}k(\vec{x} - \vec{x}_p)^2 \qquad (6.8)$$

where $\vec{x}$ represents the collective coordinates of the system, i.e. the position of all atoms, the index p indicates the product state, the index r the reactant state, and k is a force constant. The reorganization energy is given by

$$\lambda = \frac{1}{2}k(\vec{x}_p - \vec{x}_r)^2 \qquad (6.9)$$

Since the electron transfer reaction is accompanied with large changes in the electrostatic potential because of the transferred charge, the effects on the medium surrounding the redox-active sites can be rather long range (on the order of 20 Å). Therefore, it is appropriate to separate the reorganization energy into an inner sphere reorganization

energy λ_i and an outer sphere reorganization energy $\lambda_\circ$

$$\lambda = \lambda_i + \lambda_\circ \qquad (6.10)$$

The inner sphere reorganization energy is mainly connected to changes in the redox active site itself and can be calculated by an expression that is similar to Eq. 6.9. The outer reorganization energy is connected to changes in the surrounding medium such as the solvent or the protein environment. Marcus suggested that the outer sphere reorganization energy can be calculated on the basis of a continuum electrostatic model (Marcus 1956). Namely, the idea is to separate the fast electronic response $(10^{-15} \cdots 10^{-16} \, \text{s})$ from the slow molecular (orientational) response $(10^{-11} \cdots 10^{-14} \, \text{s})$ as explained in Fig. 6.3. This part can be described by an electrostatic model as will be discussed below (section "III.B. Calculating the Outer Sphere Reorganization Energies").

D. Reaction Free Energy

The reaction free energy ΔG° is often approximated by the difference between the donor and acceptor redox groups. Even if this approximation is often justified, it is not always correct, since the interaction between the redox active groups influences the reaction free energy (Ullmann and Bombarda 2013). The situation becomes even more complex when the redox reaction is coupled to proton binding or the protein contains many redox active cofactors. Instead, it is always correct to consider the reaction free energy as the energy difference between the product state and the reactant state:

$$\Delta G = G_p - G_r \qquad (6.11)$$

The microstate model (Becker et al. 2007; Bombarda and Ullmann 2011; Ullmann and Bombarda 2013), which will be explained in section "III.D. Interacting Redox Active Groups," takes this energy difference correctly into account.

E. Moser-Dutton Ruler

A practical although not exclusive way of calculating electron transfer rate constants is the Moser-Dutton ruler (Moser et al. 1992; Page et al. 1999), which represents an empirical formula for electron transfer rates that relies on the theoretical basis of Marcus theory. The parameters were obtained by fitting known electron transfer rates to a linear equation of the form

$$\lg k_{et}^{ex} = A - B(R - R_\circ) - C \frac{(\Delta G^\circ + \lambda)^2}{\lambda} \qquad (6.12)$$

A was found to be 13.0; B has an average value of $0.6 \, \text{Å}^{-1}$ (a more complex expression can be used if the packing density is considered (Page et al. 1999)); C has a value of $3.1 \, (\text{eV})^{-1}$. $R_\circ$ is the van der Waals contact distance, which was assumed to be 3.6 Å. For endergonic reaction, Eq. 6.12 was extended to account for the uphill step (Eq. 6.13) with D = 0.06 eV.

$$\lg k_{et}^{ex} = A - B(R - R_\circ) - C \frac{(-\Delta G^\circ + \lambda)^2}{\lambda} - \frac{\Delta G^\circ}{D} \qquad (6.13)$$

The justification of Eq. 6.13 has been discussed in the literature and an alternative formulation based on the Marcus equation was proposed (Crofts and Rose 2007).

There are several other methods to estimate electron transfer rates. One of the more popular methods is the pathway model that was developed by Gray, Beratan, Onuchic, and coworkers (Gray and Winkler 1996). This model estimates the electronic coupling as a product of through-bond/through-space/through-hydrogen-bond couplings. Other methods rely on quantum chemical methods (Stuchebrukhov 2003). However, a major advantage of the Moser-Dutton ruler is its simplicity and its clear connection to molecular structures. The parameter R is the edge-to-edge distance between the cofactors involved in the electron transfer process. This distance can be estimated from

crystal structures of the relevant proteins. The other two parameters, namely the reorganization energy λ and the reaction free energy $\Delta G°$, can be also estimated from the structure either by molecular mechanics calculations (Beveridge and DiCapua 1989; Muegge et al. 1997) or by continuum electrostatic calculations (Sharp 1998; Ullmann and Knapp 1999).

F. Tuning of Electron Transfer Rates in Proteins

The protein can tune electron transfer rate constants by adapting the three energetic parameters of the Marcus equation: the electronic coupling, the reorganization energy and the reaction free energy. As discussed above, the electronic coupling between the redox cofactors can only be efficiently varied by changing the distance between them. Changing protein residues in the electron pathways between the donor moiety and the acceptor moiety has only minor effects presumably because the packing between the donor and the acceptor does not change very much and thus the electron always finds pathways that are equally well-suited to transfer the electron (Ullmann and Kostić 1995). Thus, in a given protein fold, the electron coupling cannot be much changed unless larger conformational changes occur. An example of such a conformational change affecting electron transfer reactions is the movement of the Rieske-head domain in cytochrome bc_1 shown in Fig. 6.4 (Zhang et al. 1998).

As discussed above, another parameter of the Marcus equation is the reorganization energy. The inner sphere reorganization energy is mainly connected to the nature of the redox active group (Olsson et al. 1998; Ryde and Olsson 2001). The protein can influence this parameter only to some extent, for instance through strain exerted on the redox center. The outer sphere reorganization energy is connected to the solvent and protein reorganization. Thus, this parameter cannot also be
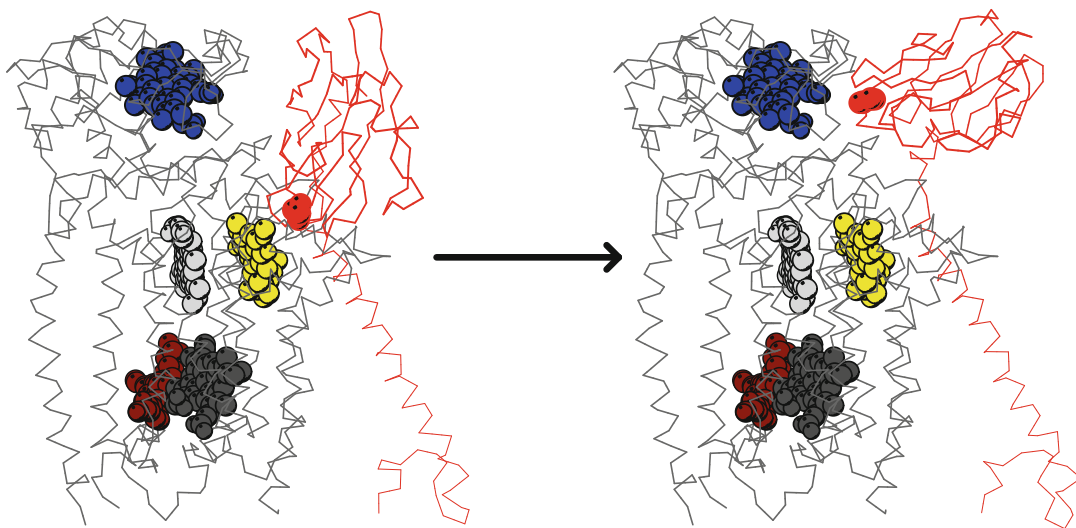
Fig. 6.4. Movement of the Rieske-Domain in cytochrome bc_1. The Rieske iron sulfur center (*red balls*) receives an electron from an ubiquinol (*yellow*) and transports this electron to cytochrome c_1. The second electron of the ubiquinol is transferred to the heme b_p (*light grey*) and from there further to the heme b_n (*dark grey*) and a quinone (*brown*). The subscript p or n indicate whether the heme is closer to the electrochemically positive or negative side of the membrane, respectively. The conformational change of the Rieske domain (shown in *red*) facilitates the electron transfer from the Rieske iron sulfur center to cytochrome c_1 by changing the electronic coupling between the redox centers. The movement of the Rieske domain is a key element of the Q-cycle through which the cytochrome bc_1 complex can generate a proton gradient.

much influenced in a given protein scaffold, since the protein has a low polarizability. Nevertheless, the outer sphere reorganization energy is influenced by the solvent exposure of the redox active group and the polarity of the protein medium.

The parameter that is most easily influenced by the protein environment is the reaction free energy. Biological systems have several ways to tune the redox properties of a particular site. The nature of the redox active site is the most crucial. Depending on whether an iron-sulfur cluster or a heme is chosen as a redox site, the redox potential will be different. Also the nature of the ligands of the metal center influences the redox properties. Thus, for instance it is known from model compounds of heme proteins, that the change from a histidine-histidine ligated heme to a histidine-methionine ligated heme decreases the redox potential from -70 to -220 mV (Wilson 1983). The protein environment is probably most important for the fine tuning of redox properties of a redox-active site (Zheng and Gunner 2009). The protein environment can tune the redox potential by (i) placement of charges or dipoles in the vicinity of the redox active site (for instance hydrogen bonds or salt bridges), (ii) changing the solvent accessibility of the redox active site, and (iii) coupling of the reduction to the protonation of a nearby site (redox-Bohr effect).

III. Electrostatic Methods for Estimating Reaction Free Energies and Reorganization Energies

The protein can electrostatically tune the redox potential of a redox active group. This tuning can be understood theoretically by using a continuum electrostatic model of the protein.

A. The Poisson-Boltzmann Equation

Electrostatic interactions are the most important interactions in biomolecules. Most of the effects in biochemical systems are dominated by electrostatics. It is therefore not too surprising that an electrostatic model can describe very well many features of biomolecular systems. This model relies on the Poisson-Boltzmann equation.

The basic idea of the Poisson-Boltzmann model is to describe the protein as a region with a low dielectric permittivity which is embedded in a region of high dielectric permittivity (aqueous solvent). The charge distribution of the protein is described by a fixed charge distribution in the low dielectric region, which is given by the molecular structure of the protein. Charges and dipoles are modeled by (fractional) point charges that are placed at the center of the atoms. The dissolved ions are represented by a Boltzmann-distributed charge density. The boundary of the low dielectric region is defined by the solvent accessible surface of the protein (Connolly 1983). Mathematically, the whole system is described by the Poisson-Boltzmann equation (Eq. 6.14) (Warwicker and Watson 1982; Honig and Nicholls 1995),

$$\nabla\left[\varepsilon(\mathbf{r})\nabla\phi(\mathbf{r})\right] = -\rho_f(\mathbf{r}) - \sum_{i=1}^{K} c_i^{\text{bulk}} Z_i e_{\text{o}}$$

$$\times \exp\left(\frac{-Z_i e_{\text{o}}\phi(\mathbf{r})}{RT}\right) \qquad (6.14)$$

where ∇ is the differential operator, $\varepsilon(\mathbf{r})$ is the spatially varying dielectric permittivity, $\rho_f(\mathbf{r})$ is the spatially fixed charge density which is usually defined by point charges at the positions of the nuclei of the solute molecule; c_i^{bulk} is the bulk concentration of the ionic species i with the charge Z_i (e_{o} is the elementary charge). The sum runs over all K ionic species in the solvent and describes a Boltzmann distribution of the ions in the electrostatic potential $\phi(\mathbf{r})$ of the protein. The electrostatic potential $\phi(\mathbf{r})$ can be obtained by solving Eq. 6.14.

Generally, non-linear partial differential equations like the one in Eq. 6.14 are difficult to solve even numerically. However by approximating the exponential term as a

series, Eq. 6.14 can be linearized. With the common definitions of the ionic strength $I = \frac{1}{2} \sum_{i=1}^{K} c_i^{\text{bulk}} Z_i^2$ and the modified inverse Debye length $\bar{\kappa} = \sqrt{\frac{2 N_A e_o^2 I}{k_B T}}$ the linearized Poisson-Boltzmann equation assumes the form that is found in biophysics text books (Eq. 6.15).

$$\nabla \left[\varepsilon(\mathbf{r}) \nabla \phi(\mathbf{r}) \right] = -\rho_{prot}(\mathbf{r}) + \bar{\kappa}^2(\mathbf{r}) \phi(\mathbf{r}) \quad (6.15)$$

In Eq. 6.15, the term $\varepsilon(\mathbf{r})$ reflects the spatially varying permittivity (or dielectric constant). The first term on the right hand side describes the spatially fixed charge distribution in the protein and the second term describes the charge distribution due to the mobile ions which adopt a Boltzmann distribution in the field of the protein.

The Poisson-Boltzmann equation in its linearized form can be solved analytically only for particular geometries. However, there are several methods that can solve the Poisson-Boltzmann equation numerically for arbitrary geometries. The solution of the Poisson-Boltzmann equation can be expressed as a potential that is composed of two contributions:

$$\phi(\mathbf{r}) = \sum_{i=1}^{M} \frac{q_i}{4\pi \varepsilon_p \mid \mathbf{r} - \mathbf{r}_i' \mid} + \phi_{\text{rf}}(\mathbf{r}) \quad (6.16)$$

First, the Coulomb potential at the position $\mathbf{r}$ caused by M point charges q_i at positions $\mathbf{r}_i'$ in a medium with a permittivity ε_p, and second, the reaction field potential arising from the M point charges q_i and the dielectric boundary between the protein and the solvent, as well as from the distribution of ions in the solution. The reaction field potential originates from the polarization of the solvent by the solute. Usually, the reaction field potential stabilizes charged states. At the molecular level, a smaller part of this polarization originates from the deformation of the electron density of the solvent due to the presence of the solute, while the larger part originates from the orientational polarization of the solvent molecules.

The total electrostatic energy of a system in aqueous solution consists of two parts: the interaction between the components of the system and the interaction of the system with the solvent. The first contribution is obtained by charging the charge set ρ_2 in the presence of the electrostatic potential caused by charge ρ_1. Assuming that the charge set ρ_2 consists of a single charge q_f, the interaction energy becomes

$$G_{\text{inter}} = \int_0^{q_f} \phi(\rho_1, \mathbf{r}_q) \mathrm{d}q = \phi(\rho_1, \mathbf{r}_q) q_f \quad (6.17)$$

Since the potential $\phi(\rho_1, \mathbf{r}_q)$ at the position $\mathbf{r}_q$ of the charge q_f is totally independent of the charge q_f itself, the integration in Eq. 6.17 reduces to a simple multiplication. Equation 6.17 can be generalized to the interaction between two disjunct sets of charges $\{q\}$ and $\{p\}$, which is given by

$$G_{\text{inter}} = \sum_{i=1}^{N_q} q_i \, \phi(\{p\}, \mathbf{r}_{q_i}) = \sum_{i=1}^{N_p} p_i \, \phi(\{q\}, \mathbf{r}_{p_i})$$
$$(6.18)$$

where N_q and N_p are the number of charges in the charge sets $\{q\}$ and $\{p\}$, respectively, $\phi(\{p\}, \mathbf{r}_{q_i})$ is the potential caused by the charge set $\{p\}$ at the position of the charge q_i and $\phi(\{q\}, \mathbf{r}_{p_i})$ is the potential caused by the charge set $\{q\}$ at the position of the charge p_i. As can be seen from Eq. 6.18, this interaction energy is symmetric.

The second contribution is the reaction field energy, which is also sometimes called "self-energy". This energy originates from the interaction energy of the charge set $\{q\}$ with its own reaction field potential ϕ_{rf}. To obtain this energy, one imagines the charging of a particle in a dielectric medium, and asks what is the energy of this charging process. Analogous to Eq. 6.17, we can write

$$G_{\text{rf}} = \int_0^{q_f} \phi_{rf}(q, \mathbf{r}_q) \mathrm{d}q \quad (6.19)$$

In contrast to the situation in Eq. 6.17, the reaction field potential ϕ_{rf} depends on the

charge of the particle. For simplicity, one assumes a linear response, i.e. $\phi_{rf}(q_f, \mathbf{r}_q) = Cq_f$. From Eq. 6.19, we obtain

$$G_{\mathrm{rf}} = \int_0^{q_f} Cq \; \mathrm{d}q = \frac{1}{2}Cq_f^2 = \frac{1}{2}\phi_{rf}(q_f, \mathbf{r}_q)q_f$$

(6.20)

Equation 6.20 can be generalized to obtain the reaction field energy of a charge set $\{q\}$

$$G_{\mathrm{rf}} = \frac{1}{2}\sum_{i=1}^{N_q} q_i \; \phi_{\mathrm{rf}}(\{q\}, \mathbf{r}_{q_i})$$

(6.21)

As shown above, the factor $\frac{1}{2}$ in this equation is a consequence of the linear response approach.

The reaction field energy can be used to calculate the electrostatic contribution of the solvation energy, which is the energy to transfer from vacuum into a solvent with a given dielectric constant ε_s. This approach was used by Max Born to calculate the solvation energy of a ion with the radius r and the charge Z (Born 1920)

$$\Delta G_{solv} = -\frac{1}{4\pi\varepsilon_\circ}\frac{(Ze_\circ)^2}{2r}\left(1 - \frac{1}{\varepsilon_s}\right)$$

(6.22)

This equation is part of an expression for the outer reorganization energy derived by Marcus (1956), which will be discussed in the next section.

Although the continuum electrostatic approach is relatively simple, it is surprising how well it works to understand the energetics of biochemical systems (Ullmann et al. 2008). It can be used to analyze electron and proton transfer reactions as will be explained in the following.

B. Calculating the Outer Sphere Reorganization Energies

By separating the fast and the slow polarization, Marcus derived a simple expression for the outer sphere reorganization energy for the transfer of the charge Δe between two spherical ions with the radii a_1 and a_2
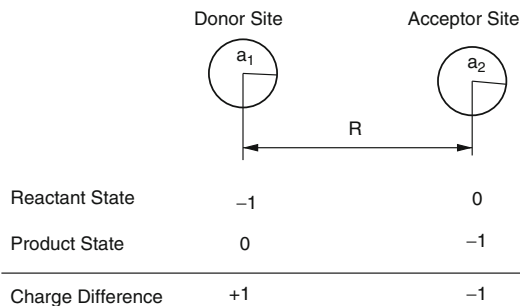
Fig. 6.5. Model of the charge transfer between two spheres at a separation R with the radii a_1 and a_2, respectively. The charge difference between the product state and the reactant state can be used to calculate the outer sphere reorganization energy and leads to Eq. 6.23.

at a distance R (Marcus 1956), leading to Eq. 6.23

$$\lambda_o = \frac{\Delta e^2}{4\pi\varepsilon_\circ}\left(\frac{1}{2}\left(\frac{1}{a_1} + \frac{1}{a_2}\right) - \frac{1}{R}\right)\left(\frac{1}{\varepsilon_{op}} - \frac{1}{\varepsilon_s}\right)$$

(6.23)

where ε_{op} is the optical dielectric constant and ε_s is the dielectric constant of the solvent (Fig. 6.5). The optical dielectric constant takes into account only the electronic polarization and thus has a value of about 2.

Equation 6.23 can be interpreted as having two contributions: (i) the difference between the solvation energy of ions in a low dielectric medium with a purely electronic polarization (ε_{op}) and a solvent with a higher dielectric constant ε_s and (ii) interaction energy of the charge difference between the reactant and the product state. Since the charge difference is considered, the charge at the donor site and the acceptor site is $+1$ and -1, respectively. The interaction between these charges gives rise to the $1/R$ term. The solvation energy difference is related to the Born formula (Eq. 6.22). Thus, the influence of the reaction field originating from the donor site is neglected at the acceptor site and vice versa. This approximation is valid if R is large. The outer sphere reorganization energy is always a positive number, since the optical dielectric constant, which

accounts only for the purely electronic polarization, is much smaller than the solvent dielectric constant and the separation between the charges is much larger than the radius of the spheres.

The idea that underlies Eq. 6.23 can be generalized such that it can be used to calculate the reorganization energy of a protein (Sharp 1998). For proteins, the numerical solution of the Poisson-Boltzmann equation must be used instead of the Born formula. The outer sphere reorganization energy in this frame work is given by

$$\lambda_o = \frac{1}{2}\sum_i \Big(\phi_{r*-r}(\{\Delta q\}, \mathbf{r_i}) - \phi_{p-r}(\{\Delta q\}, \mathbf{r_i})\Big)\Delta q_i \qquad (6.24)$$

where the charge set $\{\Delta q\}$ is the difference between charges assigned to the product state and the reactant state, respectively

$$\Delta q_i = q_i^p - q_i^r \qquad (6.25)$$

In Eq. 6.24, the term $\phi_{r*-r}(\{\Delta q\}, \mathbf{r_i})$ reflects the potential considering the fast electronic relaxation and is the numerical solution of Eq. 6.26

$$\nabla \varepsilon_{op} \nabla \phi_{r*-r}(\{\Delta q\}) = -\rho(\{\Delta q\}) \qquad (6.26)$$

The term $\phi_{p-r}(\{\Delta q\}, \mathbf{r_i})$ represents the potential considering slow orientiational relaxation which is given by the numerical solution of Eq. 6.27

$$\nabla \varepsilon(\mathbf{r}) \nabla \phi_{p-r}(\{\Delta q\}) = -\rho(\{\Delta q\}) + \bar{\kappa}^2 \phi_{p-r}(\{\Delta q\}) \qquad (6.27)$$

With this approach, the reorganization energy of a charge transfer reaction can be easily calculated on the basis of molecular structures using the numerical solution of the Poisson-Boltzmann equation. Interestingly, in this approach the outer sphere reorganization energy depends only on the charge difference between the reactant state and the donor state and to a minor extent on the shape of the low dielectric region defined by the protein. Thus, in the frame work of this continuum electrostatic model, the surrounding protein residues do not have much influence on the outer sphere reorganization energy except by defining the dielectric boundaries. Consequently, one would expect that the reorganization energy cannot be much altered by mutations.

C. Calculating Redox Potentials of Proteins

The redox potential of a group characterizes the energetics of an electron transfer reaction, since it is proportional to the energy change upon electron release or uptake. The calculation of absolute redox potentials requires very high-level quantum chemical calculations and is often not very accurate. However, changes in the redox potentials arising from the protein are mainly caused by electrostatic interactions. Thus, it is possible to determine the changes of the redox potential of a group A due to the protein environment compared to a model compound of the group for which the redox potential $E^{\mathrm{o}}_{\mathrm{model},A}$ is known. There are two contributions to the redox potential shift, one originates from the changes of the reaction field when the model compound is moved from the aqueous solution into the protein ($\Delta\Delta G^{\mathrm{redox}}_{\mathrm{Born},A}$). The other originates from the interaction of the redox active group with the charges and dipoles of the protein ($\Delta\Delta G^{\mathrm{redox}}_{\mathrm{back},A}$). The redox potential in the protein $E^{\mathrm{o}}_{\mathrm{intr},A}$ is thus expressed by

$$E^{\mathrm{o}}_{\mathrm{intr},A} = E^{\mathrm{o}}_{\mathrm{model},A} - \frac{1}{F}(\Delta\Delta G^{\mathrm{redox}}_{\mathrm{Born},A} + \Delta\Delta G^{\mathrm{redox}}_{\mathrm{back},A}) \qquad (6.28)$$

where

$$\Delta\Delta G^{\mathrm{redox}}_{\mathrm{Born,A}}$$

$$= \frac{1}{2}\sum_{i=1}^{N_{Q,A}} Q^{\mathrm{red}}_{i,A}[\phi_p(\mathbf{r}_i; Q^{\mathrm{red}}_A) - \phi_m(\mathbf{r}_i; Q^{\mathrm{red}}_A)]$$

$$- \frac{1}{2}\sum_{i=1}^{N_{Q,A}} Q^{\mathrm{ox}}_{i,A}[\phi_p(\mathbf{r}_i; Q^{\mathrm{ox}}_A) - \phi_m(\mathbf{r}_i; Q^{\mathrm{ox}}_A)]$$

$$(6.29)$$

$\Delta\Delta G_{\text{back,A}}^{\text{redox}}$

$$= \sum_{i=1}^{N_p} q_i[\phi_p(\mathbf{r}_i; Q_A^{\text{red}}) - \phi_p(\mathbf{r}_i; Q_A^{\text{ox}})]$$

$$- \sum_{i=1}^{N_m} q_i[\phi_m(\mathbf{r}_i; Q_A^{\text{red}}) - \phi_m(\mathbf{r}_i; Q_A^{\text{ox}})]$$

(6.30)

The summations in Eq. 6.29 run over the $N_{Q,A}$ atoms of group A that have different charges $Q_{i,A}^{\text{ox}}$ and $Q_{i,A}^{\text{red}}$ in the oxidized (ox) and in the reduced (red) state, respectively. The first summation in Eq. 6.30 runs over the N_p charges of the protein that belong to atoms in non-titratable groups. The second summation in Eq. 6.30 runs over the N_m charges of atoms of the model compound that do not have different charges in the different redox states. The terms $\phi_m(\mathbf{r}_i, Q_A^{\text{ox}})$, $\phi_m(\mathbf{r}_i, Q_A^{\text{red}})$, $\phi_p(\mathbf{r}_i, Q_A^{\text{ox}})$, and $\phi_p(\mathbf{r}_i, Q_A^{\text{red}})$ denote the values of the electrostatic potential at the position $\mathbf{r}$ of the atom i. The electrostatic potential can be obtained by solving the Poisson-Boltzmann equation numerically using the shape of either the protein (subscript p) or the model compound (subscript m) as dielectric boundary and assigning the charges of the redox-active group A in either the oxidized (Q_A^{ox}) or the reduced (Q_A^{red}) form to the respective atoms.

The background energy $\Delta\Delta G_{\text{back,A}}^{\text{redox}}$ reflects the interaction of the redox-active group with charges of the protein. Thus, it is this term that is mainly influenced by mutations of residues or by variations of the protonation state of nearby residues. The Born term $\Delta\Delta G_{\text{Born,A}}^{\text{redox}}$ is sensitive to changes in the protein shape and thus it may change in case the border between protein and solvent is modified by mutations or by conformational changes in the vicinity of the active site.

The equilibrium between the redox couple $A_{\text{ox}}/A_{\text{red}}^-$ is defined by

$$A_{\text{ox}} + e^- \overset{K_{ET}}{\rightleftharpoons} A_{\text{red}}^-; \qquad K_{ET} = \frac{[A_{\text{red}}^-]}{[A_{\text{ox}}][e^-]}$$

(6.31)
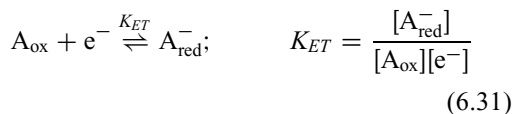
where K_{ET} is the equilibrium constant. One can define the solution redox potential E and the redox potential E^{o} of the redox couple $A_{\text{ox}}/A_{\text{red}}^-$ as $E = -RT/F \ln \gamma[e^-]$ and $E^{\text{o}} = RT/F \ln K_{ET}$, respectively. With the factor RT/F, where F is the Faraday constant, one obtains E and E^{o} in the units volts; γ is the activity coefficient. The relation between E^{o} and the standard reaction free energy $G_{\text{redox}}^{\text{o}}$ is given by $G_{\text{redox}}^{\text{o}} = FE^{\text{o}}$. With these definitions and Eq. 6.31, one obtains the Nernst equation for a single electron reduction:

$$E = E^{\text{o}} + \frac{RT}{F} \ln \frac{[A_{\text{ox}}]}{[A_{\text{red}}^-]}$$

(6.32)

The probability $\langle x \rangle$ that a redox-active group is in its oxidized state is defined as $\langle x \rangle = [A_{\text{ox}}]/([A_{\text{red}}^-] + [A_{\text{ox}}])$. The relation between the probability $\langle x \rangle$, the solution redox potential E, and the redox potential E^{o} is given by

$$\langle x \rangle = \frac{\exp\left(\frac{F}{RT}(E - E^{\text{o}})\right)}{1 + \exp\left(\frac{F}{RT}(E - E^{\text{o}})\right)}$$

(6.33)

Consequently, the free energy G_{redox} required to oxidize a one-electron redox-active group at a given redox potential of the solution E is given by Eq. 6.34.

$$G_{\text{redox}} = -F(E - E^{\text{o}}) = -RT \ln \frac{\langle x \rangle}{1 - \langle x \rangle}$$

(6.34)

D. Interacting Redox Active Groups

In many redox proteins, there is more than one redox active group and these redox active groups interact. Because of these interactions, the titration curves of the individual redox active sites can become irregular. These irregular titration curves originate from the population of several microstates. The simplest case is a system with two interacting sites.

Such a system has four possible microstates: fully reduced, reduced at one group, reduced at the other group, and fully oxidized (Fig. 6.6). These states can be respectively described by their redox
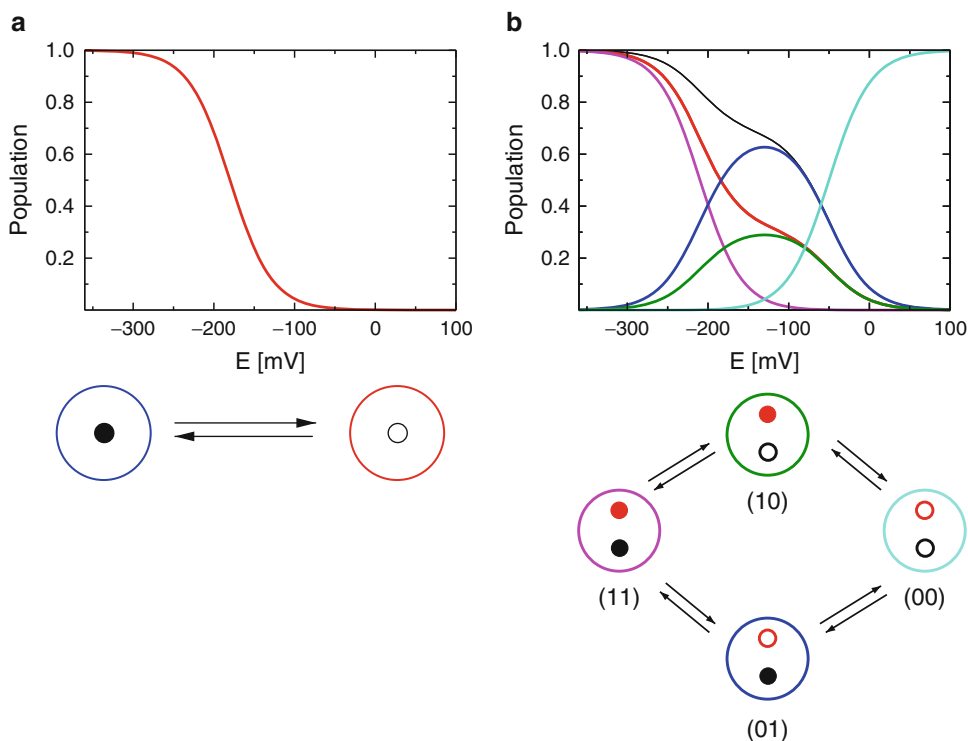
Fig. 6.6. Titration behavior of molecules with one and two redox-active groups. (**a**) Titration curve of a single redox-active group. The titration curve has a sigmoidal shape. (**b**) Titration curve of a molecule with two titratable groups. The contributions of the populations of the different microstates lead to non-sigmoidal titration curves. The microstates and their populations, the titration curves and the sites are color coded in the reaction scheme and in the diagram. For instance, the titration curve of the *red* site is the sum of the probability of the microstate population curve of the state (11) in *magenta* and the microstate population curve of state (10) in *green*.

state vectors: 11, 10, 01, and 00, where 1 marks that the group is reduced and 0 that it is oxidized. In order to obtain the titration curve of group one, the probabilities of all microstates in which this group is reduced need to be added. For instance the titration curve of group one is given by $\langle x_1 \rangle = \langle 10 \rangle + \langle 11 \rangle$. The titration curves of such groups can considerably deviate from standard sigmoidal titration curves and can show highly irregular features because of electrostatic interactions between the groups. The assignment of an E° value to one particular group is in such cases difficult if not impossible. To eliminate the difficulties, the problem can be reformulated. Instead of considering a protein as a system of groups with a certain probability of being reduced, the problem can be formulated in terms of

well-defined microstates of the protein which have a certain probability of occurrence as will now be outlined.

Let us consider a system that possesses K redox-active sites. Such a system can adopt $M = 2^K$ states assuming that each sites can exist in two forms. The interaction between them can be modeled electrostatically. Each state of the system can be written as a K-dimensional vector $\vec{x} = (x_1, \cdots, x_K)$, where x_i is 1 or 0 if site i is reduced or oxidized, respectively. Each state of the system has a well-defined energy which depends on the redox energetics of the individual sites (E_i^{intr}) and the interaction between sites (W_{ij}). The energy of a state $\vec{x}_\nu$ is given by (Bashford and Karplus 1990; Ullmann and Knapp 1999; Ullmann 2000; Gunner et al. 2006; Nielsen and McCammon 2003):

$$G(\vec{x}_\nu) = \sum_{i=1}^{K}(x_{\nu,i} - x_i^\circ)F(E - E_i^{\text{intr}})$$

$$+ \frac{1}{2}\sum_{i=1}^{K}\sum_{j=1}^{K}(x_{\nu,i} - x_i^\circ)(x_{\nu,j} - x_j^\circ)\,W_{ij} \quad (6.35)$$

where R is the gas constant, T the absolute temperature, and F the Faraday constant; $x_{\nu,i}$ denotes the redox form of the site i in state $\vec{x}_\nu$, x_i° is the reference form of site i; E_i^{intr} is the redox potential that site i would have if all other sites are in their reference state (this value is also called the intrinsic redox potential; see section "III.C. Calculating Redox Potentials of Proteins"); E is the reduction potential of the solution; W_{ij} represents the interaction energy between site i and j. It can be calculated from Eq. 6.36.

$$W_{ij} = \sum_{A=1}^{N_{Q,A}}[Q_{i,A}^{\text{red}} - Q_{i,A}^{\text{ox}}][\phi_p(\mathbf{r}_A, Q_j^{\text{red}}) - \phi_p(\mathbf{r}_A, Q_j^{\text{ox}})]$$

$$(6.36)$$

    Equation 6.35 can be used to calculate either microscopic redox potentials or also microscopic reaction free energies by subtracting the appropriate microstate energies from each other. For example, if we consider a system with two sites as depicted in Fig. 6.6, we can calculate a microscopic redox potential from the energy difference between the states (1,0) and (1,1). This microscopic redox potential will differ from the microscopic redox potential between the states (0,0) and (0,1) because of electrostatic interactions between the two groups. The reaction free energy is given by the energy difference between the states (0,1) and (1,0). This reaction free energy can be used in Marcus theory to calculate electron transfer rates.

    The equilibrium properties of a physical system are completely determined by the energies of its states. To keep the notation concise, states will be numbered by Greek indices in the subscript, i.e., for state energies we write G_ν instead of $G(\vec{x}_\nu)$. For site indices, the roman letters i and j will be used.

The equilibrium probability of a single state $\vec{x}_\nu$ is given by

$$p_\nu^{eq} = \frac{e^{-\beta G_\nu}}{Z} \quad (6.37)$$

with $\beta = 1/RT$ and Z being the partition function of the system.

$$Z = \sum_{\nu=1}^{M} e^{-\beta G_\nu} \quad (6.38)$$

The sum runs over all M possible states. Properties of single sites can be obtained from Eq. 6.37 by summing up the individual contributions of all states. For example, the probability of site i being reduced is given by

$$\langle x_i \rangle = \sum_{\nu}^{M} x_{\nu,i} p_\nu^{eq} \quad (6.39)$$

where $x_{\nu,i}$ denotes the redox form of site i in the charge state $\vec{x}_\nu$. For small systems, this sum can be evaluated explicitly. For larger systems, Monte-Carlo techniques can be used to determine these probabilities (Beroza et al. 1991; Ullmann and Ullmann 2012).

    In general, the titration curves in such a system do not have to be sigmoidal and can even be non-monotonic (Onufriev et al. 2001). However as a consequence of statistical thermodynamics, it can be proven that the macroscopic titration curve of a system can be always decomposed into standard titration curves (Nernst functions) as long as there is no positive cooperativity (Ullmann 2003; Ullmann and Bombarda 2013).

    In principle, 2^{K-1} different microscopic redox potentials can be assigned to each redox-active group in a protein, where K is the number of redox active groups in the molecule. Since however, most of the interactions are relatively weak, most of these microscopic redox potentials will be very similar. But in case of strong interactions, a larger variation of the redox potential can be observed. As an example, Fig. 6.7 shows the calculated redox potentials of the hemes
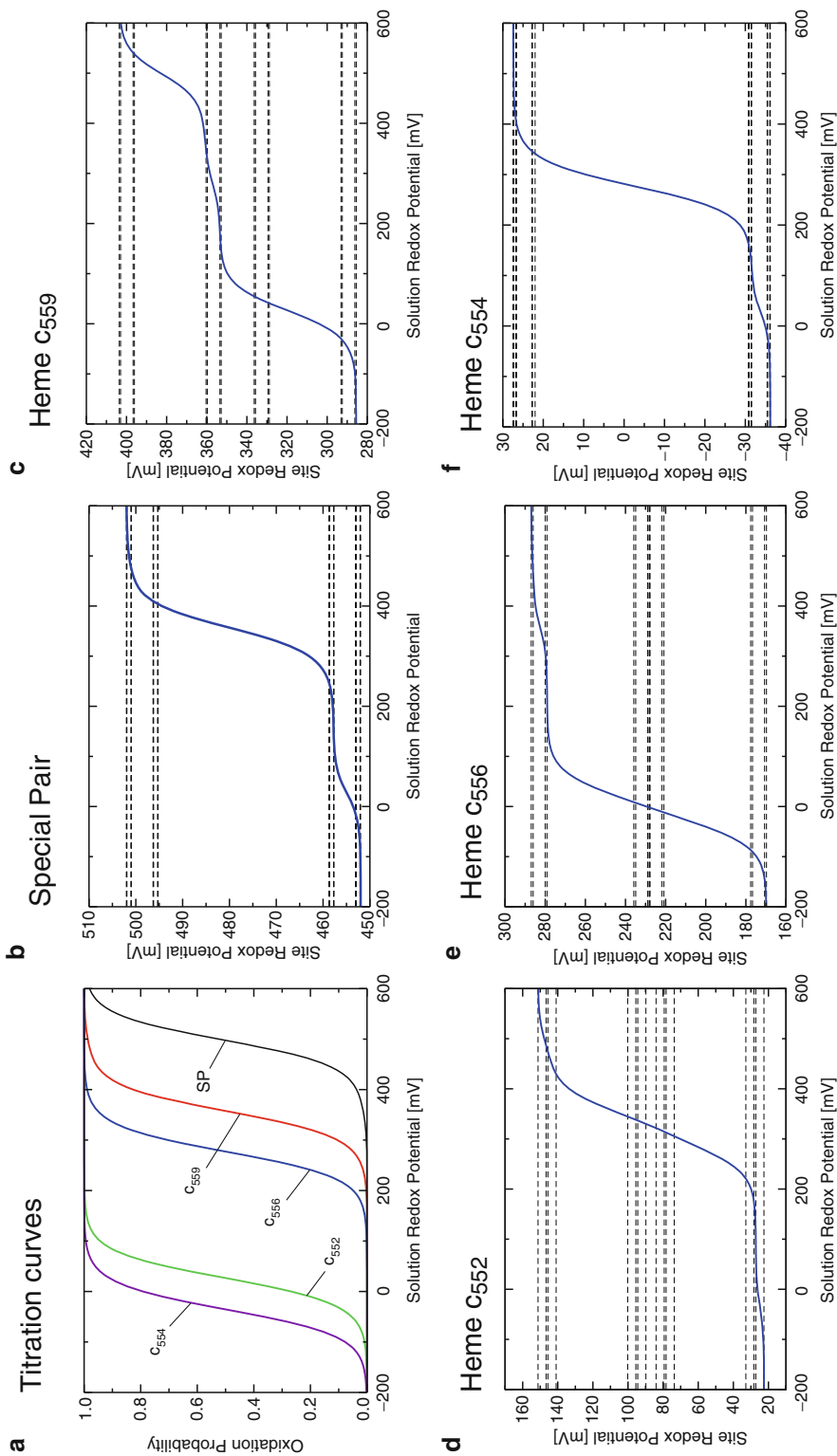
Fig. 6.7. Calculated redox titration behavior of special pair (SP) and the hemes of the RC from *B. viridis.* (**a**) Probability of the oxidized form of the redox-active sites; (**b**) to (**f**) solution redox-potential dependence of the site redox potential of the special pair (**b**), heme c_{559} (**c**), heme c_{552} (**d**), heme c_{556} (**e**), heme c_{554} (**f**). The *dashed lines* show the different microscopic redox potentials, the *blue lines* show the average redox-potential which depends on the solution redox potential.

and the special pair of bacteriochlorophylls in the photosynthetic reaction center from *B. viridis*. This protein possesses four heme groups that facilitate the rereduction of the special pair after photooxidation (Fig. 6.10). As can be seen from the graphs in Fig. 6.7, the microscopic redox potentials may vary quite substantially and cannot necessarily be read as midpoint potentials directly from the titration curves which are depicted in Fig. 6.7a. An effective redox potential or midpoint potential can be defined from the redox potential-dependent reduction probability

$$E_i^\circ = E - \frac{RT}{F}\left(\ln\frac{\langle x_i\rangle}{1 - \langle x_i\rangle}\right) \qquad (6.40)$$

This effective midpoint potential can depend on the solution redox potential because of the interaction between the redox active sites. It represents an equilibrium redox potential (Ullmann and Bombarda 2013). The value of this effective midpoint potential varies between the most extreme microscopic redox potentials (see Fig. 6.7). However, it must be underlined that this effective midpoint potential is not necessarily the functionally relevant redox potential at which an enzyme is working as will be shown later (section "IV. Complex Electron Transfer Proteins").

E. Looking at the Coupling of Reduction Reactions to Protonation Reaction

Redox reactions are often coupled to protonation reactions. Such a coupling can be very strong, meaning that as soon as an electron binds a proton also binds stoichiometrically. In other cases, the coupling is relatively weak, meaning that the coupling is substoichiometric. Biologically, this coupling of protonation and reduction can help on the one hand to tune the redox potential of a redox active group; on the other hand, it is essential for the electron transfer driven proton transfer that plays a central role in biological energy transduction (Nicholls and Ferguson 2013).

Since protonation and redox reactions can be considered in general as binding reactions, they are basically all described by the same underlying theory. Figure 6.8 summarizes and compares the similarity among the description of protonation reactions by the Henderson-Hasselbalch equation, redox equilibria by the Nernst equation, and a general thermodynamic description of binding equilibria. Because of this resemblance, protonation equilibria can be described by the same theoretical tools as redox equilibria. Since there are usually several protonatable groups in a protein, one has to consider the interaction between the protonatable groups and the mutual interaction between the protonatable groups and the redox active groups. The state vector defined above needs to be extended to a 2^{N+K} dimensional vector where N is the number of protonatable groups and K is number of redox active groups. Equation 6.35 needs to be extended to include the effect of the pH.

$$
\begin{aligned}
G(\vec{x}_\nu) &= \sum_{i=1}^{N}(x_{\nu,i} - x_i^\circ)RT\ln 10\,(\mathrm{pH} - \mathrm{p}K_{a,i}^{\mathrm{intr}}) \\
&+ \sum_{i=1}^{K}(x_{\nu,i} - x_i^\circ)F(E - E_i^{\mathrm{intr}}) \\
&+ \frac{1}{2}\sum_{i=1}^{N+K}\sum_{j=1}^{N+K}(x_{\nu,i} - x_i^\circ)(x_{\nu,j} - x_j^\circ)\,W_{ij}
\end{aligned} \quad (6.41)
$$

In this equation, $\mathrm{p}K_{a,i}^{\mathrm{intr}}$ is the intrinsic $\mathrm{p}K_a$ value, i.e., the $\mathrm{p}K_a$ value that the site would have if all other titratable groups are in their respective reference state. By analogy to the intrinsic redox potential, the intrinsic $\mathrm{p}K_a$ value is calculated using an appropriate model compound and takes into account the protonation energy shift due to the change in the solvation energy ($\Delta\Delta G_i^{\mathrm{Born}}$) and the change due to the interaction with non-titrating background charges ($\Delta\Delta G_i^{\mathrm{back}}$).

$$
\begin{aligned}
\mathrm{p}K_{a,i}^{\mathrm{intr}} &= \mathrm{p}K_a^{\mathrm{model}} - \frac{1}{RT\ln 10}(\Delta\Delta G_i^{\mathrm{Born}} \\
&\quad + \Delta\Delta G_i^{\mathrm{back}})
\end{aligned} \qquad (6.42)
$$

Protonation	Reduction	General Binding
$HA \overset{K_a}{\rightleftharpoons} A^- + H^+$	$A_{ox} + e^- \overset{K_{ET}}{\rightleftharpoons} A_{red}^-$	$R + L \overset{K}{\rightleftharpoons} RL$
Henderson-Hasselbalch Equation	Nernst Equation	Binding Equilibrium
$pK_a = pH - \lg \frac{[A^-]}{[HA]}$	$E^o = E + \frac{1}{\beta F} \ln \frac{[A_{red}^-]}{[A_{ox}]}$	$G^o = \mu_L + \frac{1}{\beta} \ln\left(\frac{[R]}{[RL]}\right)$
pH Value	Solution Redox Potential	Chemical Potential
$pH = -\lg\left(\gamma[H^+]\right)$	$E = -\ln\left(\gamma[e^-]\right)$	$\mu_L = \mu_L^o + \ln\left(\gamma[L]\right)$
Protonation Energy at	Reduction Energy at	Binding Energy at
a given pH value	a given solution redox potential	a given chemical potential
$G^{prot} = -\beta^{-1}\ln 10(pK_a - pH)$	$G^{red} = -F(E^o - E)$	$G^{bind} = -(G^o - \mu_L)$
Protonation Probability	Reduction Probability	Binding Probability
$\langle x \rangle = \frac{e^{-\ln 10(pH-pK_a)}}{1+e^{-\ln 10(pH-pK_a)}}$	$\langle x \rangle = \frac{e^{-\beta F(E-E^o)}}{1+e^{-\beta F(E-E^o)}}$	$\langle x \rangle = \frac{e^{-\beta(G^o-\mu_L)}}{1+e^{-\beta(G^o-\mu_L)}}$

Fig. 6.8. Comparison of the theoretical formulations of protonation equilibria, redox equilibria and general binding equilibria. The direct comparison shows the equivalence of the formulations and the relationship between the corresponding terms. Moreover, it allows to see the relation protonation reactions and reduction reactions to a more general binding formalism. The meaning of the symbols is explained in the text.

These energy shifts as well as the interaction energy W_{ij} between two groups can be calculated on the basis of the Poisson-Boltzmann equation as described above (Bashford and Karplus 1990; Ullmann and Knapp 1999).

The formalism described here is applicable only to sites that have two states, i.e., either protonated and deprotonated or oxidized and reduced. For some redox active sites such as quinones or flavins, the situation is more complicated, because they have multiple redox and protonation states for just one site. Furthermore, changes in the conformation of the amino acids side chain (rotamers) as well as larger conformational changes may

need to be considered. For all such cases, the formalism needs to be extended (Ullmann and Ullmann 2012). The equations become slightly more complicated; nevertheless, the basic philosophy stays the same and the microstate model can be applied.

F. Influence of Membrane Potential and Transmembrane Gradients

Many electron transfer proteins are embedded in membranes and generate an electrochemical gradient consisting of a proton gradient and a membrane potential across these membranes. This electrochemical

transmembrane gradient is used by ATP-synthase membrane protein complex to transduce the electrochemical energy into chemical energy in the form of ATP. Thus, the effect of transmembrane gradients and membrane potentials also need to be considered to describe energy transduction processes properly.

The influence of a membrane potential on the energetics of a membrane protein can be incorporated in the Poisson-Boltzmann theory (Roux 1997). The linearized Poisson-Boltzmann equation (see section "III.A. The Poisson-Boltzmann Equation") of a membrane system with a membrane potential Ψ present is given by

$$\nabla\left[\varepsilon(\mathbf{r})\nabla\phi(\mathbf{r})\right] = -\rho_f(\mathbf{r}) + \varepsilon(\mathbf{r})\overline{\kappa}(\mathbf{r})^2 \left[\phi(\mathbf{r}) - \Psi\Theta(\mathbf{r})\right] \quad (6.43)$$

where $\Theta(\mathbf{r})$ is a simple step function which is equal to zero at the extracellular side and equal to one at the cytoplasmic side (Heaviside step function). The potential $\phi(\mathbf{r})$ which is obtained as the numerical solution of Eq. 6.43 can be expressed as (You and Bashford 1995; Roux 1997)

$$\phi(\mathbf{r}) = \sum_{i=1}^{M} \frac{q_i}{\varepsilon_p \mid \mathbf{r} - \mathbf{r}_i' \mid} + \phi_{\text{rf}}(\mathbf{r}) + \Psi\chi_{\text{mp}}(\mathbf{r}) \quad (6.44)$$

As in Eq. 6.16, the first term describes the Coulomb electrostatic potential at the position $\mathbf{r}$ caused by M point charges q_i at positions $\mathbf{r}_i'$ in a medium with a dielectric permittivity ε_p. The second term describes the reaction field potential $\phi_{\text{rf}}(\mathbf{r})$ originating from the dielectric boundary between the protein and the solvent as well as from the distribution of ions in the solution, and the third term describes the potential originating from the transmembrane potential. In Eq. 6.44, $\chi_{\text{mp}}(\mathbf{r})$ is a dimensionless function, which depends on the dielectric properties of the system and the ion distribution in the medium but not on the charge distribution within the protein. The function $\chi_{\text{mp}}(\mathbf{r})$ has

the property that $0 \leq \chi_{\text{mp}}(\mathbf{r}) \leq 1$ (Roux 1997). This function $\chi_{\text{mp}}(\mathbf{r})$ describes how the membrane potential is modulated inside the membrane protein and can deviate from a simple linear function (Roux 1997).

The ions, protons and electrons that are transferred across the membrane are also influenced by the membrane potential. By analogy to the chemical potential μ_c of a substance c, one can define the electrochemical potential $\overline{\mu}_c$ for the ionic species c with charge z_c and activity $\gamma[c]$ in a system with a membrane potential Ψ:

$$\overline{\mu}_c = \mu_c + z_c F\Theta(\mathbf{r})\Psi \quad (6.45)$$

with

$$\mu_c = \overline{\mu}_c = \mu_c^\circ + RT \ln \gamma[c] \quad (6.46)$$

The energy for moving an ion across a membrane has thus two contributions: one arising from the work against the concentration difference between the two sides of the membrane and a second arising from work against the membrane potential Ψ (Cramer and Knaff 1991; Nicholls and Ferguson 2013). The work required to transfer an ion from one side of the membrane to the other is given by the difference between the electrochemical potential at the two sides of the membrane.

In order to account for the membrane potential in the energy calculation of a microstate, it is necessary to include the effect of the membrane potential on the binding energetics. Moreover, also the ligand concentrations on the two sides of the membrane may be different. Thus, a generalized version of Eq. 6.41 which uses binding free energies and electrochemical potentials instead of redox potentials, pK_a values and pH values defines the state energy for a transmembrane protein with N sites connected to the extracellular region (EC), and K sites connected to the cytoplasmic region (CP) (Calimet and Ullmann 2004; Bombarda et al. 2006) is

$$G_n(\overline{\mu}_{c,i}^{EC}, \overline{\mu}_{h,j}^{CP})$$

$$= \sum_{i=1}^{N}(x_i^n - x_i^{\circ})(\overline{G}_{\mathrm{intr},i}^{\circ} - \overline{\mu}_i^{EC})$$

$$+ \sum_{j=1}^{K}(x_j^n - x_j^{\circ})(\overline{G}_{\mathrm{intr},j}^{\circ} - \overline{\mu}_j^{CP})$$

$$+ \frac{1}{2}\sum_{i=1}^{N+K}\sum_{j=1}^{N+K} W_{ij}(x_i^n - x_i^{\circ})(x_j^n - x_j^{\circ})$$

(6.47)

where $\overline{\mu}_i^{EC}$ and $\overline{\mu}_j^{CP}$ are the electrochemical potentials of the ligand (for instance electrons or protons) binding to site i at the extracellular and site j at the cytoplasmic side, respectively. As before, the intrinsic binding energy $(\overline{G}_{\mathrm{intr},i}^{\circ})$ is the binding energy that the site i would have in the presence of a membrane potential if all the other titratable sites are in their reference state. The intrinsic binding energy $\overline{G}_{\mathrm{intr},i}^{\circ}$ has several contributions (For sites j, an analogous expression has to be used):

$$\overline{G}_{\mathrm{intr},i}^{\circ} = G_{\mathrm{model},i}^{\circ} + \Delta\Delta G_i^{\mathrm{solv}} + \Delta\Delta G_i^{\mathrm{back}} + \Delta\Delta G_i^{\Psi}$$
(6.48)

i.e., in addition to the terms in Eq. 6.28, Eq. 6.48 includes the interaction with the membrane potential $\Delta\Delta G_i^{\Psi}$, which can be obtained from

$$\Delta\Delta G_i^{\Psi} = F\Psi\,\Gamma_i$$

$$= F\Psi \sum_{k=1}^{M_i} \chi_{mp}(\mathbf{r}_k)(Q_{k,i}^b - Q_{k,i}^u)$$

(6.49)

where the quantity Γ_i describes the relative effect of the membrane potential on the energy; $\chi_{\mathrm{mp}}(\mathbf{r})$ is the dimensionless function defined in Eq. 6.44; M_i is the number of charges of residue i that change during the reaction; $Q_{k,i}^b$ and $Q_{k,i}^u$ are the charges of atom k of residue i in the bound and unbound form, respectively.

It is important to realize that due to the membrane potential, there is an important
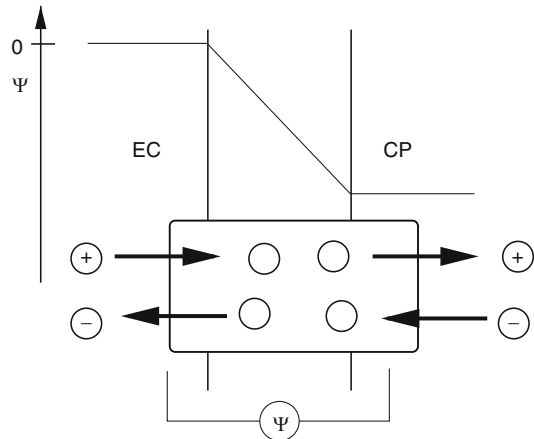
Fig. 6.9. Schematic representation of the ion, proton or electron flow in a membrane. In this scheme the membrane potential $\Psi = \psi_{CP} - \psi_{EC} < 0$, with ψ_{CP} and ψ_{EC} being the potential in the cytoplasm (CP) and in the extracellular space (EC), respectively. In case of protons, one could make the assumption that the hydrogen bond network connects the titratable sites to either the EC region or to the CP region (i.e. protons cannot flow through the protein). Proton displacement along one hydrogen bond network is favored in the direction of decreasing potential, electrons would flow in the opposite direction. Under the condition that the protons cannot pass the membrane, the membrane potential will increase the protonation probability of a titratable site receiving a proton from the EC region and it will decrease the protonation probability of a titratable site receiving a proton from the CP region. The opposite is true for electrons.

difference between the sites connected to the cytoplasm and the sites connected to the extracellular region (Bombarda et al. 2006). For example, a negative membrane potential as in Fig. 6.9 favors the protonation of sites connected to the extracellular side, since proton uptake is energetically downhill with regard to the membrane potential (Fig. 6.9). In contrast, protonation of sites connected to the cytoplasm is hindered in the presence of a negative membrane potential, since proton uptake is uphill with regard to the membrane potential (Bombarda et al. 2006). The opposite behavior will be found for electrons, i.e. the reduction from the extracellular side will be disfavored in the presence of a negative membrane potential, but favored from the cytoplasmic side.

IV. Complex Electron Transfer Proteins

A. Simulating Complex Electron Transfer Networks

To explore possible mechanisms of large protein complexes such as for instance the photosynthetic reaction center or also cytochrome bc complexes, it is often required to examine many different possibilities. Sometimes, even many mechanisms may be possible simultaneously and a single answer may not exist. Such complex reaction schemes can be investigated using the microstate model introduced in section "III.D. Interacting Redox Active Groups". The kinetics of such reactions can be simulated by a master equation approach. The rate constants which are required for such simulations can be calculated using the methods introduced above. Thus, combined with a master equation approach, continuum electrostatics offers a possibility to access the non-equilibrium behavior of biomolecular systems. In the microstate formalism, charge transfer events are described as transitions between well-defined microstates of a system. The time dependence of the population of each microstate can be simulated using a master equation

$$\frac{d}{dt}p_\nu(t) = \sum_{\mu=1}^{M} k_{\nu\mu}p_\mu(t) - \sum_{\mu=1}^{M} k_{\mu\nu}p_\nu(t) \quad (6.50)$$

where $p_\nu(t)$ denotes the probability that the system is in charge state ν at time t, $k_{\nu\mu}$ denotes the probability per unit time that the system will change its state from μ to ν. In Eq. 6.50, first sum includes all the reactions that form state ν, the second sum includes all the reactions that annihilate state ν. The summations run over all possible states μ. Simulating charge transfer by Eq. 6.50 assumes that these processes can be described as a Markovian stochastic process. This assumption implies that the probability of a given charge transfer depends only on the current state of the system and not on the pathway in which the system has reached this state. The system given by Eq. 6.50 is a system of coupled linear differential equations with constant coefficients, which can be written in the form

$$\frac{d\,\mathbf{p}(t)}{dt} = \mathbf{A}\,\mathbf{p}(t) \quad (6.51)$$

The diagonal element $A_{\nu\nu}$ of the matrix $\mathbf{A}$ is the negative of the sum over all the rate constants $k_{\mu\nu}$ annihilating the state ν. The off-diagonal element $A_{\nu\mu}$ is the rate constant $k_{\nu\mu}$ for the conversion of state μ to state ν (Becker et al. 2007). The analytical solution for such equations can be written as (Ferreira and Bashford 2006; Becker et al. 2007)

$$p_\nu(t) = \sum_{\mu}^{M} c_\mu v_{\mu,\nu} e^{-\alpha_\mu t} \quad (6.52)$$

where α_μ is the μth eigenvalue of the matrix $\mathbf{A}$, and $v_{\mu,\nu}$ is the νth element of the μth eigenvector of matrix $\mathbf{A}$; c_μ are integration constants determined from the initial probabilities $\mathbf{p}$ at $t = 0$ (i.e. all the terms $e^{\alpha_\mu t} = 1$).

$$\mathbf{c} = \mathbf{V}^{-1}\mathbf{p}(0) \quad (6.53)$$

where $\mathbf{V}^{-1}$ is the inverse of the matrix containing the eigen vectors of $\mathbf{A}$.

Equation 6.52 describes the time dependence of the probability distribution of microstates of the system. For these microstates, energies G_ν and transition probabilities $k_{\nu\mu}$ can be assigned unambiguously. The time-dependent probability of finding a single site in a particular form can be obtained by summing up individual contributions from the time-dependent probabilities $p_\nu(t)$.

$$\langle x_i \rangle(t) = \sum_{\nu}^{M} x_{\nu,i}p_\nu(t) \quad (6.54)$$

If the reactions are electron transfer reactions, their rate constants can be estimated using, for example, the rate laws developed

by Moser and Dutton (Moser et al. 1992; Page et al. 1999) described in section "II.E. Moser-Dutton Ruler".

B. Analyzing Calculated Electron Transfer Networks

For the analysis of a complex charge transfer system, it is of particular interest to follow the flow of charges through the system, i.e., the charge flux. The flux from state ν to state μ is determined by the population of state ν times the probability per unit time that state ν will change into state μ, i.e., by $k_{\mu\nu}P_\nu(t)$. The net flux between states μ and ν is thus given by

$$J_{\nu\mu}(t) = k_{\nu\mu}P_\mu(t) - k_{\mu\nu}P_\nu(t) \qquad (6.55)$$

$J_{\nu\mu}$ (Eq. 6.55) is positive if there is a net flux from state μ to state ν. This flux analysis allows deduction of the reaction mechanism from even very complex reaction schemes (Becker et al. 2007).

In cases where the number of possible microstates is too large, the differential equation can not be solved analytically. Thus, approximations and simulations need to be applied. One attractive simulation is a dynamic Monte Carlo simulation scheme (Gillespie 2001; Till et al. 2008), which allows the simulation of very complex reaction mechanisms such as proton transfer through a protein matrix. Again, the reaction parameter can be obtained using simple approximations such as continuum electrostatics. Each simulation describes one particular reaction path through the possible states of the system. A reaction mechanism can then be inferred from the analysis of many such trajectories. Up to now, this method was only applied to relatively simple systems (Till et al. 2008). However, future application to enzymes which involve chemical transformation, proton and electron transfer, as well as conformational changes, seems possible and is a promising future road for analyzing enzyme functions.

C. Electron Transfer from the C-Subunit of the Photosynthetic Reaction Center to the Special Pair

The microstate model described above has been used to simulate the kinetics of electron transfer between the tetraheme-subunit and the special pair of the photosynthetic reaction center of *Blastochloris viridis* (Becker et al. 2007; Bombarda and Ullmann 2011). The comparison with the experimental data (Ortega and Mathis 1993) shows that continuum electrostatic calculations can be used in combination with the empirical rate law of Eq. 6.12 to reproduce measurements on the re-reduction kinetics of the photo-oxidized special pair (SP) in the bacterial photosynthetic reaction center.

Re-reduction of the SP in *B. viridis* is facilitated by the cytochrome C-subunit
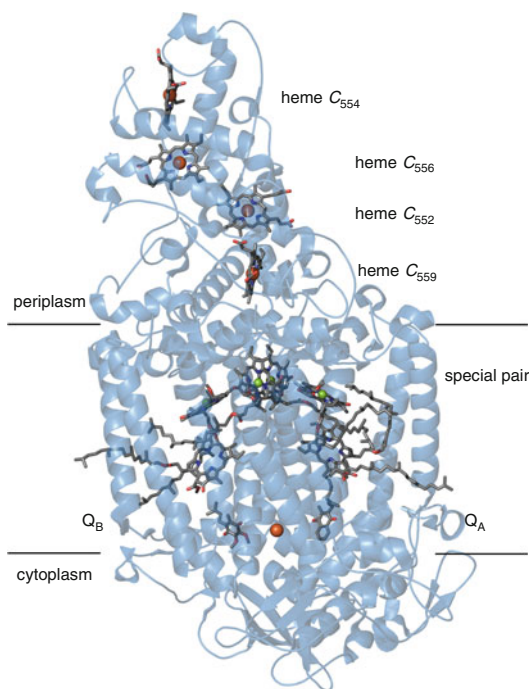
Fig. 6.10. Photosynthetic reaction center from *B. viridis*. In addition to the special pair, the accessory chlorophylls, the pheophytins and the quinones, this reaction center possesses four heme groups that re-reduce the special pair after photooxidation. The re-reduction kinetics of this protein has been well investigated experimentally.

(Fig. 6.10). The C-subunit, contains four heme cofactors forming a transfer chain. The heme groups are commonly labeled according to their absorbance maxima (subscripts) as heme c_{559}, heme c_{552}, heme c_{556} and heme c_{554}. To simulate electron transfer within the C-subunit and between the C-subunit and the SP, a model was constructed consisting of the five redox-active groups. Redox potentials of these groups and interaction energies for pairs of these groups were calculated using the model with pure electrostatic interaction. In addition, the available structural information was used to calculate reorganization energies for all different pairs of redox-active groups (Becker et al. 2007; Bombarda and Ullmann 2011).

In the experimental studies, Ortega et al. (1993) exposed the reaction center of *B. viridis* to different redox-potentials. The system was prepared experimentally in charge configurations with 4, 3 and 2 electrons distributed over the system consisting of the four hemes and the SP. The re-reduction kinetics of the SP was measured after photo-induced oxidation. These experimental setups can be mimicked by simulations. To illustrate the kinetics seen in such simulations, the result obtained for a system starting from 3 electrons distributed over the four heme groups is shown in Fig. 6.11. In Fig. 6.11a, the time-dependent probability distribution of the accessible microstates is shown. The corresponding oxidation probabilities for the hemes and the SP are shown in Fig. 6.11b. It can be seen that only a limited number of microstates contribute significantly to the probability distribution in the pico- to microsecond time domain. However, this feature does not imply that only this limited number of microstates is important for the kinetics of the system. The detailed information available from the simulation data allows to track the electron fluxes between microstates and thus electron movements between individual sites in a reaction scheme (Fig. 6.12).

The redox microstates can be denoted as vectors of 1 (reduced sites) and 0 (oxidized sites). The first element denotes the redox state of the special pair, the next four elements denote redox states of the hemes in the order of their distance to the SP, starting from the nearest. The kinetics depicted in Fig. 6.11 suggests a rather simple picture for the time dependence of the population of accessible microstates. Starting from a population of the two microstates (0,1,1,1,0) (90%) and (0,1,0,1,1) (10%) after photooxidation, the system relaxes towards an equilibrium distribution which is mainly given by one microstate (1,1,0,1,0). However, the underlying transfer dynamics of the system as depicted in Fig. 6.12 is considerably more complex. The highly populated initial state (0,1,1,1,0) can rapidly decay into the final state via just one intermediate, (1,0,1,1,0). In contrast, the initial state (0,1,0,1,1) has to relax towards the final state via a succession of several intermediates. These intermediate states are only transiently populated. Each flux into one of the intermediates is accompanied by an equally high flux out of these intermediates. For example, the transition from the initial state to the intermediate (1,0,0,1,1) is rapidly followed by a transition to a second intermediate state (1,0,1,0,1). This intermediate state in turn either decays into state (1,0,1,1,0) via an electron transfer from heme c_{554} to heme c_{556}, or alternatively to state (1,1,0,0,1) via electron transfer from heme c_{552} to heme c_{559}.

The procedure outlined here can also be applied to proton transfer reactions. Thus, the strategy to combine continuum electrostatics with the master equation represents an important method to understand the mechanism of complex charge transfer systems.

V. Conclusions

In this article, we reviewed how our understanding of the electron transfer in proteins on the basis of Marcus theory can help to analyze the mechanism of complex protein machines that can couple redox reactions to proton transfer reactions. Even the effect of membrane potentials and transmembrane
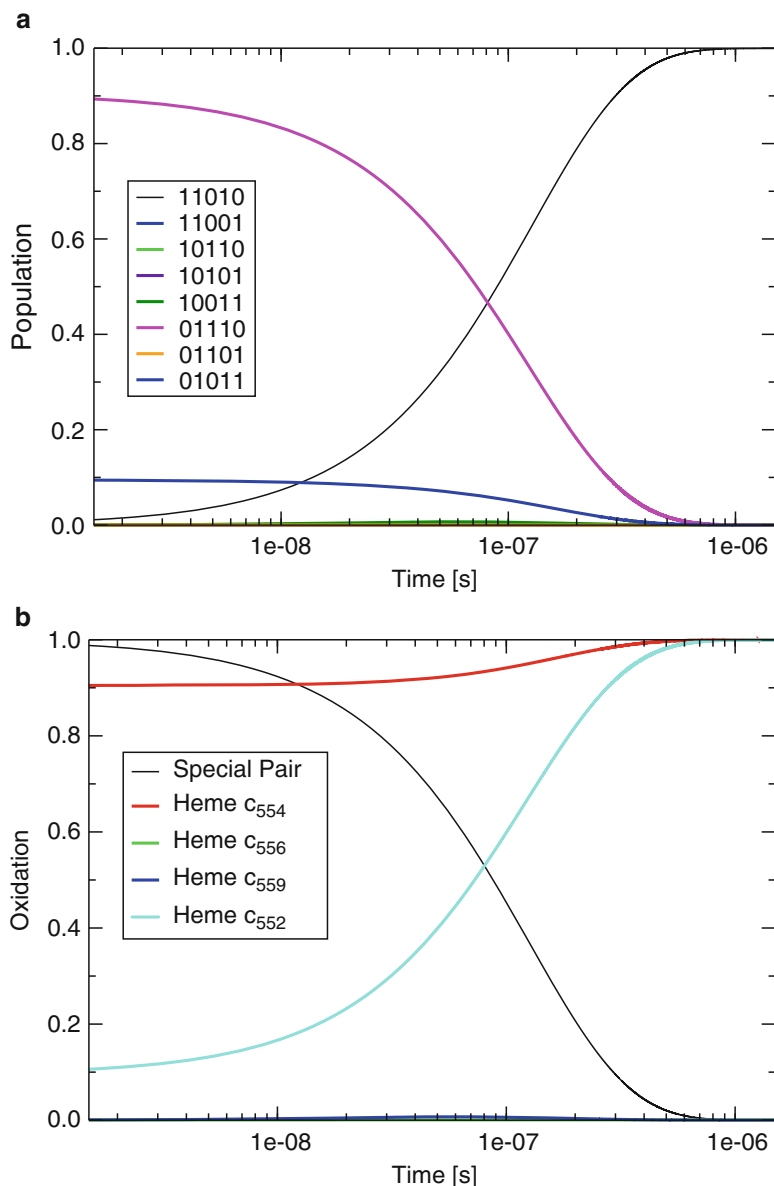
Fig. 6.11. Simulation of the re-reduction kinetics of the special pair of the photosynthetic reaction center in a system with three electrons. (**a**) The time-dependent probability distribution of microstates after photo-oxidation of the SP. The state vector is given in the order (SP, heme c_{559}, heme c_{552}, heme c_{556}, heme c_{554}). In the state vector, 1 denotes a reduced site and 0 an oxidized site. States that are not shown are not significantly populated. (**b**) Oxidation probabilities of the four hemes and the SP. Initially, three electrons are distributed over the four hemes.

concentration gradients can be included in the calculations. The theoretical basis of the analysis of complex protein machines is the microstate model. In this model, the protein is divided into several functional groups, which can be, for instance, redox-active centers (like hemes or iron sulfur clusters), protonatable residues (like histidine or glutamate), or enzymatic active sites. The part of the protein that does not belong to a functional group is considered a background that interacts with the functional groups.
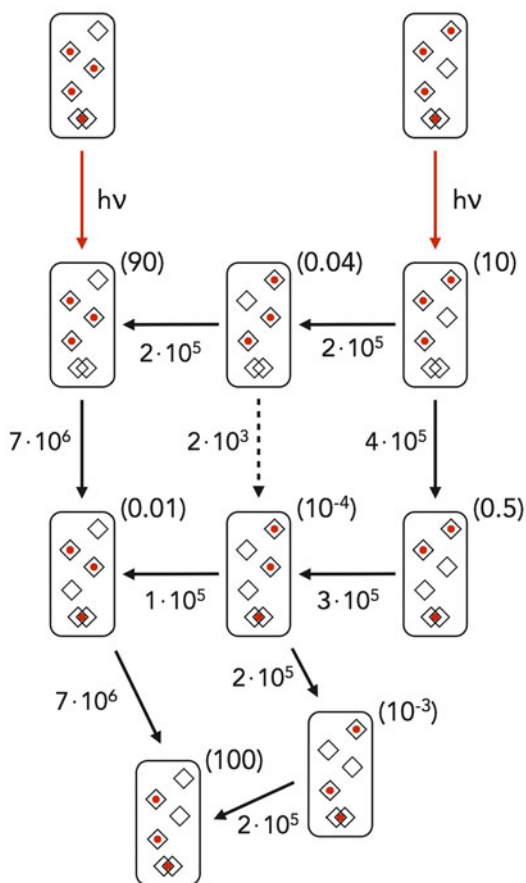
Fig. 6.12. Reaction scheme for the rereduction kinetics of the special pair of the photosynthetic reaction center in a system with three electrons. The reaction scheme is deduced from the flux analysis. Each *rectangle* represents a microstate of the photosynthetic reaction center, the *rhombi* inside the *rectangle* represent the hemes (the order top to bottom: heme c_{554}, heme c_{556}, heme c_{552}, heme c_{559}), the overlapping *rhombi* represent the special pair. The reduced state of the cofactors is indicated by a *red sphere*. After photooxidation of the special pair (*red arrows*), several electron transfer reactions lead to the new equilibrium. The microstates representing the starting and the final configurations of the system in the simulation are depicted in the top and the bottom row, respectively. The values in parentheses denote the maximal probability observed during the simulation. Only fluxes (in s^{-1}) contributing significantly are indicated by *arrows* and their maximum value is given.

The state of the protein is characterized by defining the state of each functional group (for instance redox state or protonation state). In this picture, a protein with N sites has many states (precisely $\prod_{i=1}^{N} s_i$, where s_i is the number of states of site i). The energy of a particular state is defined by the sum of the energies of each particular site and the interactions between the sites. In this energy sum, the interaction with membrane potentials or other external fields can also be included.

Thermodynamic properties can be calculated by averaging over all possible states of the systems using statistical thermodynamics. If the system has so many states that explicit averaging is not possible, Metropolis Monte Carlo can be applied. To simulate the kinetic behavior of the protein, a master equation including all transitions between the states can be used. A flux analysis can help to extract mechanistic information from the simulation.

The microstate model is fairly general and does not rely on any particular way by which the state energies or the transition rate constants are calculated. In this article, we showed that the use of continuum electrostatic theory on the basis of the Poisson-Boltzmann equation is particularly useful, since, even for very large systems, the calculations are still tractable and lead to biophysically and biochemically meaningful results. The energies and rate constants of the microstate model could also be calculated with more accurate methods. However, it is important to realize that proteins that contain many cofactors cannot be regarded as a sum of these cofactors. Instead such proteins can exist in many states. Which states are functionally important, for instance, in catalytic cycles can be revealed by the analysis described in this article. We are convinced that the methods described here will allow analysis of many complex enzymatic reactions.

Acknowledgements

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Part III

Molecular Structures and Functions of Cytochrome Complexes

A. Photosynthetic Reaction Centers and Linked Cytochromes (Chapters 7 and 8)

B. Structure-Function of the Cytochrome bc_1 b_6f Complexes (Chapters 9, 10, 11, 12, 13, 14 and 15)

C. Cytochrome Oxidases (Chapters 16, 17, 18 and 19)

Higher Plant and Cyanobacterial Photosystem I: Connected Cytochrome Pathways

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Summary

Oxygenic photosynthesis is the principal converter of sunlight into chemical energy on earth. The conversion of solar energy is catalyzed by four multi-subunit membrane protein complexes: photosystem I (PSI), photosystem II (PSII), the cytochrome b6-f complex $(cytb_6f)$ and ATP-synthase (F_OF_1). These protein complexes are connected by soluble electron carriers that are vital not only for the proper function of ATP and NADPH production but also to render the system highly efficient in different organisms and various environments, some of which are quite harsh. While the main fabric of the membrane complexes is highly conserved, their surfaces and interaction with the soluble factors provide the specificity and fine regulation of the operating system. One of the prime examples for this phenomenon is the cyanobacterial photosynthetic electron transport chain that is situated alongside with respiratory complexes, yet it stays unique by virtue of the interacting soluble components. Cyanobacteria contain many different cytochromes that potentially can donate electrons to both PSI and cytochrome oxidase, yet the two systems can operate separately in a synchronous mode. The crystal structure determination of photosynthetic and respiratory protein complexes shed light on the various partial reactions and explains how they can function alongside each other.

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I. Introduction

Over 3 billion years ago our planet was under anaerobic conditions and most of the elements were present in their reduced-valence states (Barber 2008; Nelson 2011). Thus, the atmosphere was rich in nitrogen in the form of ammonia (NH_3) or N_2, carbon as CO or CO_2, and oxygen as H_2O (Bendall et al. 2008), and the Earth's surface was covered by water that contained not only minerals but also organic molecules. Porphyrins may have been one of the earliest complex organic molecules present on earth. Porphyrins can be synthesized abiotically in a primordial soup Urey-Miller type experiments (Szutka 1966), and can bind a wide variety of metal ions over a range of oxidation states. The biosynthetic pathways leading to chlorophylls and cytochromes are still connected, stemming from a common precursor, 5-aminolevulinic acid (ALA) (Smith and Witty 2002).

In spite of their common evolutionary origin, presently hemes and chlorophylls serve very different functions. Chlorophylls, by far the most abundant porphyrins in nature, serve mainly as light harvesting pigments, while heme-containing cytochromes participate in electron transfer reactions. Of course, within all photosynthetic reaction centers, special chlorophylls participate in very fast charge separation and electron transfer reactions as well.

The hallmark of oxygenic photosynthesis is the coupling of two unique reaction centers within the same membrane system. In spite of the fact that oxygenic photosynthesis evolved early in the evolution of the biosphere, approximately 3 billion years ago, today we can still point to cyanobacteria as the evolutionary ancestors of all known oxygenic photosynthetic membrane systems. The link between the two photosystems is mainly provided by the cytochrome b_6f and the small soluble Cyt c_6 or Pc proteins.

II. Cytochrome *bc* Complex and Its Central Role in Photosynthetic and Respiratory Electron Transport Chains

The cytochrome bc complex that operates at mild redox potentials has homologs in most photosynthetic and respiratory electron transport chains. Initial biochemical studies have determined the composition of the mitochondrial bc1 complex that contains two heme b, one heme c and a non-heme iron cluster. Preparation of isolated complex contained all those factors and was shown to function in a quinone-cytochrome c oxydoreduction reaction (Hurt and Hauska 1981). The use of detergents for solubilizing membranes was introduced and the isolation of functional cytochrome bc1 complex was aided by the strong spectroscopic background of scientists in the area of bioenergetics (Rieske et al. 1964; Chance et al. 1967; Fan and Cramer 1970). The study of cytochrome b6f complex of oxygenic photosynthesis was hampered by the fact that the same detergents used in mitochondria were useless for photosynthetic membranes and the spectroscopic studies were hampered by the high absorbance of the numerous pigments, especially chlorophylls, present in photosynthetic membranes. Eventually cytochrome b6f complex was isolated and was shown to be spectroscopically highly similar to its mitochondrial counterpart (Fan and Cramer 1970; Nelson and Neumann 1972). Albeit the two are quite similar, the main difference was the electron acceptor of the complex that is cytochrome c in mitochondria and plastocyanin in chloroplasts (Hauska et al. 1971). Those two soluble factors totally differentiated between the two systems. In any case, in plants the two systems are distinguished by the two separate compartments that are separated by 1.5 billion years of evolution

Abbreviations: ALA – 5-aminolevulinic acid; Cyt c6 – Cytochrome C6; Fd – Ferredoxin; NDH1 – NADH:ubiquinone oxidoreductase I; Pc – Plastocyanin; PSI – Photosystem I; PSII – Photosystem II; PTOX – Plastoquinol terminal

(Hauska et al. 1971). Cyanobacteria went against this dogma by having the two reactions in similar compartments and using soluble cytochromes for both photosynthesis and respiration. Thus the fine tuning of respiratory and photosynthetic electron transport activity required a richer gallery of soluble c-type cytochromes.

The existence of respiratory complexes in the thylakoid membranes of both cyanobacteria and plants has been known for quite some time. Cyanobacteria in particular contain what appears to be a complete respiratory chain in their thylakoid membrane (Peschek et al. 2004). The electron flow rate through this chain is much lower (1–5 %) than the rate of the photosynthetic electron transport. However, under particular physiological conditions, prolonged dark growth or in various stress conditions, this rate can increase substantially (Molitor et al. 1986; Peschek et al. 2004). Plants and algae contain NDH1, the complex I equivalent of photosynthesis. The function of respiratory electron chains in the thylakoid membranes of cyanobacteria is usually associated with various forms of cyclic electron flow, mostly revolving around the NDHI complex (Battchikova et al. 2010).

III. Oceanic Photosynthesis

Earth's oceans contribute 50 % of the global photosynthetic activity to our biosphere. The majority of this contribution is carried out by microorganisms, either cyanobacteria or algae. Here we will focus on oceanic cyanobacteria. The global oceanic population of cyanobacteria is mostly made up of two very similar genera, the *Prochlorococcus* and *Synechococcus*. *Synechococcus* dominate in nutrient rich waters close to coastal areas while *Prochlorococcus* dominates the vast, oligotrophic regions that encompass most of the ocean (Partensky et al. 1999; Scanlan et al. 2009). These bacteria can reach significant densities (up to 10^5 cells/ml in certain depths) in spite of the inhospitable nature of their environment

and can inhabit waters up to 200 m deep. The end result of these two facts is that an incredibly large population of these cyanobacteria exists globally ($\sim 10^{27}$ cells) and this population makes a significant contribution to the Earth's oxygen and carbon cycle (Partensky et al. 1999). While *Prochlorococcus* and *Synechococcus* are quite similar, very significant differences exist between them. The most noticeable among them is the size, *Prochlorococcus* is the smallest known photosynthetic cell, the small size (~ 0.6 μm) is accompanied by the smallest genome (~ 1.7 Mbp) of any oxygenic photosynthesizer (Dufresne et al. 2003; Rocap et al. 2003; Partensky and Garczarek 2010). In addition, *Prochlorococcus* synthesizes unusual a- and b-type chlorophylls (Chisholm et al. 1992) and, in contrast, to most other cyanobacteria does not contain phycobilisomes but relies on membrane bound IsiA type antennas for both of its photosystems (Bibby et al. 2003). The antenna genes for *Prochlorococcus* evolved to accommodate the different light regimes (in both intensity and spectrum), and the full complexity of their interaction with the photosynthetic machinery is still not sufficiently explored (Garczarek et al. 2000). It is clear that *Prochlorococcus* contains interesting adaptations to its environment, thus enabling its success. Some of these adaptations can be found in its photosynthetic genes and they provide unique versions of the otherwise highly conserved photosynthetic complexes.

An important element within the environment of oceanic cyanobacteria are cyanophages. The phage population in the ocean outnumbers their hosts ten to one (Suttle 2007). This, together with the highly diverse nature of their genomes points to this population as a huge source for genetic innovation, which can flow into their host population. A particularly relevant example is the presence of photosynthetic genes in the genomes of a large fraction of the oceanic phages. The initial discovery was made in 2003 after the full sequence of one such virus revealed the presence of both D1 and D2 (the reaction

center polypeptides of photosystem II) in its genome (Mann et al. 2003). Since then, many experiments were done on phage-host interactions and on the phylogenetic relationships between viruses and between their photosynthetic genes. Several important facts rose from these studies. First, it was found that the viral D1 and D2 genes are expressed and translated by the cyanobacterium host (Lindell et al. 2005). Secondly, it became clear that certain phage families can infect both *Prochlorococcus* and *Synechococcus*, while other families have a narrow host range (Sullivan et al. 2003). Third, the viral D1 and D2 proteins are distinct from the host proteins (Zeidner et al. 2005). However, presently, there is no known instance where a phage mutation/variant is associated with a unique function (or dysfunction). In fact, due to the highly conserved nature of both D1 and D2, it is still not certain that the viral proteins can fully replace the host D1 and D2. Fourth, from sequence analysis it seems that the viral genes can recombine with host genes and thus increase the genetic variability of the D1 and D2 pool (Zeidner et al. 2005).

In the past decade genomic studies conducted in the ocean added large amounts of sequence information to the database (Rusch et al. 2007; Dinsdale et al. 2008). The improved sampling of the ocean's genetic landscape resulted in a much richer picture of the gene content of phages and expanded their putative interaction with the host photosynthetic and respiratory electron transport chains well beyond the initial observation. The current list of phage-encoded photosynthesis-related genes includes D1 and D2, plastoquinol terminal oxidase (PTOX), Ferredoxin (Fd), Plastocyanin, some NDHI, and FoF1 subunits, and an entire PSI operon which will be presented in depth in the following sections of this chapter (Sullivan et al. 2006; Sharon et al. 2009, 2011; Mazor et al. 2014). Initially, the rationale employed to explain the role of viral photosystem II subunits was focused on the short half-life of D1, making it the most vulnerable point of the photosynthetic electron transport chain

during phage infection (since phage proteins are transcribed and translated preferentially during infection). This line of thinking is definitely valid for Photosystem II. However, most of the other virally encoded photosynthetic chain members are not particularly short-lived in known photosynthetic model organisms. An additional explanation for the function of the photosynthetic phage-encoded proteins is that they induce an alternative cyclic flow of electrons in the photosynthetic electron transport chain. This is mainly based on the occurrence of the NDHI subunits and on the unusual composition and sequence of the virally encoded PSI operon (Sharon et al. 2009; Alperovitch-Lavy et al. 2011).

IV. Interactions Between Photosystem I and Small Electron Donors

Compared with the active sites of "simple", single subunit enzymes, the large complexes, which participate in electron transfer reactions, contain extremely complicated active "sites", usually spanning the entire membrane. In the case of Photosystem I, the active "site" can be thought of as a molecular wire selectively linking two poles across the membrane (Fig. 7.1). Charge separation originates at P700 or, according to more recent findings at the electron acceptor A0 (Muller et al. 2003), and then travels across several cofactors to finally reach F_B, the reducing terminus of PSI (Fig. 7.1). The oxidized P700+ is reduced back by plastocyanin (Pc) or cytochrome c6 (Cyt c6).

A large body of work has accumulated on the interaction between Pc/Cyt c6 and PSI leading to the identification of the N-terminus of PsaF and a hydrophobic patch juxtaposition to P700 as the most important elements of this interaction (Farah et al. 1995; Sun et al. 1999; Sommer et al. 2002). One of the main differences between cyanobacteria and eukaryotes is their interaction with the small protein electron donor. In the eukaryotic system, a fast phase
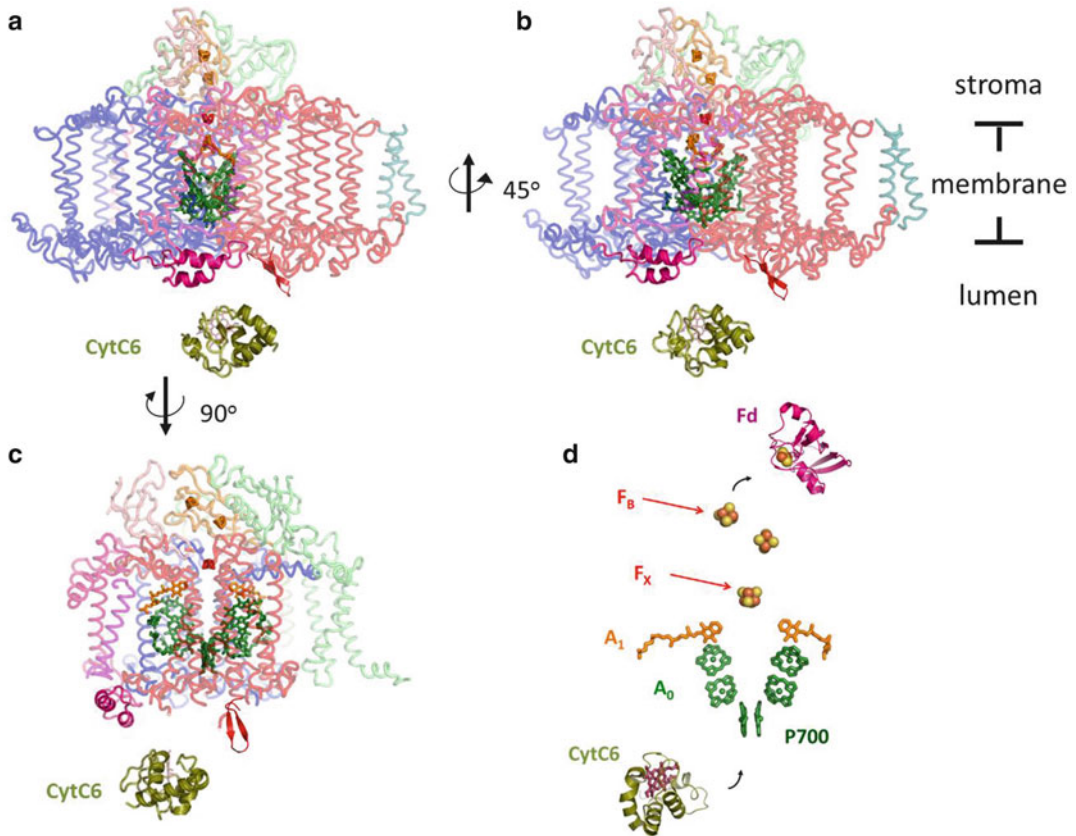
Fig. 7.1. Electron transport in PSI. Ribbon representation of photosystem I subunits. Only the central ligands, involved in electron transport are shown. PsaA is colored in *red*, PsaB in *blue* and PsaF in *pink*. The N-terminus of PsaF and the luminal loop of PsaA are shown as cartoons at the bottom of every complex to illustrate their potential in controlling the Cyt c_6 binding site. Cyt c_6 (colored in *olive*) was manually placed close to its binding site and its orientation does not reflect any experimental data. (**a**) and (**b**) side views of PSI from the membrane plane. (**c**) a 90° rotation compared to A. with a portion of PsaA removed to reveal the components of the ETC. (**d**) representation of the ETC in the same orientation as c, showing the flow of electrons from Cyt c_6 to Fd (PDB codes for PSI and Cyt c_6 are 1JB0 and 4EIC respectively).

(t1/2, several μs) in PSI reduction kinetics has been taken as the manifestation of a stable PSI-Pc complex (Bottin and Mathis 1985; Delosme 1991). In cyanobacteria, this PSI-Pc complex is not as common and several modes of interaction were observed, in line with the highly diverse nature of this group (Baymann et al. 2001; Hervas et al. 2005). However, there is evidence for a stable complex between PSI and Cyt c6 in some cyanobacteria and a fast phase should not be taken as a strictly "eukaryotic" property (Baymann et al. 2001).

The key to the differences between cyanobacteria and eukaryotes can be found in two places. First, the N-terminus of PsaF has changed considerably in both structure and physical properties (Fig. 7.2) and secondly the small donors are very similar in eukaryotes while being very diverse in cyanobacteria (Table 7.1) (Jordan et al. 2001; Ben-Shem et al. 2003). The electrostatic differences between the cyanobacterial PSI and the eukaryotic one are shown in Fig. 7.2, in which the luminal face of PSI is colored according to electrostatic
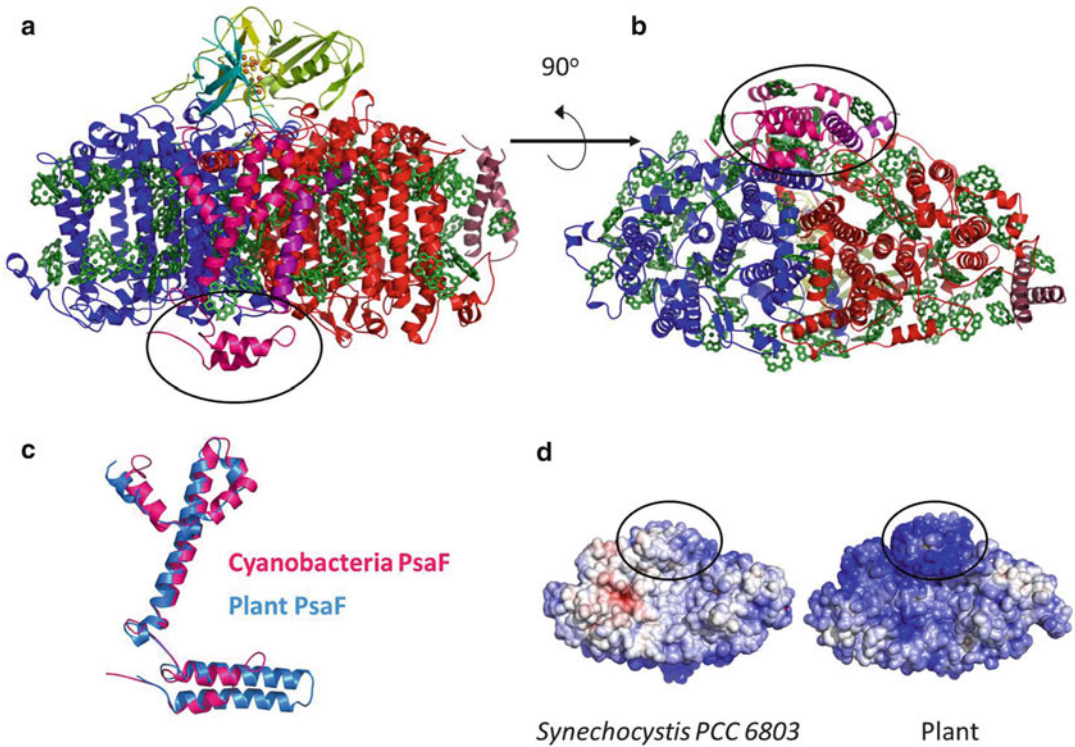
Fig. 7.2. PsaF determines the shape and charge of the Pc/Cyt c_6 binding surface in PSI. (**a**) PSI from *Synechocystis* is shown, in view parallel to the plane of the membrane, with chlorophylls represented as rings only, colored in *green*. (**b**) The luminal face of PSI with the N-terminus of PsaF encircled with a *black ellipse*. (**c**) The PsaF subunits of cyanobacteria (*pink*) and plant (*blue*), superimposed, showing the two helix extension in the eukaryotic PsaF subunit. (**d**) Electrostatic properties of the luminal surface of PSI showing the positive N-terminal domain in the eukaryotic complex. Electrostatic surfaces were calculated using the APBS plugin for Pymol (Baker et al. 2001), and colored *blue* for positive and *red* for negative. The LHC belt was omitted from the eukaryotic complex for ease of comparison (its occlusion did not affect the charge around the PsaF subunit). PDB id for cyanobacteria and plant PSI are 4KT0 and 3LW5.

potential. It is clear that a patch of positive charge, shielding a segment that is negatively charged, has been added in eukaryotes. This is accompanied by the extension of the two-helix domain of PsaF shown in Fig. 7.2. Cross linking experiments showed the Pc and Cyt c6 bind this positive patch directly in Chlamydomonas and higher plants (Wynn and Malkin 1988; Hippler et al. 1996) and that positively charged residues are important for binding (Hippler et al. 1998). In contrast to the clear findings in *Chlamydomonas*, PsaF mutants in *Synechocystis* show no obvious phenotype in vivo or in vitro

(Chitnis et al. 1991; Farah et al. 1995; Hippler et al. 1996; Mazor et al. 2014). The N-terminus of cyanobacterial PsaF still carries some positive charges and this together with highly acidic donors can lead to stable complex between PSI and its donor. This suggests that the evolution of reaction mechanisms in cyanobacteria mostly took place on the donor side of the two partners (Hervas et al. 2005).

The mechanism of interaction between PSI and Pc/CytC6 was explored in detail using mutants of each of the small donors. The results revealed two sites, one involved

Table 7.1. Calculated PI of Cyt c_6/Pc. PI values were calculated using the Compute pI/MW tool on ExPASy (http://web.expasy.org/compute_pi/). Only completely sequenced organisms were used in the table, allowing us to reach a conclusion on the absence of Pc or Cyt c_6. Sequences were retrieved from multiple blast searches using various query sequences and aligned using MUSCLE to assign cytochrome type (Edgar 2004). Cyt c_{6A} sequences are highlighted in *yellow*, Cyt c_{6B-C} in *purple* and Cyt c_M in *red*.

Occurrence of small electron donors within photosynthetic organisms.

cyanobacteria	CytC6				CytC$_M$	Pc
Synechocystis sp. PCC 6803	6.7				6.2	6.3
Prochlorococcus marinus str. MIT9202					5.8	
Prochlorococcus marinus str. CCMP1986					6.9	4.7
Prochlorococcus marinus str. MIT9215					5.4	4.7
Prochlorococcus marinus str. MIT9301		9.5			5.4	4.7
Prochlorococcus marinus str. MIT9211		7.8			6.1	5.0
Prochlorococcus marinus str. MIT9312		9.5			6.4	4.9
Prochlorococcus marinus str. NATL1A		8.3			5.3	4.9
Prochlorococcus marinus str. CCMP1375		9.2			4.9	5.0
Prochlorococcus marinus str. NATL2A		8.8			5.3	4.9
Prochlorococcus marinus str. MIT9303	5.0	8.8			6.0	4.5
Prochlorococcus marinus str. MIT9313	5.0	9.2			5.8	4.5
Synechococcus sp. BL107	4.5	6.0			4.9	4.7
Synechococcus sp. CB0101	5.4	6.1			8.6	no PC
Synechococcus sp. CC9311	4.9	5.7	5.9	6.0	8.6	4.9
Synechococcus sp. CC9605	4.8	6.1			4.9	5.4
Synechococcus sp. JA-3-3Ab	8.6				6.2	9.3
Synechococcus sp. RCC307	5.1	5.3			6.5	no PC
Synechococcus sp. RS9916	4.5	9.2	4.7	5.3	6.2	5.0
Synechococcus sp. WH5701	5.4	9.6			9.2	4.8
Synechococcus sp. WH7803	4.4	5.5	4.8		9.2	4.9
Synechococcus sp. WH8016	5.2	6.5	5.6		6.8	4.9
Synechococcus sp. WH8102	4.7	7.8			5.4	4.9
Synechococcus elongatus PCC 7942	6.0	9.1	9.8		7.8	5.5
Synechococcus sp. PCC 7335	4.9	9.6			no cM	6.7
Synechococcus sp. PCC 6312	6.5				7.9	5.7
Synechococcus sp. PCC 7002	6.5	9.3			7.8	no PC
Thermosynechococcus sp. BP-1	7.7				7.8	no PC
Anabaena variabilis ATCC 29413	6.1	9.4	9.3		7.8	9.1
Cyanothece sp. ATCC 51142	6.5	9.2			6.0	5.5
Cyanothece sp. PCC 7425	8.6	7.8			5.8	no PC
Gloeobacter violaceus PCC 7421	5.6	9.6			6.6	9.4
red algae						
Calliarthron tuberculosum	4.6					no PC
Chondrus crispus	4.9					no PC
Cyanidioschyzonmerolae	5.5					no PC
Nannochloropsis gaditana	4.7	9.9				no PC
Porphyra purpurea	4.8					no PC
green algae						
Chlamydomonas reinhardti	8.7	7.7				6.3
Chlorella variabilis	5.7					4.2
Volvox carteri	9.1					5.6
Micromonas pusilla CCMP1545	9.1					5.5
green plants						
Theobroma Cacao	8.5					4.8
Vitis vinifera	8.2					5.3
Lotus japonicus	9.1					5.0
Medicagotruncatula	8.2					5.0
ArabidopsisThaliana	5.1					5.7
Cicer arietinum	9.1					5.0
Cucumis sativus	8.5					4.9

in electrostatic interaction and one involved in hydrophobic interactions located on analogous patches on both proteins (De la Cerda et al. 1997, 1999). These results match the sites identified on PSI, creating a complementary fit (Hervas et al. 2003).

The calculated PI of a large number of small PSI electron donors from cyanobacteria, red and green algae and higher plants, with the purpose of illustrating the most common and divergent points in the architecture of the small electron donors, has been compiled (Table 7.1).

The most diverse group in this table is clearly cyanobacteria with many species containing multiple Cyt c6 species, alongside with examples of complete loss of either Pc or Cyt c6.

Land plants contain a single Cyt $c6_A$ like protein called Cyt $c6_A$. The function of this cytochrome is still under debate. Initial claims to its involvement in photosynthetic electron transport proved to be wrong (Molina-Heredia et al. 2003; Weigel et al. 2003). Its relatively low Em (0.14 V) suggests that it cannot accept electrons from the b_6f complex and its electron donation to PSI was shown to be negligible (Molina-Heredia et al. 2003). Cyt $c6_A$ contains a small insertion loop with an S-S bond and it was suggested that this S-S bond may play a role in oxidative stress resistance (Marcaida et al. 2006). Currently, the function of this cytochrome in plant and green algae is still not clear. During the evolution of land plants, the functional Cyt c6 of green algae was therefore lost. The reasons for this loss are not entirely clear. Red algae, a diverse and successful group, appear to have gone the opposite way, losing Pc from their genomes (Table 7.1). It therefore seems unlikely that environmental stresses contributed to these loss events and we think that they simply represent random events fixed by evolution.

Pc and Cyt c6 are interchangeable but cyanobacteria cannot survive without at least one of them (Duran et al. 2004). The relative levels of Pc and Cyt c6 are affected by the iron and cupper concentrations in some species. As can be seen in Table 7.1, Pc and

Cyt c6 have a similar charge in *Synechocystis* PCC 6803. In the case of *Synechocystis* these values were determined experimentally and the values in the table are in reasonable agreement with experimentally determined values ($\sim$5.5 for Cyt c6, Cyt c_M and Pc). The similar charge of Pc and Cyt c6 is reflects their similar function. However, as more and more genomes are sequenced the full richness of the Cyt c6 pool in photosynthetic organisms begins to be appreciated and opens possible routes to new functions.

Two new groups of c6 type cytochromes were found, Cyt $c6_B$ and Cyt $c6_C$ (Bialek et al. 2008). These two groups are colored separately in Table 7.1 and we have somewhat expanded the analysis to include additional sequences (Bialek et al. 2008). The differences between Cyt c6 and $c6_{B-C}$ were shown to be highly significant. The PI of the Cyt $c6_B$ from *Synechococcus* sp. PCC 7002 is basic at around 9 and its Em ($\sim$0.15 V) is significantly lower than that of Cyt c6 (which is around 0.35 V) (Bialek et al. 2008). The heme pockets of Cyt $c6_{A-C}$ are related and this is the reason for their low and similar Em values (Worrall et al. 2007; Zatwarnicki et al. 2013). As with Cyt $c6_A$, it is not likely that Cyt $c6_B$ or Cyt $c6_C$ can receive electrons from the b_6f complex and their involvement in photosynthetic electron transport remains to be determined.

Most of the Cyt $c6_B$ and Cyt $c6_C$ sequences are found in marine *Synechococcus* and *Prochlorococcus* (Table 7.1). In the case of *Prochlorococcus*, they are the only cytochromes present (with the exception of the MIT9313 and MIT9303 strains which are very similar to *Synechococcus*). If, indeed, they do not donate electrons to PSI then *Prochlorococcus* as a family seem to utilize only Pc in its photosynthetic electron transport chain, in line with the minimalist nature of these organisms, as exemplified in their minimalist genome.

Another cytochrome with a cryptic function is Cyt c_M. This cytochrome was discovered well before the completion of the Synechocystis genome sequence (Malakhov et al. 1994). However, its function is still

unknown. Cyt c_M is present in virtually all cyanobacterial species and thus it probably carries some unique function (Bernroitner et al. 2009). The Em of Cyt c_M as measured for the *Synechocystis* protein is ∼0.15 V and so, like Cyt c_{6A-C}, it is not a good candidate for an electron acceptor from the b_6f complex (Cho et al. 2000; Molina-Heredia et al. 2002). The expression of Cyt c_M is induced under stress conditions and in vitro it can donate electrons to the terminal oxidase as efficiently as Cyt c6/Pc (Malakhov et al. 1999; Bernroitner et al. 2009).

To summarize, there is a large group of cytochrome c6-like proteins. These proteins are probably imported into the luminal and periplasmic space of cyanobacteria. Their Em prevents them from receiving electrons from the b_6f complex and their interaction with the PSI complex is less efficient than that of Cyt c6/Pc. These cytochromes may transfer electrons from the PQ pool to any one of the terminal oxidases in cyanobacteria in times of stress, and may donate electrons to PSI under rare conditions. The nature of the electron donor to this cytochrome pool is still a mystery. This putative activity, which bypasses the b_6f complex, is probably transient in nature, stems from the PQ pool, and should be tightly controlled. As we discussed in the following section, some of this control may be found in the PSI complex.

V. Phage Encoded PSI Complex

The GOS expedition and other initiatives dramatically increased the amount of sequence information available in the form of environmental samples. Searching these databases for PSI related sequences revealed a phage-encoded PSI operon (Sharon et al. 2009). The phage operon codes for PsaA, PsaB, PsaC, PsaD, PsaE, PsaJF and PsaK. The operon is unique in both its subunit composition and the sequence of some individual subunits. The presence of 7 subunits out of the known 11 or 12 PSI subunits in cyanobacteria is unusual. However the present subunits can form a fully functional, "minimal" complex,

which probably functions as a monomer in its host membrane (Mazor et al. 2012, 2014). Two noticeable alterations in the sequence of the phage PSI operon provide clues to its function.

The first is the fused PsaJF subunit, in which the PsaJ subunit is fused with a PsaF subunit, which lacks the entire N-terminal domain. As mentioned previously, the function of the cyanobacterial PsaF is not entirely clear as it was shown to be dispensable for Pc/Cyt c6 binding in vitro and in vivo. We proposed that deleting the N-terminus of PsaF will results in a promiscuous PSI complex, a complex that can accept electrons from a wide spectrum of donors. A phage mimetic complex constructed in Synechocystis proved to have faster reaction kinetic with a mammalian respiratory cytochrome (which is a very basic protein) when compared to the wild type PSI complex (Mazor et al. 2014).

In addition to the PsaJF subunit, which is only found in phages, the PsaA subunit is missing one of the most highly conserved loops in PSI (624–633 in the *Synechococcus elongatus* structure (Jordan et al. 2001)). This PSI deletion is present in all *Prochlorococcus* PsaA sequences (Mazor et al. 2012). The function of this loop has not been examined experimentally but its proximity to P700 and its high conservation from cyanobacteria to plants suggest that it plays a role in Pc/Cyt c6 binding.

The prevalence of Cyt c6-like proteins in *Prochlorococcus* may be related to this alteration in PSI and indicates that the *Prochlorococcus* PSI is inherently promiscuous, also for example, accepting electrons from the Cyt c_{6B} proteins.

To summarize, cyclic electron flow around PSI affects both the bioenergetic efficiency of photosynthetic membranes and its production of toxic radicals. The existence of respiratory activities in the photosynthetic membranes is now a proven fact, but our knowledge of all of the relevant components and their interaction with the photosynthetic electron transport chain is still lacking. The new groups of cytochrome c6-like proteins are

probably an important part of this story and future studies will surely reveal the pathways through which this pool receives and donates electrons in vivo. We suggest that some of the pathway insulation between photosynthesis and respiration is accomplished inherently by the PSI complex through the N-terminus of PsaF and through a lumen-extended loop in PsaA. Future genomics efforts will provide additional clues to the function of photosynthetic electron flow in the environment and these efforts must be accompanied by equally thorough experimental validation.

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Chapter 8

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Cytochrome b_{559} in Photosystem II

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Dedicated to the memory of Gernot Renger

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Summary

The photosystem II core complex (PSIIcc) is a complicated membrane protein occurring in oxygenic photosynthetic organisms that catalyzes a key reaction, the light-induced oxidation of water. It consists of 17 membrane-spanning α-helical and three membrane-extrinsic protein subunits and binds nearly 100 cofactors. Among them is a heme protein composed of one heme b group ligated by two low molecular weight subunits with one transmembrane helix each, referred to as cytochrome (cyt) b_{559}. Despite extensive research, the role of cyt b_{559} in PSIIcc is still not known. In the present chapter, the structure of cyt b_{559} is reviewed based on recent crystallographic work together with its unusually complex redox behavior, and some ideas are discussed concerning a possible function of this unique heme protein.

I. Introduction

Cytochrome (cyt) b_{559}[1] is a heme protein that is an essential component of the photosystem II core complex (PSIIcc). PSIIcc is one of the two light-absorbing and light-driven molecular machines situated in the thylakoid membrane that provide the energy for redox processes in oxygenic photosynthesis (Renger 2008). The activity of PSIIcc ultimately results in the net transfer of electrons and protons from water to plastoquinone (Fig. 8.1). More precisely, there are three coupled processes: The photon-triggered one-electron transfer in the reaction center (RC), the four-electron water splitting chemistry catalyzed by the water-oxidizing complex (WOC) located at the lumenal side of the RC (the donor side) and the two-electron quinone reduction chemistry at the stromal (or cytoplasmic) side of the RC (the acceptor side). According to our present knowledge, cyt b_{559} is not involved in these primary processes. Nonetheless, cyt b_{559} is essential, as PSIIcc cannot assemble without it. Although there is no lack of hypotheses and discussions, the actual purpose of cyt b_{559} in the assembled PSIIcc is still unclear. In addition, cyt b_{559} exhibits a redox behavior of bewildering complexity, existing in at least three different redox potential forms. This together with the inability to assign to cyt b_{559} a clear-cut functional role contributes to the enigmatic character of this heme protein.

The aim of the present chapter is to give an up-to-date overview of the structural and redox potential properties of cyt b_{559} and a discussion of ideas concerning its function. Earlier reviews on cyt b_{559} have been given (Cramer and Whitmarsh 1977; Faller et al. 2005; Shinopoulos and Brudvig 2012; Stewart and Brudvig 1998; Whitmarsh and Pakrasi 1996). We also mention the review by Pospisil, focusing on enzymatic properties of cyt b_{559} (Pospisil 2011), and reviews discussing low molecular weight subunits of PSIIcc (Müh et al. 2008; Shi et al. 2012; Shi

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Abbreviations: Car – β-carotene; Chl – Chlorophyll; Cyt – Cytochrome; DCMU – 3-(3,4-dichlorophenyl)-1, 1-dimethylurea; DQH$_2$ – Duroquinol; EPR – Electron paramagnetic resonance; HP – High potential; IP – Intermediate potential; LMW – Low molecular weight subunit; LP – Low potential; PQ – Plastoquinone; PQH$_2$ – Plastoquinol; PSII – Photosystem II; PSIIcc – Photosystem II core complex; RC – Reaction center; ROS – Reactive oxygen species; RR – Resonance Raman; TMH – Trans-membrane helix; VLP – Very low potential; WOC – Water-oxidizing complex

[1] The subscript number refers to the first observation of cyt b_{559} due to a difference spectrum peak at 559 nm (Boardman and Anderson 1967). Curiously, the absorption maximum was later found to be rather close to 560 nm (Tae et al. 1993), and it was proposed to use the notation "cyt b_{560}" (Cramer 2004). Nonetheless, the common nomenclature "cyt b_{559}" appears to be irreversibly established, so that we use it here.
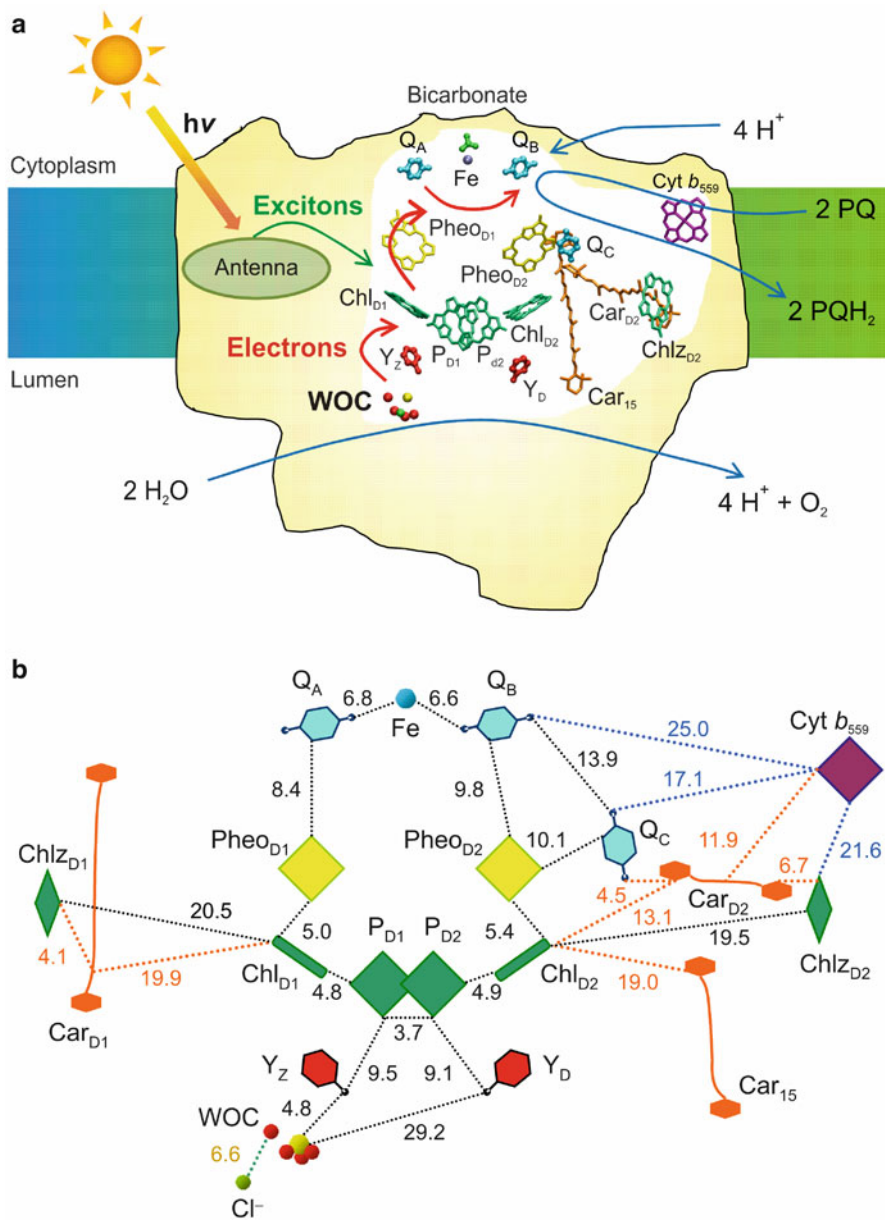
Fig. 8.1. (**a**) Arrangement of cofactors in and around PSII-RC of *T. elongatus* (made with VMD (Humphrey et al. 1996) based on PDB 3BZ1 (Guskov et al. 2009)) and relative to the perimeter of PSIIcc situated in the thylakoid membrane. Excitation by sunlight (*yellow/orange arrow*) or exciton transfer from outer antenna creates excitons in the core antenna system that are transferred to the RC (*green arrow*). Exciton-induced electron transfer in the RC (*red arrows*) ultimately leads to the reduction of quinones at the acceptor side (*top*) and oxidation of the WOC at the donor side (*bottom*). The reduced Q_B takes up protons from the cytoplasm (or stroma) forming PQH_2 and is exchanged with PQ in the thylakoid membrane (plastoquinone pool), while the oxidized WOC catalyzes the splitting of water into O_2 and protons released into the lumen (*blue arrows*). (**b**) Schematic representation of the cofactor arrangement in (**a**) along with edge-to-edge distances given in Å.

and Schröder 2004; Thornton et al. 2005) as well as a review about plastoquinone (PQ) reduction in PSIIcc that also contains a chapter about cyt b_{559} (Müh et al. 2012).

II. Structure

A. Genes and Polypeptides

PSIIcc is composed of at least 20 different polypeptide chains, a significant part of which (17) are membrane-spanning α-helical proteins. Besides the four large subunits PsbA, B, C, D comprising the RC and the core antenna of PSIIcc, there is a number of so-called low molecular weight (LMW) subunits consisting of only one trans-membrane helix (TMH) with the exception of PsbZ possessing two such helices (Müh et al. 2008; Shi et al. 2012; Shi and Schröder 2004; Thornton et al. 2005). For reasons that will become apparent below, the most interesting LMWs in the context of the present review are PsbE, F, J, X, and Y. Outstanding among these are the two polypeptides that constitute cyt b_{559} itself, PsbE and PsbF, also known as the α- and β-subunit, respectively, of cyt b_{559}. These are the subunits ligating the heme group. The crystallographic work discussed below shows that PsbJ, X and Y are located close to cyt b_{559} in PSIIcc.

In cyanobacteria and plants, the two genes *psbE* and *psbF* coding for the two cyt b_{559} subunits are adjacent and part of an operon that also includes the genes for two other LMWs, *psbL* and *psbJ* (Whitmarsh and Pakrasi 1996). The situation is different in the green alga *Chlamydomonas reinhardtii* (Mor et al. 1995). Deletion of either the entire *psbEFLJ* operon or the individual *psbE* or *psbF* genes results in a significant destabilization of PSIIcc. Studies on the mesophilic cyanobacterium *Synechocystis* PCC 6803 (henceforth referred to as *S.* 6803) showed that in these mutants the large subunit PsbD (also known as the D2 protein) is not integrated into the thylakoid membrane in a stable form, while significant amounts of the other large subunits are accumulated (Whitmarsh and Pakrasi 1996). Work on tobacco demonstrated that PsbE and PsbF each require the other for a stable insertion into the membrane, and without these subunits, PsbA (the D1 protein) is also not stable (Swiatek et al. 2003). These data clearly show that PSIIcc cannot be assembled without cyt b_{559}, and that the heme protein has at least a structure-stabilizing role.

Cyt b_{559} was first isolated and purified by Garewal and Wasserman (Garewal and Wasserman 1974a, b) and later sequenced (Herrmann et al. 1984; Widger et al. 1985). Amino acid sequences from different organisms are compiled in Fig. 8.2. Analyses of the sequences of PsbE and PsbF indicated the presence of one membrane-spanning α-helix in each of these polypeptides (Herrmann et al. 1984; Pakrasi et al. 1988), which was later confirmed by X-ray crystallography (see Fig. 8.2). The only potential heme ligands in this region are histidine residues, namely His E23 in the α-protein and His F24 in the β-protein (highlighted in orange in Fig. 8.2). Indeed, it was shown by combining optical, resonance Raman (RR), and electron paramagnetic resonance (EPR) spectroscopy, that the fifth and sixth ligands to the heme iron of cyt b_{559} are histidine nitrogens (Babcock et al. 1985) indicating that the whole heme protein consists of at least two α-helical subunits. The importance of a proper heme ligation was demonstrated by site-directed mutagenesis: The replacement of either E23 or F24 by Leu resulted in complete loss of PSII activity in *S.* 6803 (Pakrasi et al. 1991). However, there still remained the different possibilities that cyt b_{559} is a homodimer (α_2 or β_2) or an αβ-heterodimer. Chemical crosslinking experiments remained inconclusive in this respect (Moskalenko et al. 1992). Finally, X-ray crystallography unveiled cyt b_{559} to be an αβ-heterodimer, at last confirming the model proposed by Herrmann et al. (1984). Another problem that bothered researchers was the orientation of the two TMHs of cyt b_{559} in the membrane which, because of the positions of the axially ligating histidines, is intimately related to the question of whether the heme group

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PsbE
T. elongatus BP1  MAGTTGERPFSDIITS VRYWVIHSITIPALFIAGWLFVS TGLAYDVFGTPRPDSYYAQEQRSIPLVTDRFEAKQQVETFLEQLK
S.  PCC 6803      MSGTTGERPFSDIVTS IRYWVIHSITIPMLFIAGWLFVS TGLAYDAFGTPRPDEYFTQTRQELPILQERYDINQEIQEFNQ
A. thaliana          SFADIITS IRYWVIHSITIPSLFIAGWLFVS TGLAYDVFGSPRPNEYFTESRQGIPLITGRFDPLEQLDEFSRSF

PsbF
T. elongatus BP1  MTSNTPNQEPVSYPIFT VRWVAVHTLAVPTIFFLGAIAAM QFIQR
S.  PCC 6803      MATQNPNQPVTYPIFT VRWLAVHTLAVPSVFFVGAIAAM QFIQR
A. thaliana          MTIDRTYPIFT VRWLAVHGLXVPTVSFLGSISAM QFIQR

PsbJ
T. elongatus BP1      MMSEGGRIP LWIVATVAGMGVIVIVGLFFYGA YAGLGSSL
S.  PCC 6803          MFAEGRIP LWVVGVVAGIGAIGVLGLFFYGA YAGLGSSM
A. thaliana           MADTTGRIP LWVIGTVAGILVIGLIGIFFYGS YSGLGSSL

PsbX
T. elongatus BP1      MTITPSLKG FFIGLLSGAVVLGLTFAVLIAIS QIDKVQRSL
S.  PCC 6803          MTPSLAN FLWSLVLGAAIVLIPATVGLIFI SQKDKITRS
A. thaliana       AGSGISPSLKN FLLSIASGGLVLTVIIGVVVGVS NFDPVKRT

PsbY
T. elongatus BP1          M DWRVLVVLLPVLLAAGWAVRNI LPYAVKQVQKLLQKAKAA
S.  PCC 6803              M DWRVIVVVSPLLIAATWAAINI GAAAIRQLQDVLGREA
A. thaliana      A1  IQQIAQLAAANASS DNRGLALLLPIVPAIAWVLYNI LQPAINQVNKMRE
                 A2      AAEAAAASS DSRGQLLLIVVTPALLWVLYNI LQPALNQINKMRSGD
```

Fig. 8.2. Amino acid sequence alignment of low molecular weight subunits of PSIIcc related to cyt b_{559} for the thermophilic cyanobacterium *Thermosynechococcus elongatus* BP1, the mesophilic cyanobacterium *Synechocystis* PCC 6803, and the representative higher plant *Arabidopsis thaliana* (mouse-ear cress). PsbE and PsbF are the α- and β-subunit, respectively, of cyt b_{559}, ligating the heme group with the histidine residues highlighted in *orange*. Other residues forming the heme binding pocket are highlighted in *yellow* (see also Fig. 8.8). PsbJ and PsbX together with PsbE/F define the openings of the two putative plastoquinone diffusion pathways in PSIIcc (see Fig. 8.4). PsbY is in close contact with cyt b_{559} (see Figs. 8.3b and 8.5). The transmembrane regions of the peptides, as identified from the crystal structures, are *underlined*. In the case of *A. thaliana*, PsbX and PsbY are nuclear-encoded; corresponding signal sequences (Kim et al. 1996; Mant and Robinson 1998) are omitted. A1 and A2 refer to the two variants of the mature protein in *A. thaliana* contained in the precursor protein pPsbY (Thompson et al. 1999).

is located closer to the cytoplasm (stroma) or to the lumen. Various methods were applied including protease digestion (Tae et al. 1988; Tae and Cramer 1994) as well as immuno-gold labeling and electron microscopy (Vallon et al. 1989) to provide evidence that the N-termini point into the cytoplasmic phase as confirmed later by the crystal structures. Determining the number of cyt b_{559} αβ-heterodimers per PSIIcc has been another problem as discussed in greater detail by Whitmarsh and Pakrasi (1996). At least for cyanobacterial PSIIcc, this problem is solved, as the crystal structures clearly show that there is only one cyt b_{559} per RC. A crystal structure of plant PSIIcc is not yet available. However, structural information about supercomplexes at 12 Å resolution from electron microscopy (Caffarri et al. 2009) suggests that there is a high degree of homology between cyanobacterial and plant PSIIcc and that there is no space for a second cyt b_{559} in the latter.

The LMW subunit PsbJ is not only of interest here, because its gene is contained in the same operon as those of PsbE and PsbF, but also because it is structurally located close to cyt b_{559} (Fig. 8.3). Deletion of the *psbJ* gene affects the photoautotrophic growth and the PSII oxygen evolution to an extent that depends on the type of organism (Shi et al. 2012; Shi and Schröder 2004). The polypeptide possesses one TMH (Fig. 8.2), and its N-terminus points into the cytoplasm. Together with PsbE/F, it forms the entrance "gate" of one of the putative PQ diffusion channels in PSIIcc (Fig. 8.4). Thus, one can expect an influence of PsbJ on the PQ/PQH$_2$ exchange with the quinone pool in the membrane (cf. Fig. 8.1). Indeed, deletion of PsbJ in *S.* 6803 results in a longer lifetime of reduced Q_A and diminished oxygen evolution rates, although it is not essential for PSII activity (Lind et al. 1993; Ohad et al. 2004; Regel et al. 2001). In higher plants, PsbJ is indispensable (Hager et al. 2002). The latter
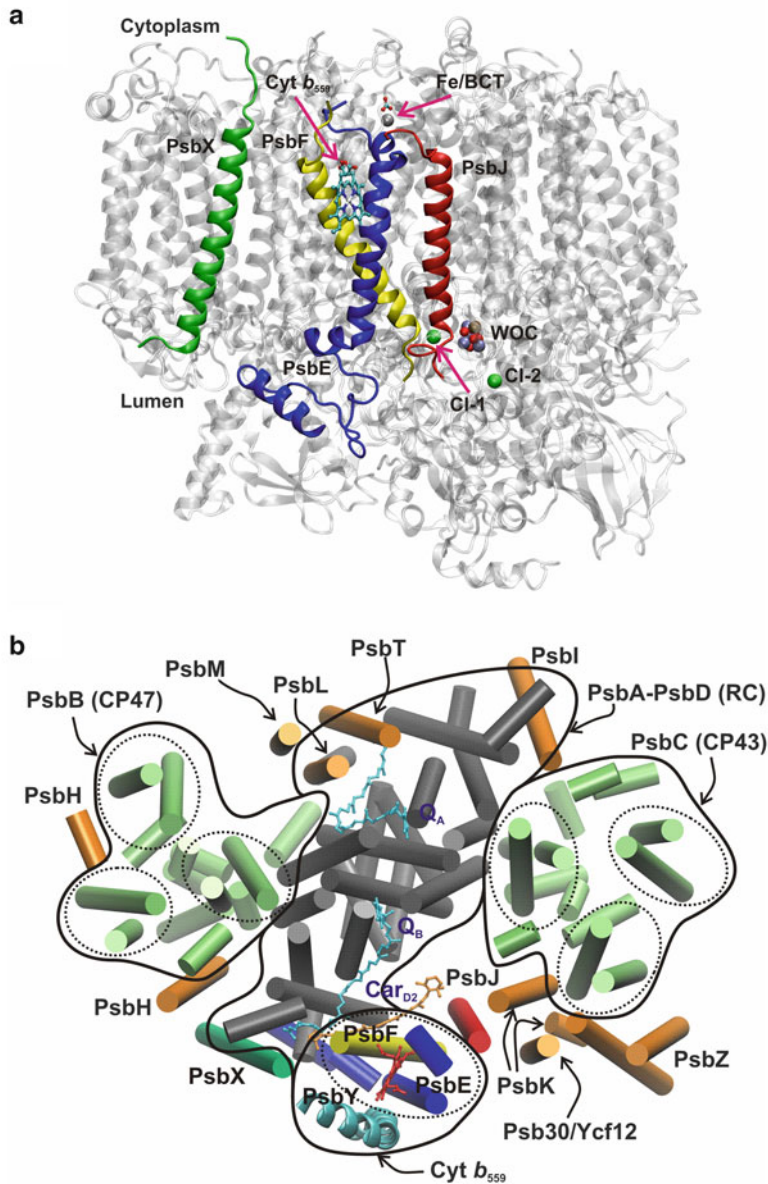
Fig. 8.3. Position of cyt b_{559} in one monomer of PSIIcc. (**a**) Ribbon representation of the protein backbone with PsbE in blue, PsbF in *yellow*, PsbJ in *red* and the remaining subunits in *light grey*; view along the membrane plane. Also shown is the heme group of cyt b_{559}, the non-heme iron with bound bicarbonate (Fe/BCT) as well as the WOC and the chloride ions close to the WOC as spheres. PsbX is shown in *green* and PsbY is omitted for clarity. (**b**) View from the cytoplasmic side showing α-helices as columns except for PsbY, which is shown in ribbon representation (*cyan*). The colors of PsbE, F, J and X are the same as in (**a**), the PsbA/D dimer (RC) is shown in *grey*, the two core antenna subunits PsbB (CP47) and PsbC (CP43) in *green*, and all other LMWs in *orange*. For clarity, the two-TMH bundles contained in PsbB, PsbC, and cyt b_{559} are enclosed by *dotted ellipses*. Also shown are the two plastoquinones Q_A and Q_B in *cyan*, the carotenoid Car_{D2} in *orange* as well as the heme group of cyt b_{559} in *red* (see also Fig. 8.1). Note that Q_B marks the position of quinone diffusion channel II (see Fig. 8.4), while channel I runs between cyt b_{559} and PsbJ. Q_C is not present in the 2.1 Å resolution structure (Koua et al. 2013), from which this figure is derived. Figure made with VMD (Humphrey et al. 1996).
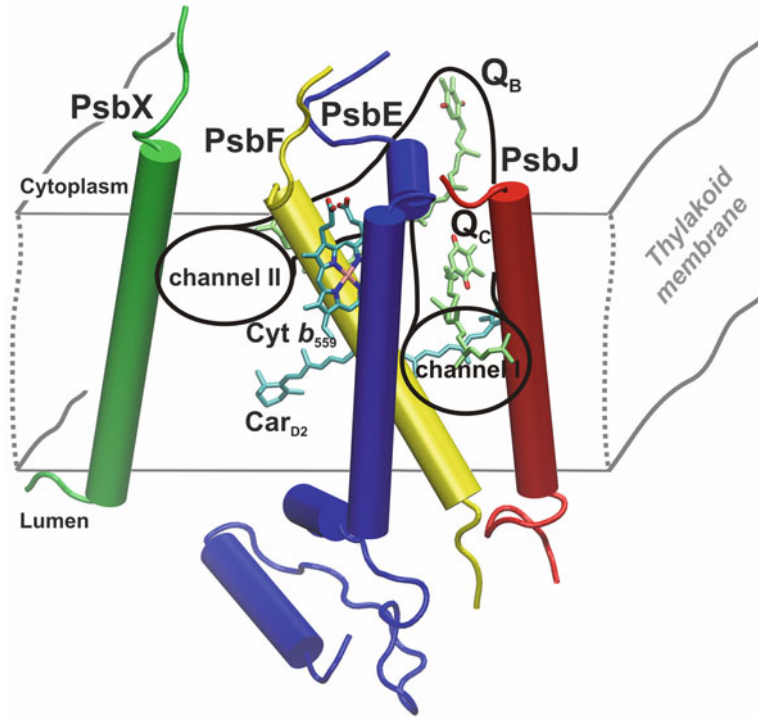
Fig. 8.4. Arrangement of cyt b_{559} (i.e., subunits PsbE, *blue*, and PsbF, *yellow*, binding the heme group) as well as subunits PsbJ (*red*) and PsbX (*green*) relative to the thylakoid membrane and the putative PQ/PQH₂ diffusion channels I and II in PSIIcc. α-Helices are represented as columns. The isoprenoid tail of the PQ occupying the Q_B site lies along channel II (in the background), while Q_C is located in channel I. Also shown is the β-carotene cofactor Car_D2. With the exception of PsbX, all N-termini of the protein subunits are located in the cytoplasm. Figure made with VMD (Humphrey et al. 1996) based on PDB 3BZ1 (Guskov et al. 2009).

fact might be related to the observation that PsbP, an extrinsic subunit pertaining to plant PSIIcc (Bricker et al. 2012), is lacking in Δ*psbJ* mutants (Hager et al. 2002; Suorsa et al. 2004).

The situation is more complicated in the thermophilic cyanobacterium *Thermosynechococcus elongatus*, as the effects of deleting PsbJ seem to depend on the type of D1 protein present. Cyanobacteria possess several genes encoding a number of PsbA-isoforms that are expressed differentially under various growth conditions (Mulo et al. 2009, 2012). There are three *psbA* genes in *T. elongatus* (Nakamura et al. 2002) that encode the three isoforms PsbA1, PsbA2, and PsbA3 (Nowaczyk et al. 2010). Under normal culture conditions, such as those used to obtain PSIIcc preparations that yield crystals (Kern et al. 2005; Zouni 2008),

PsbA1 prevails (Loll et al. 2008). PsbA3 is incorporated to about 70 % into PSIIcc under high light conditions, which is accompanied by some changes in functional properties (Kato et al. 2012; Sander et al. 2010; Sugiura et al. 2010b, 2014). The variant PsbA2 is induced under microaerobic conditions (Sicora et al. 2009), affects electron transfer reactions involving the redox-active tyrosine Y_Z at the donor side of PSIIcc (Sugiura et al. 2012), and a new hemoprotein is expressed in *T. elongatus* mutants containing only PsbA2 (Boussac et al. 2013). Interesting differences were observed when performing the *psbJ* deletion in *T. elongatus* strains expressing either only PsbA1 or PsbA3 (Sugiura et al. 2010a): While PsbA3-PSIIcc seems to be unaffected by the lack of PsbJ, PsbA1-PSIIcc shows a reduced stability and modified EPR spectra of cyt b_{559}. In

addition, many other subunits besides PsbJ are lacking in isolated PSIIcc of the PsbA1-variant. In a recent independent study, the core complex isolated from a PsbJ-deletion mutant of *T. elongatus* was found to consist mainly of a monomeric form of PSIIcc exhibiting an exchange of PsbA1 with PsbA3 despite the use of standard growth light conditions, lacking only PsbJ, and containing two additional subunits Psb27 and Psb28, which are involved in the biogenesis and repair of PSII (Nowaczyk et al. 2012).

The second putative quinone diffusion channel is located at the other side of cyt b_{559} and its mouth is flanked by the TMH of PsbX (Fig. 8.4). In line with this location is the finding that a knock-out of PsbX from *T. elongatus* results in a somewhat less efficient turnover of quinones at the Q_B site (Katoh and Ikeuchi 2001), which might be related to an influence on the diffusion of quinones to or from this site through channel II. Knock-out mutants of higher plants have not been constructed so far, but there is an *Arabidopsis thaliana* antisense plant containing less than 10 % of PsbX. These plants show a reduced level of functionally assembled PSIIcc and a severely reduced phosphorylation level of the core subunits PsbA (D1), PsbC (CP43) and PsbD (D2), which is interpreted in terms of a more oxidized quinone pool in the membrane (García-Cerdán et al. 2009). These findings suggest a structure-stabilizing role of PsbX and again an influence on the quinone turnover.

Based on studies of PsbY-deletion mutants, it was concluded that this subunit is not essential for normal function of PSII in *S.* 6803 (Meetam et al. 1999). Knock-out mutants of *A. thaliana* have been obtained and were reported to show phenotypic changes at cyt b_{559} (Shi et al. 2012) in accordance with the idea that PsbY is attached to the PsbE/F heterodimer as in cyanobacterial PSIIcc (Fig. 8.3b).

B. Insights from X-Ray Crystallography

The location of cyt b_{559} could be determined already in the first three-dimensional crystal structure of the dimeric PSII core complex (dPSIIcc) from *T. elongatus* at 3.8 Å resolution by virtue of the electron density of its heme iron (Zouni et al. 2001). It was also possible to identify helices, and the two TMHs close to the heme group were assigned to the α- and β-subunit of cyt b_{559}. The two subunits could be distinguished, because the C-terminus of PsbE extends significantly into the lumen as known from the amino acid sequence (Fig. 8.2). The electron density was consistent with an orientation of the π-plane of the heme group perpendicular to the membrane plane in agreement with EPR data (Bergström and Vänngard 1982; Crowder et al. 1982) and models based on the assignment of the axially ligating histidines (Babcock et al. 1985). What was new, however, was the observation, that the two TMHs of PsbE and PsbF are tilted with respect to the membrane normal and with respect to each other, giving the impression that they form a pair of tongs holding the heme group (see Figs. 8.3a and 8.4). Also apparent from this structure was the substantial distance between cyt b_{559} and the RC cofactors of PSIIcc (see also Fig. 8.1b).

Some assignments of α-helices to LMWs were made in this structure and the following refinements (Biesiadka et al. 2004; Ferreira et al. 2004; Kamiya and Shen 2003; Loll et al. 2005), but remained difficult until at a resolution of 2.9 Å (Guskov et al. 2009) a definite assignment was provided as shown in Fig. 8.3 for the subunits of interest here. The location of PsbY close to cyt b_{559} was in agreement with an independent crystallographic study of a PsbY-deletion mutant (Kawakami et al. 2007). A peculiar feature of the 2.9 Å resolution structure of PSIIcc was that, besides the usual primary (Q_A) and secondary (Q_B) quinones, a third PQ named Q_C was discovered ((Guskov et al. 2009), for pictures of the electron density, see (Guskov et al. 2010; Müh et al. 2012)). It is located in one of the two putative quinone diffusion channels that were identified on the basis of a theoretical analysis of the crystal structure (Guskov et al. 2009). It is of particular interest that these channels are located

on both sides of the PsbE/PsbF-heterodimer (Fig. 8.4), suggesting that the transport of PQ and/or PQH_2 between the Q_B site (the active site of PQ reduction) and the thylakoid membrane (PQ pool) is somehow related to cyt b_{559}. Also, the close proximity of PsbJ and PsbX to the channel mouths (Fig. 8.4) suggests an influence of these LMWs on quinone turnover, which is indeed observed as discussed above. It is not known, how the quinone transport itself takes place. An attractive hypothesis is that one channel is used by PQ, the oxidized form, to approach the Q_B site, whereas after light-induced reduction, PQH_2 uses channel II to reach the PQ pool, but there are other possibilities (Müh et al. 2012).

In contrast to PsbE, F, and J, the N-termini of PsbX and Y point into the lumen. In the case of PsbY, the electron density was still poor at 2.9 Å resolution. The assignment of this LMW and its orientation was made on the basis of characteristic bends in the helix due to proline residues (for amino acid sequence, see Fig. 8.2) in agreement with an earlier proposal (Kashino et al. 2007) and the above-mentioned crystal structure of a deletion mutant (Kawakami et al. 2007). Nonetheless, PsbY could only be modeled as a poly-Ala chain without structural details. It was clearly a major breakthrough in the structural biology of photosynthesis that the crystal structure of dPSIIcc from *T. vulcanus* could be improved to a resolution of 1.9 Å (Umena et al. 2011). Unfortunately, PsbY is missing in this structure. Subsequently, a crystal structure of dPSIIcc from *T. vulcanus*, in which the calcium ion of the WOC was replaced with strontium, was obtained at 2.1 Å resolution (Koua et al. 2013). This structure features PsbY in one monomer and allows a first detailed look at this subunit.

C. Subunit Interactions and Structural Relationships

Cyt b_{559} is located on the periphery of PSIIcc. This is not only apparent from the cofactor arrangement (Fig. 8.1), but also immediately evident from the α-helix array shown for one PSIIcc monomer in Fig. 8.3b (Note that not only TMHs are shown). When PSIIcc forms dimers (or rods of dimers as in the thylakoid membrane of cyanobacteria (Mörschel and Schatz 1987) or supercomplexes as in plant thylakoids (Caffarri et al. 2009)), cyt b_{559} is still at the outside. This location is meaningful, as cyt b_{559} marks the openings of the quinone diffusion channels, which have to be free to allow for quinone turnover. The central part of PSIIcc is dominated by the two large RC subunits PsbA and PsbD, each with five TMHs (grey) that are flanked by the two large antenna subunits PsbB and PsbC (green). The six TMHs of each of the latter are grouped in antiparallel two-helix bundles that are enclosed by dotted ellipses in Fig. 8.3b. The inner core of these four subunits is decorated at the outside with the various LMWs (orange). According to the present structural information, cyt b_{559} is actually a three-helix bundle composed of the heme-coordinating subunits PsbE (blue) and PsbF (yellow) forming a parallel two-helix bundle (enclosed by a dotted ellipse in Fig. 8.3b) as well as the additional subunit PsbY (cyan ribbon) which is oriented antiparallel to PsbE. Notably, cyt b_{559} makes contact with the inner core of PSIIcc only via PsbD, i.e., there is mainly an interaction between the TMH of PsbF and TMH-a, the first TMH along the amino acid sequence of the D2 protein. The putative quinone diffusion channel II as indicated by the isoprenoid tail of Q_B in Fig. 8.3b is located between these two TMHs. The channel opens to the lipid phase of the thylakoid membrane between the TMHs of PsbE and PsbX, while channel I opens between the TMHs of PsbE and PsbJ (Fig. 8.4). The region between PsbJ and PsbC is effectively filled up by the LMWs PsbK, Psb30 (also known as Ycf12) and, more peripheral, PsbZ, which thus block quinone diffusion in this direction (Fig. 8.3b). Thus, on the basis of the crystal structure, it seems that the possible quinone transport pathways are designed to make PQ or PQH_2 pass close by cyt b_{559} and that some of the LMWs are crucial in this

respect. The protein-free region between these LMWs and the large subunits is filled with lipids (not shown). In particular, there is a "bilayer island" composed of eight lipid molecules that is enclosed by protein, thus forming a kind of internal piece of membrane within PSIIcc (Guskov et al. 2009; Kern and Guskov 2011). Perpendicular to the channels and within the membrane plane lies the β-carotene cofactor Car_{D2} (Figs. 8.3b and 8.4), which sticks out from all carotenoids bound to PSIIcc because of its unusual orientation and relative vicinity to the RC cofactors (Fig. 8.1). This carotenoid is believed to be involved in slow electron transfer processes involving cyt b_{559} and the RC (Brose et al. 2013; Faller et al. 2005; Frank and Brudvig 2004; Renger and Schlodder 2011; Shinopoulos and Brudvig 2012). Recent studies on site-directed mutations provide further evidence for this involvement (Shinopoulos et al. 2014).

The two TMHs of PsbE and PsbF cross at an angle of –40°. The minus sign implies that, when looking on the PsbE/PsbF heterodimer from the PsbE side as in Fig. 8.5a, the TMH of PsbE is rotated clockwise with respect to that of PsbF. This follows the usual sign convention for rotation angles (Chothia et al. 1977). The particular arrangement of the two helices is also referred to as a right-handed crossing. Note that it implies a form of chirality: A counter-clockwise rotation of PsbE with respect to PsbF would have created the mirror image, a left-handed crossing motif. An angle of about –40° is frequently found in crossing motifs of parallel TMHs (Walters and DeGrado 2006). Helix-helix interactions are found to be promoted by typical amino acid sequences such as the prototypical GxxxG-motif of glycophorin A, an erythrocyte protein of unknown function (Langosch and Arkin 2009; Moore et al. 2008). The glycine residues may be replaced by other residues with small side chains such as Ala or Ser, which is why the motif, in the case of right-handed crossing, is also referred to as GAS_{Right} motif (Moore et al. 2008). The GAS_{Right} motif may be

supplemented by aromatic residues such as Trp or may be extended to an FxxGxxxG motif (Langosch and Arkin 2009). Indeed, the motifs found in cyt b_{559} are exactly like that: The sequence $^{31}FxxGWxxV^{38}$ in PsbE interacts with the homologous sequence $^{32}FxxGxxxA^{39}$ in PsbF (see the central part of Fig. 8.5c). The two glycine residues Gly E34 and Gly F35 define the region of closest approach of the two TMHs. The side chains of the two phenylalanine residues Phe E31 and Phe F32 are symmetry-related to each other and are attached to the Gly residues of the respective other subunit (blue in Fig. 8.5c), presumably by a $C_\alpha - H \cdots \pi$ interaction (not shown, but see (Brandl et al. 2001) for prototypical examples of such interactions). Further downstream the amino acid sequence in the interaction motif, the side chains of Trp E35 and Val E38 form a pair of tongs holding the side chain of Ala F39 by van-der-Waals interactions (see lower inset to Fig. 8.5a). These interactions very likely already determine the crossing angle of –40°. Notwithstanding, binding of the heme group will further stabilize the structure. This view is supported by recent EPR studies of a spin-labeled β_2-homodimer referred to as cyt $b_{559'}$, showing that heme binding is not needed for dimerization, but tightens the heme binding site (Akdogan et al. 2012). In the native αβ-heterodimer, this stabilization of the structure is accomplished not only by the ligation of the central iron of the heme by the two histidine side chains E23 and F24 as well as the formation of salt bridges between the propionate substituents of the heme and the two arginine residues E18 and F19 (see below), but also by the additional formation of a hydrogen bond between one of these arginines (F19) and the backbone carbonyl of Arg E8 in the cytoplasmic extension of PsbE (see the upper inset to Fig. 8.5a). The latter bond establishes a further direct interaction between the two subunits.

When the crossing angle of two interacting TMHs is close to 0°, the interaction motif has to cover the full length of the trans-membrane domain. Typical for
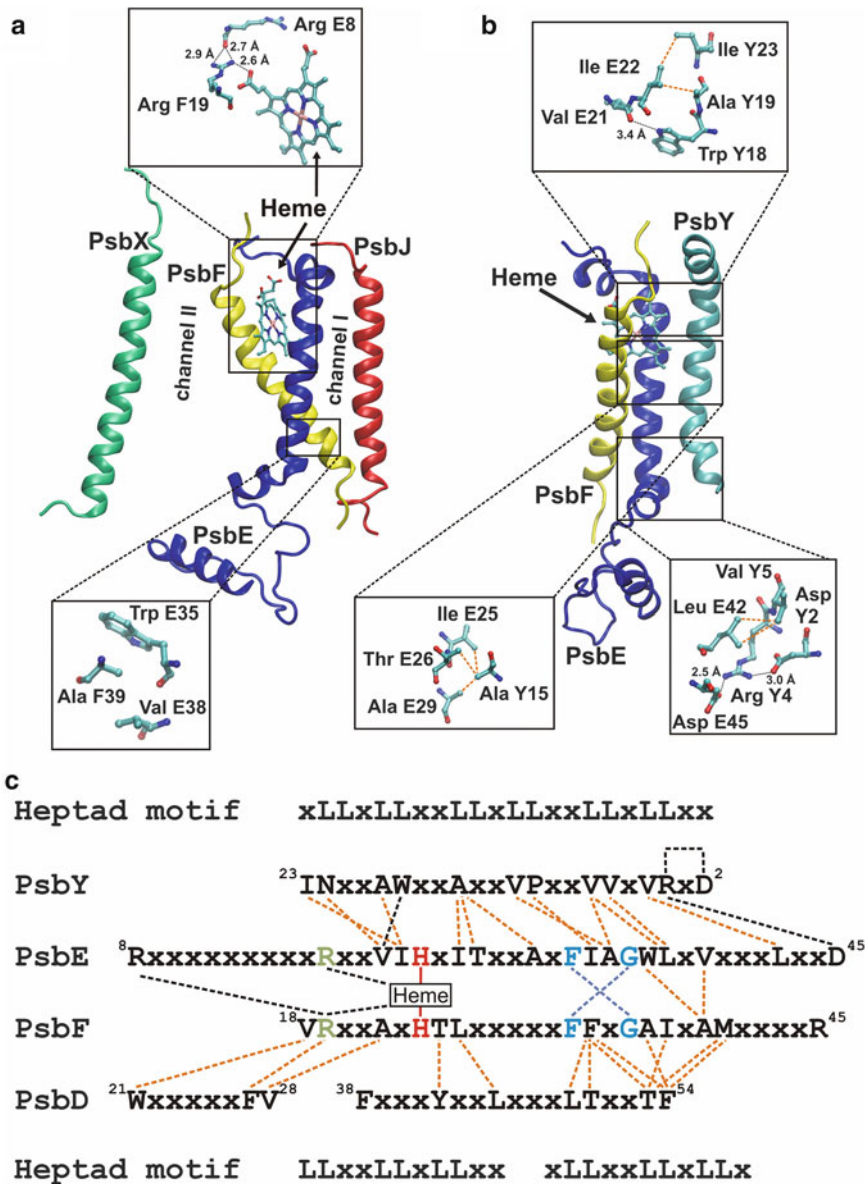
Fig. 8.5. Interactions between protein subunits of cyt b_{559}. α-Helices are represented as ribbons. View along the membrane plane with cytoplasm at the *top* (**a**) Relative arrangement of PsbE (*blue*), PsbF (*yellow*), PsbJ (*red*), and PsbX (*green*) (**b**) Relative arrangement of PsbE (*blue*), PsbF (*yellow*), and PsbY (*cyan*). Insets show enlarged views of specific amino acid interactions (**c**) Alignment of amino acid sequences in the interaction region of protein subunits PsbD, PsbE, PsbF, and PsbY. The sequence of the latter is shown in reverse order, because the TMH of PsbY is oriented antiparallel to those of the other subunits. The two histidine residues ligating the central iron ion of the heme are colored in *red* and the two arginine residues forming salt bridges with propionate substituents of the heme in *green*. Residues colored in *blue* belong to the central interaction region at the crossing point of PsbE and PsbF (GAS$_{Right}$ motif). *Dashed lines* indicate C$_\alpha$−H···π interactions (*blue*), van-der-Waals interactions (*orange*), and hydrogen bonds (including salt-bridges, *black*). For comparison, prototypical heptad repeat motifs characteristic for membrane-spanning leucine zippers are shown in the *top* and in the *bottom*. Figures made with VMD (Humphrey et al. 1996) based on PDB 4IL6 (Koua et al. 2013).

interactions of this kind are heptad repeat motifs of the form LLxxLLxLLxxLLx, where Leu residues are located at positions suitable for interaction with their counterpart in the second helix, while the "x" residues point into a different direction (Gurezka et al. 1999). Such motifs are referred to as membrane-spanning leucine zippers. However, there is no strict requirement for the residues at the "L" positions to be leucines (Gurezka and Langosch 2001) and not even to be apolar (Choma et al. 2000; Zhou et al. 2000). A leucine zipper has been identified in the center of the PSIIcc dimer, where the two PsbM subunits interact (Guskov et al. 2009). Here, the interaction motif is [6]LxxLIxxxLxxLVxxVxxIIxxV[27]. Similar heptad motifs are found in the interaction region of PsbE and PsbY, although the helices run antiparallel. This is the reason, why the sequence of PsbY is shown in reverse order in Fig. 8.5c. The reverse zipper sequence [19]AxxxAxxVPxxVV[7] in PsbY contacts the sequence [22]IxxITxxAxxIAxxL[36] in PsbE. The occurrence of the more polar threonine in the latter sequence might be surprising, but in fact, it behaves like an apolar residue by contributing a methyl group to van-der-Waals interactions (see the lower left inset to Fig. 8.5b). These transmembrane helix-helix contacts between PsbE and PsbY are supplemented by further interactions at both ends of the helices that also involve hydrogen bonds and salt bridges (see the upper and lower right inset to Fig. 8.5c).

Cyt b_{559} makes contact with the inner core of PSIIcc only at TMH-a of PsbD (Fig. 8.6). The interaction between this TMH and that of PsbF is special: When looking along a certain direction in the membrane plane, one notices that the crossing angle is 0°, i.e., the TMHs run parallel as the N-termini of both subunits lie in the cytoplasm (Fig. 8.6b). However, when looking from a perpendicular direction in the membrane plane (Fig. 8.6a), one readily sees that the TMHs make an angle, i.e., they are further apart towards the cytoplasm than towards the lumen. Most

likely, the reason for this arrangement is to clear a space for quinone diffusion. In fact, the region between these two TMHs is a bottleneck of channel II. The interactions between the protein subunits are described accordingly. There is contact between heptad motifs only in the lower part of the helices towards the lumenal side (Fig. 8.5c). In the middle of the helices, there is a special interaction between Tyr D42 and Thr F25 without hydrogen bonding (Fig. 8.6c). This point marks the lower boundary of channel II. Additional interactions in the cytoplasmic loops of PsbD and PsbF contribute to maintain the angle between the helices and thus to keep the channel open. This region is particularly interesting for two reasons, as we shall discuss in the remainder of this subsection: (i) the possible existence of a PQH_2/herbicide binding site and (ii) a possibly crucial role in PSIIcc assembly/repair.

1. A PQH_2/Herbicide Binding Site Between PsbD and PsbF?

Several studies point to the possibility that there are more quinone binding sites in PSIIcc than just the two of Q_A and Q_B. A third PQ labeled Q_C (see Fig. 8.4) was identified in the crystal structure at 2.9 Å resolution (Guskov et al. 2009), but not in any other structure. Kruk and Strzalka investigated electron transfer between cyt b_{559} and exogenous quinones and found that the low-potential form of cyt b_{559} (see Sect. III) interacts with the quinones in a way that suggests the presence of two additional distinct binding sites termed "Q_C" and Q (Kruk and Strzalka 2001). Since DCMU, a herbicide known to occupy the Q_B pocket ((Müh and Zouni 2013) and references therein), has no influence on these electron transfer reactions, it was concluded that Q_B is not involved. Furthermore, the new binding sites showed a preference for polar quinones. In contrast, the crystallographically detected Q_C resides in a fairly apolar environment and is located relatively far away from cyt b_{559}. Therefore, we think – in agreement with recent sugges-
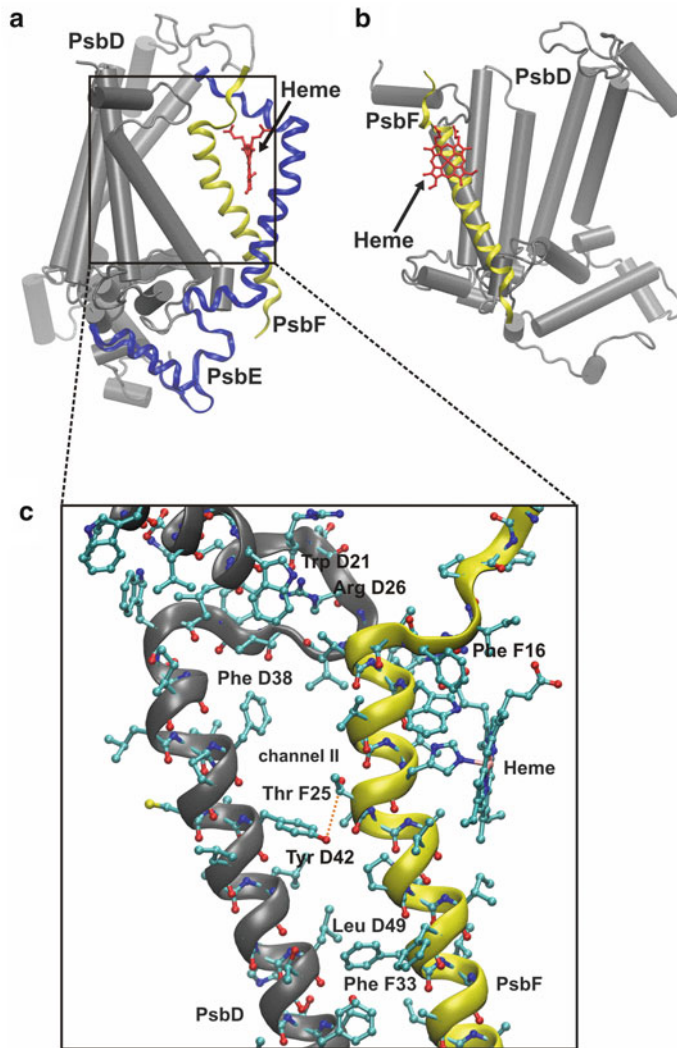
Fig. 8.6. Interaction of cyt b_{559} with the N-terminal part of the RC subunit PsbD (D2 protein) (**a**) Location of TMHs of PsbE (*blue ribbon*) and PsbF (*yellow ribbon*) relative to those of PsbD (*gray columns*). The helix of PsbD closest to PsbF is TMH-a. The heme group of cyt b_{559} is shown in *red*. View along the π-plane of the heme lying *vertical* and along the plane of the thylakoid membrane lying *horizontal*. The *black rectangle* indicates the region shown enlarged in (**c**) (**b**) View rotated by 90° relative to (**a**) demonstrating that the TMH of PsbF (*yellow ribbon*) and TMH-a of the PsbD (*gray column*) have the same orientation in this direction. The heme group of cyt b_{559} is shown in *red*. PsbE is omitted for clarity. (**c**) Region of closest interaction between PsbD and PsbF in the thylakoid membrane. The only polar groups of the two subunits facing each other inside the membrane are Tyr D42 and Thr F25 approaching hydrogen bonding distance, but without forming such a bond (The indicated distance is that between the hydroxyl oxygen of Tyr and carbon C_{β} of Thr). Note that channel II (see Fig. 8.4) runs above these two residues and is relatively narrow in this region (see also Fig. 8.8d in (Müh et al. 2012)). Figures made with VMD (Humphrey et al. 1996) based on PDB 4IL6 (Koua et al. 2013).

tions (Cardona et al. 2012; Kaminskaya and Shuvalov 2013) – that the "Q_C" site proposed by Kruk and Strzalka (2001) is different from the Q_C site identified by Guskov et al. (2009).

The herbicides DCMU and dinoseb were found to influence the redox potential of the high-potential form of cyt b_{559}, suggesting that they bind close to cyt b_{559} (Kaminskaya

et al. 2007). Since both reagents also bind to the Q_B pocket, it was suggested that the new site close to cyt b_{559} may also accommodate the structurally similar PQ or PQH_2. More recently, the photoreduction of cyt b_{559} in PSII membrane fragments was investigated and shown to be due to PQH_2 from the quinone pool (Kaminskaya and Shuvalov 2013). Cyt b_{559} can also be reduced by the analogous duroquinol (DQH_2), and dinoseb competes with DQH_2, whereas DCMU does not. These data suggest the existence of a binding site close to cyt b_{559} specific for quinone-like molecules and accessible from the membrane phase, but different from the crystallographic Q_C site. It was proposed to call this site Q_D (Kaminskaya and Shuvalov 2013).

As discussed elsewhere (Müh et al. 2012), the idea exists that channel II is specific for PQH_2. It is then reasonable – as a first guess – to search for the Q_D site along this channel. In this respect, the region around the bottleneck between TMH-a of PsbD and the TMH of PsbF is an interesting candidate, because it likely constitutes a barrier to quinol diffusion and features a number of specific side chains pointing into the channel that may interact with PQH_2. For example, the hydroxyl group of Thr F25 is one of only a few possibilities to form a hydrogen bond (Fig. 8.6c). Also, of the few aromatic side chains pointing into the channel, which might bind quinols by π-stacking (Waters 2002), some are located in this area such as Phe D38 or Tyr D42. The region is located close to the heme group suitable for electron transfer and is near the membrane surface, which is more polar than the membrane interior and thus suitable for quinols or more polar quinones (Nowicka and Kruk 2010). Finally, some titratable groups such as Arg D26 (Fig. 8.6c) or Asp X35 (not shown) are nearby in the cytoplasmic region. These groups are potential proton acceptors that might be important to support one-electron oxidation of a quinol (Buser et al. 1992; Kaminskaya and Shuvalov 2013). As dinoseb competes with quinols for the binding place,

a crystal structure of PSIIcc subjected to this herbicide would be of interest to identify the Q_D site.

2. A Role of PsbD-PsbF Interaction in PSIIcc Assembly or Repair?

According to current models of PSIIcc assembly (see (Nixon et al. 2010) for a review), the subunits PsbD, PsbE, and PsbF, on one hand, as well as PsbI and a precursor of PsbA (pD1, containing an extended C-terminus that has to be removed to allow assembly of the WOC) on the other hand, form pre-complexes in the thylakoid membrane: the D2-cyt b_{559} and pD1-PsbI complex, respectively, which subsequently combine to a larger RC complex. Given the subunit interactions in the fully assembled PSIIcc (Fig. 8.3b), the existence of a D2-cyt b_{559} complex is reasonable. Then, it is obvious that the peculiar interaction between TMH-a of PsbD and the TMH of PsbF is already present in this sub-complex, and that the involved amino acid residues are important for its assembly. Based on the structure of PSIIcc, one may further assume that the PsbD-PsbF interaction is kept up during the PSII repair cycle, in which the damaged D1 protein is replaced (Nixon et al. 2010). Provided the Q_D site discussed above is indeed located in this region, it is likely also present in the D2-cyt b_{559} complex and during D1-exchange. This would enable electron transfer reactions between quinones/quinols and cyt b_{559} under these conditions with possible functional implications. We would like to stress that the search for a functional role of cyt b_{559} should not be restricted to the fully assembled PSIIcc, but a role may well be found in subcomplexes related to PSIIcc assembly and repair.

D. A Closer Look at the Heme Site

The heme group of cyt b_{559} is well shielded from the environment by the protein, as is apparent from the space-filling representation in Fig. 8.7. The major part of this enclo-
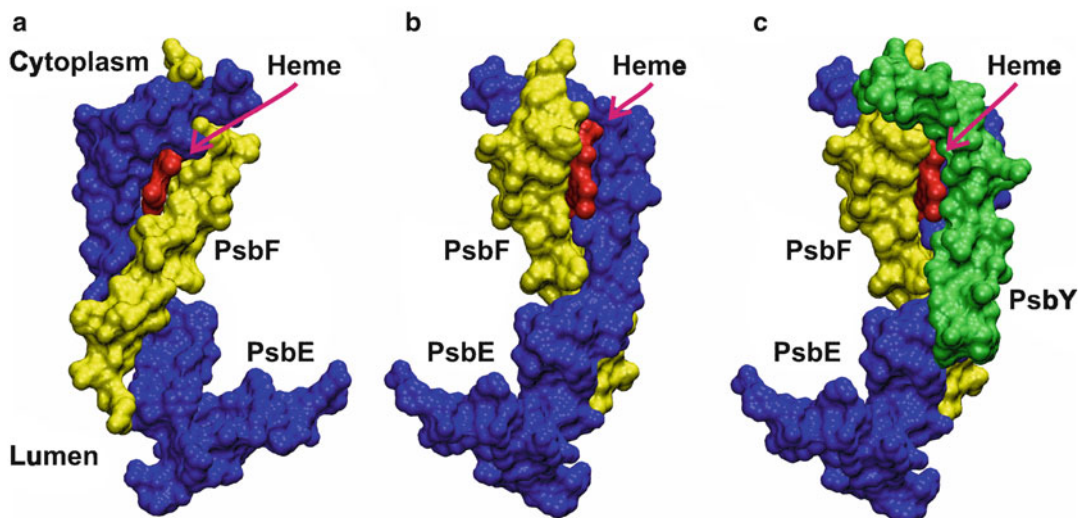
Fig. 8.7. Space filling representation of cyt b_{559} with the heme group in *red*, PsbE in *blue*, PsbF in *yellow*, and PsbY in *green*. (**a**) and (**b**) show views from different directions in the absence of PsbY. The view in (**c**) is the same as in (**b**), but with PsbY present. Figures made with VMD (Humphrey et al. 1996) based on PDB 4IL6 (Koua et al. 2013).

sure is already provided by PsbE and PsbF, including an effective shielding against the cytoplasmic phase. However, the shielding by just two TMHs is unavoidably incomplete, as can be seen by the red spots that show the heme in Figs. 8.7a, b, indicating contact of the heme with the membrane phase along the π-plane of the heme group. Notably, a significant fraction of the contact surface at the outside of PSIIcc is covered by PsbY (Fig. 8.7c). Thus, we propose that the role of PsbY is to protect cyt b_{559} against unwanted side reactions due to contact with redox active molecules in the thylakoid membrane, e.g., quinones or dioxygen.

A closer look at the heme itself reveals that it is distorted in an unusual way (Fig. 8.8a). The two pyrrole rings pointing towards the lumen are practically within the π-plane of the tetrapyrrole, whereas those facing the cytoplasm are significantly bent out of this plane giving the heme a propeller-like appearance. Clearly, the reason for this non-planarity is the interaction of the two propionate substituents with arginine residues in the two protein subunits (Arg E18 and F19). In this way, it appears that the heme conformation is coupled to the crossing angle of the

two TMHs of PsbE and PsbF. It is unclear, however, to what extent this angle is variable and whether a conformational distortion of the heme is of functional importance as has been discussed for other heme proteins (Shelnutt et al. 1998).

The π-systems of the two axially ligating histidines (E23 and F24) are almost in plane, i.e., make an angle of ∼30° (Fig. 8.8a), which is not generally the case for *b*-type cytochromes (for a recent counter-example, see the structure of cyt b_{561} from *A. thaliana*, where the angle is close to 90° (Lu et al. 2014)). A possible meaning of this angle is discussed in Sect. III below. Another special feature is the π-stacking of the axially ligating histidines with other aromatic residues as shown for the PsbF-side in Fig. 8.8b. Note that there are various forms of π-stacking (Waters 2002), implying that the π-systems of the stacking side chains need not be in plane, but may also be oriented perpendicular to each other as seen for Trp F20 and Phe F16. These interactions link the orientation of the histidine π-systems with respect to that of the heme to the protein conformation, but the functional significance of such a link is not clear.
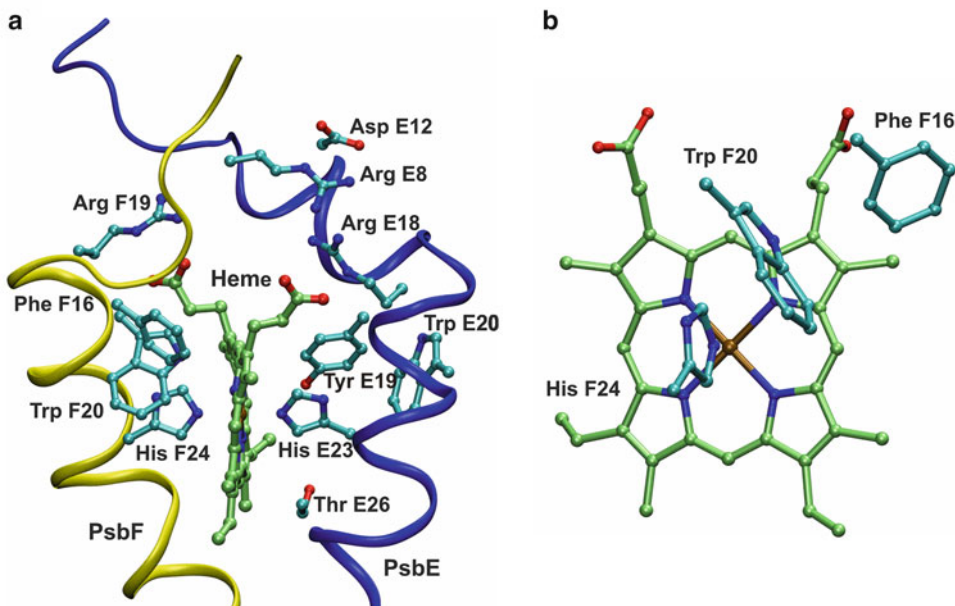
Fig. 8.8. A closer look at the heme site of cyt b_{559}. (**a**) View along the π-plane of the heme (*carbon atoms green*) with selected amino acid side chains (*carbon atoms cyan*) and the peptide backbone (*ribbons*) of PsbE (*blue*) and PsbF (*yellow*) (**b**) View on top of the π-plane of the heme with selected amino acid side chains from PsbF (same colors as in **a**). Figures made with VMD (Humphrey et al. 1996) based on PDB 4IL6 (Koua et al. 2013).

III. Redox Properties

A. Different Redox Potential Forms

The redox midpoint potential of *b*-type cytochromes is normally found in the range of -150 mV $< E_m < +150$ mV (Bérczi and Moller 2000; Cramer and Whitmarsh 1977; Gámiz-Hernández et al. 2010). Indeed, the redox potential of the so-called low-potential (LP) form of cytochrome b_{559} is in this range (Iwasaki et al. 1995; Kaminskaya et al. 1999; McNamara and Gounaris 1995; Ortega et al. 1988; Roncel et al. 2001; Shuvalov et al. 1994; Thompson et al. 1989). However, in PSII-enriched membrane preparations and untreated chloroplasts of higher plants, the prevailing form is the high-potential (HP) form with 350 mV $< E_m < 450$ mV (Horton and Croze 1977; Iwasaki et al. 1995; Kaminskaya et al. 1999, 2007; McNamara and Gounaris 1995; Ortega et al. 1988; Roncel et al. 2001; Thompson et al. 1989). Some authors distinguish in

addition an intermediate potential (IP) form with $E_m \sim +200$ mV (Ahmad et al. 1993; Horton and Croze 1977; Kaminskaya et al. 2005, 2007; Ortega et al. 1988; Roncel et al. 2001; Shuvalov et al. 1994; Thompson et al. 1989) and a very low potential (VLP) form with $E_m \sim -200$ mV (Kaminskaya et al. 1999; Shuvalov 1994). An overview is given in Table 8.1. It is evident that a variety of conditions exists, under which the different forms are observed.

In samples that are close to physiological conditions, i.e. untreated chloroplasts or membrane fragments, one usually observes a mixture of HP, IP, and LP form, indicating that all these forms are related to the native behavior of cyt b_{559}. Although the HP form is prevailing in intact membranes, it has been found to be very labile in the dark toward conversion to a form with a lower potential (Cramer and Whitmarsh 1977). There is a clear tendency to reduce the amount of the HP form, when the isolation procedure is pushed forward, i.e., to

Table 8.1. Redox potential forms of cyt b_{559}.

Organism	Sample type	Preparation conditions	pH	Form	E_m (mV)	Reference
Pea	RC	2 mM βDM[a]	8.0	HP 28 %	430	Ahmad et al. (1993)
				IP 62 %	180	
				LP 10 %	25	
	PSII membranes	Kuwabara and Murata (1982)	5.0–10.0	IP → LP	203 → 110	Roncel et al. (2001)
	Chloroplasts	Untreated	7.5	HP 58 %	383	Horton and Croze (1977)
				LP 31 %	77	
		NH$_2$OH-treated(dark)		IP 58 %	240	
				LP 42 %	93	
		NH$_2$OH-treated (light)		LP 95 %	90	
Spinach	RC	0.1 % (w/v) bDM[a]	7.2	HP 4 %	380	Shuvalov et al. (1994)
				IP 48 %	150	
				LP 48 %	−45	
			9.4	HP 4 %	400	
				IP 48 %	150	
				LP 24 %	40	
				VLP 24 %	−220	
	RC	Nanba and Satoh (1987)	4.0–9.0	LP 100 %	115 → 50	Shibamoto et al. (2008)
	PSIIcc	0.025 % (w/v) βDM[a]	6.5–9.3	LP 86 %	118	Kaminskaya et al. (1999)
				VLP 14 %	−147	
	PSII membranes	Kuwabara and Murata (1982)	5.0–10.0	HP	360	Ortega et al. (1988); Roncel et al. (2001); Thompson et al. (1989)
				IP → LP	203 → 110	
		Berthold et al. (1981); Beck et al. (1985)	6	HP 44 %	375	
				IP 31 %	228	
		Untreated		LP 25 %	57	

(continued)

Table 8.1. (continued)

Organism	Sample type	Preparation conditions	pH	Form	E_m (mV)	Reference
		NaCl-washed		HP 18 %	375	McNamara and Gounaris (1995)
				IP 42 %	171	
				LP 40 %	3	
Spinach	PSII membranes	Chapman et al. (1988)				
		Untreated oxidative	6.0	HP 90 %	353	
				LP 10 %	81	
		Untreated reductive		HP 49 %	390	
				LP 51 %	125	
		In the presence of 0.05 % βDM a		LP 100 %	96	
		Treated with 0.5 % Triton X-100		LP 100 %	109	
		Treated with NaCl in the dark		HP 75 %	398	
				LP 25 %	149	
		Treated with NaCl in dim light		IP 100 %	179	
		Treated with MgCl2		IP 100 %	167	
		Treated with CaCl2		IP 100 %	169	
		Radmer et al. (1986)				Iwasaki et al. (1995)
		Untreated	6.5	HP 66 %	437	
				IP 19 %	259	
				LP 14 %	26	
		NH2OH-treated		HP 54 %	435	
				IP 24 %	237	
				LP 22 %	45	
		NH2OH-treated and exposed to heat-filtered white light at 22 °C		HP 24–54 %	435	
				IP' 17–41 %	342–360	
				IP 24–54 %	175–237	
				LP 19–56 %	28–61	

Organism	Sample	Condition/Reference	pH	Fraction	E_m (mV)	Reference
Sugar beet	RC	0.1 % (w/v) βDM[a]	6.5–9.3	LP 100 %	60	Kaminskaya et al. (1999)
			9.3–9.7	LP 100 %	40–60	
		Liposomes	6.8–9.5	LP 100 %	25–100	
	PSII membranes	Berthold et al. (1981); Völker et al. (1985)	6.0–9.2	HP 64 → 34 %	351–387	
				IP 21 → 47 %	196–242	
				LP 15 → 19 %	11–101	
Sugar beet	PSII membranes	Berthold et al. (1981); Völker et al. (1985)	6.5	HP 70–75 %	386–404	Kaminskaya et al. (2007)
				IP 10–15 %	169–228	
		Without additive		LP 10–20 %	0–109	
		With 640 μM DCMU[b]		HP 75 %	355	
				IP 15 %	225	
				LP 10 %	100	
		With 110 μM dinoseb[c]		HP 81 %	318	
				IP 8 %	217	
				LP 11 %	101	
		With 30 μM TPB[d]		HP 54 %	265	
				IP 24 %	171	
				LP 22 %	-7	
		With 20 μM CCCP[e]		HP 66 %	316	
				IP 11 %	107	
				LP 23 %	-12	
Phormidium laminosum	PSII membrane	Stewart and Bendall (1980)	7.5	HP (variable amounts)	330–350	Stewart and Bendall (1980)
				LP (small amounts)	0–100	
Synechocystis 6803	PSII membranes	Ortega et al. (1994)	7.5	IP 100 %	170	Ortega et al. (1994)

Table 8.1. (continued)

Organism	Sample type	Preparation conditions	pH	Form	E_m (mV)	Reference
T. elongatus	PSIIcc	0.02 % (w/v) βDM[a] (Kern et al. 2005)	6.5	IP 100 %	244	Kaminskaya et al. (2005)
		0.03 % (w/v) βDM[a]	6.5–8.5			Roncel et al. (2003)
		His-tagged (Sugiura and Inoue 1999)		HP 50 %	390	
		Untreated		IP 50 %	275	
		Untreated	9.5	HP 30 %	380	
				IP 70 %	260	
		Tris-washed[f]	6.5	HP 30 %	380	
				IP 70 %	277	
		After mild heating	6.5	HP 15 %	390	
				IP 85 %	250	
T. elongatus	PSIIcc	0.03 % (w/v) bDM[a]	6.5	HP 85 %	394	Guerrero et al. (2014)
		His-tagged (Sugiura and Inoue 1999)		IP 15 %	240	

Note that some authors call LP what we here refer to as IP for consistency

[a] n-dodecyl-β-D-maltoside, a detergent used to solubilize the thylakoid membrane
[b] 3-(3,4-dichlorophenyl)-1,1-dimethylurea, a herbicide
[c] 2,4-dinitro-6-sec-butylphenol, a herbicide
[d] tetraphenylboron
[e] carbonylcyanide-*m*-chlorophenyllhydrazone
[f] tris(hydroxymethyl)aminomethane

isolated core complexes or RC preparations. This is an indication that interaction with the detergent used to solubilize the membrane is one factor destabilizing the HP form. In particular, the VLP form is only observed in these preparations and preferably at high pH (Shuvalov et al. 1994). It is of note that crystallizable cyanobacterial PSIIcc (isolated from *T. elongatus*) only exhibits the IP form with $E_m = 244$ mV at pH = 6.5 (Kaminskaya et al. 2005), although preparations from other strains have been reported to also show the HP form (Guerrero et al. 2014; Roncel et al. 2003). These results have to be taken into account, when trying to link the redox properties of cyt b_{559} to the crystal structure.

Various treatments aiming at a manipulation of the donor side of PSIIcc have been reported to change the composition of redox potential forms of cyt b_{559} in the samples. These treatments include washing with NaCl to remove some of the extrinsic protein subunits of PSIIcc at the lumenal side or incubation with NH_2OH to reduce and eventually destroy the WOC. Since all these experiments have been performed on plant material, and plant PSIIcc differs from cyanobacterial PSIIcc with respect to the extrinsic protein subunits (Bricker et al. 2012; Bricker and Burnap 2005), it is presently difficult to draw any conclusions from the crystal structures concerning these treatments. In the case of cyanobacterial PSIIcc, washing with the buffer Tris at pH 9.0 was reported to also destabilize the HP form (Roncel et al. 2003). This treatment is expected to remove manganese from the WOC and thus to modify the donor side of PSIIcc (Yamashita 1986). It is not clear, how this could affect the redox potential of cyt b_{559}. However, due to its lumenal extension (Fig. 8.3a), PsbE may be sensitive to manipulations of the donor side.

B. Factors Influencing the Redox Potential

Long-standing questions concerning the redox behavior of cyt b_{559} are: Why is the redox potential of the HP form so high? And how is the HP form converted to the other forms? There are still no clear-cut answers to these questions, but a number of factors that may influence the redox potential of cyt b_{559} have been discussed in the literature. In the following, we shall go through a list of these factors, but also add some points that have not been mentioned in earlier reviews.

1. pH Dependence

The redox potential of the HP form appears to be pH-independent (Ahmad et al. 1993; Ortega et al. 1988; Roncel et al. 2001, 2003), although Kaminskaya et al. (1999) observed a linear drift from 387 to 351 mV between pH 6.0 and 9.2. The behavior of the IP and LP forms is more complicated. In cyanobacterial PSIIcc, Roncel et al. (2003) observed what they call LP to be pH-independent (We call it IP, because $E_m \sim 275$ mV; see Table 8.1). With plant material, Kaminskaya et al. (1999) demonstrated the pH-dependence of the IP and LP forms to vary in a complicated manner with sample types and conditions. A somewhat simpler picture emerges from the experiments by Ortega et al. (1988) and Roncel et al. (2001) on plant PSII-membranes: The data suggest that the IP and LP form are connected, i.e., that they are just the low-pH and high-pH variant, respectively, of the same form. The results of Roncel et al. (2001) are reproduced in the upper part of Fig. 8.9. The dashed curve is a fit to the Nernst-Clark equation:

$$E_m = E_0 - \frac{RT}{F} \ln \left(\frac{K_{a,\text{ox}} + a_H}{K_{a,\text{red}} + a_H} \right) \qquad (8.1)$$

describing the pH-dependence of the midpoint potential E_m of a redox-active group that is electrostatically coupled to a titratable group, i.e., a group that can release a proton. Here, E_0 is the midpoint potential of the redox-active group at low pH, R is the gas constant, T the temperature, a_H is the proton activity in the medium ($a_H = 10^{-pH}$), and $K_{a,\text{ox}}$ and $K_{a,\text{red}}$ are the equilibrium constants for the deprotonation of the titratable group, when the redox-active group is oxidized and reduced, respectively, related to the
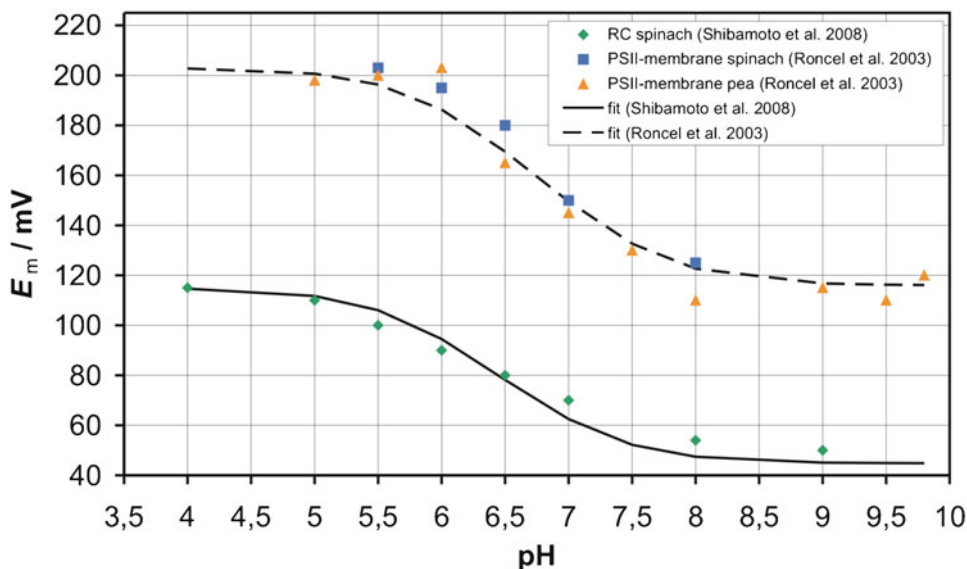
Fig. 8.9. pH-dependence of the redox midpoint potential E_m (versus normal hydrogen electrode) of cyt b_{559} in spinach PSII-enriched membranes according to Roncel et al. (2003) and in spinach PSII-RC complexes according to Shibamoto et al. (2008). The *curves* are fits to the Nernst-Clark equation.

corresponding pK_a values p$K_{a,ox}$ and p$K_{a,red}$. Based on such a fit, the data can be interpreted in terms of a single titratable group interacting with cyt b_{559} with p$K_{a,ox} = 6.0$ and p$K_{a,red} = 7.5$. However, the fit can be improved by considering at least two titratable groups interacting with cyt b_{559} (Roncel et al. 2001). Based on a chemical modification of histidine residues with the agent diethylpyrocarbonate, a model was proposed, according to which the deprotonation of one of the axial histidyl ligands to the heme is responsible for the pH-dependent transformation from the IP to the LP form, while the HP form originates from a reorientation of the protonated histidine side chain and the formation of a hydrogen bond accompanied by structural changes (Roncel et al. 2001). This model is interesting, but problematic in view of the crystal structure (see below, Sect. III.B.3), and the question remains unanswered, why this pH-dependence is not observed in cyanobacterial cyt b_{559} (Roncel et al. 2003).

In 2008, the pH-dependence was reinvestigated by thin-layer cell spectroelectrochemistry using plant PSII-RCs. The obtained data are shown in the lower part of Fig. 8.9 (Shibamoto et al. 2008) and are consistent with those of Roncel et al. (2001) concerning the pH-dependence, if one allows for slight changes of p$K_{a,ox}$ and p$K_{a,red}$ due to the isolation of the RC. It is striking, however, that both the low- and the high-pH form have a significantly lower redox potential in the RC compared to native membrane samples. This result suggests that there is at least one other factor that influences the redox potential independent of the titratable groups.

2. Polarity and Position Relative to α-Helices

There is a substantial electrostatic field in proteins due to polar groups (carrying partial charges), titratable residues (in their charged protonation state) and the fields induced by this permanent charge distribution in the polarizable medium (reaction fields). These fields on the whole determine the redox potential of a protein-bound heme group and not just those of the titratable groups causing the pH-dependence discussed above. This influence is due to the fact that

the heme group itself changes its charge and charge distribution when going from the reduced to the oxidized state. The two charge distributions interact differently with the permanent charge distribution of the surrounding protein and induce different reaction fields in the polarizable medium. In particular, the redox potential of a heme group is increased in an apolar medium compared to a polar medium due to the loss of the electrostatic solvation energy. The latter can be estimated based on the Born formula for ion solvation

$$\Delta G_{\text{Born}} = \frac{(Ze)^2}{8\pi\varepsilon_0 r}\left(\frac{1}{\varepsilon_m} - \frac{1}{\varepsilon_w}\right) \qquad (8.2)$$

where e is the elementary charge, Z the charge number of the ion (or charge difference between redox states, i.e., $Z = 1$ for the one-electron oxidation of the heme group), r is the ion radius (or effective radius assumed for a heme group), ε_0 is the vacuum permittivity, and ε_m and ε_w are the static dielectric constants of the membrane and the aqueous phase, respectively. This formula describes the free energy change for shifting an ion from water into the membrane interior. Using $r = 6.5$ Å, $\varepsilon_m = 4$ and $\varepsilon_w = 80$, one can estimate the contribution of the low dielectric constant of the membrane phase to the redox potential increase to be $\sim$260 mV. Similar values were obtained based on a simple geometric model of two TMHs binding a heme group in a lipid bilayer that also takes into account the polarizability of the protein part and the positioning of the heme close to the dielectric boundary between lipid and aqueous phase (Krishtalik et al. 1993). However, medium polarizability is unlikely to fully explain the potential difference between the HP form and the IP or LP forms of cyt b_{559}, as this would require substantial structural changes that lead to a shift of the heme group from an apolar medium to an essentially aqueous phase. The latter may occur, when cyt b_{559} disintegrates and the heme is at least partially released. It is thus possible that changes in medium po-

larizability contribute to the observation of lower potentials in damaged or aged samples depending on the degree of heme release and its effect on the absorption spectrum. Certain types of treatments such as trypsinization or heating may also cause a heme release, and one has to be careful with the interpretation of the observed low-potential forms. On the other hand, a distinct structural change from an intact HP form to intact IP or LP forms is unlikely to cause such drastic changes in the polarizability of the heme environment, so that the contribution of polarizability to a transition between these forms can be expected to be smaller than 260 mV. A precise determination of this contribution is impossible without detailed structural information about the different forms of cyt b_{559}.

An important aspect in protein electrostatics is that not only the side chains of polar amino acid residues create electrostatic fields, but also the protein backbone due to the dipole moment of the peptide bond. The form of the fields created by the backbone depends on the secondary structure. In particular, α-helices create a strong dipole field because of the practically parallel alignment of their peptide bonds. The electrostatic potential due to the backbone of an α-helix is similar to that of two partial charges of opposite sign (i.e., about $\pm0.5\ e$) positioned at the ends of the helix (Sheridan et al. 1982). Hence, a molecule experiences a different field depending on whether it is located close to the N-terminus, the center or the C-terminus of the helix (Krishtalik et al. 1993). In cyt b_{559}, the heme group is bound close to the N-termini of the TMHs belonging to PsbE and PsbF. Krishtalik et al. (1993) estimated an increase of the redox potential by about 200 mV due to this position based on their structural model, but also discussed carefully the limits of the model. Indeed, the value of 200 mV is uncertain for the following reasons: (i) The heme group is positioned somewhat farther away from the N-termini of the helices in the crystal structure than in the model, which may decrease the potential upshift due to the helix dipoles. (ii) The TMHs

are tilted with respect to each other, which on the basis of model calculations by Krishtalik et al. (1993) may increase the redox potential by up to 100 mV for a tilt angle of $30°$. Based on the tilt angle of actually $40°$ (see above), the upshift may be even larger. (iii) The TMH of PsbY is close by and contributes the field of a helix dipole in antiparallel orientation. This may in fact decrease the redox potential by a yet unknown amount. In addition, as stated above, one has to take into account the electrostatic field of the protein as a whole, so that it is anyway unclear, whether the dipoles of TMHs play a dominating role in determining the redox potential of cyt b_{559}. More detailed calculations based on the recent crystal structures will be necessary to resolve this issue.

3. Axial Ligation and Interaction with Aromatic Amino Acid Residues

There is now doubt that the axial ligands of the central iron ion influence the redox potential of the heme group. According to earlier spectroscopic work (Babcock et al. 1985) and the crystal structures, cyt b_{559} has a typical bis-histidine ligation. The question is, whether this ligation is changed in a transition from one to another redox potential form of cyt b_{559}. The enzymatic activity discussed for cyt b_{559} (Pospisil 2011) indeed may involve the displacement of one axial ligand to pave the way for a substrate such as superoxide to ligate to the iron. However, nothing of the sort appears to be responsible for the transition between redox potential forms, i.e., the iron remains ligated by two histidines. Rather, it has been proposed that the orientation of the two histidine ligands with respect to each other is different in the HP and LP forms (Babcock et al. 1985). On the basis of the crystal structure, though, such a reorientation is difficult to envision due to the strong interaction of the histidine imidazole rings with the protein environment. In particular, there are hydrogen bonds between the second imidazole nitrogen (i.e., not coordinating the iron) of each ligand

with a peptide backbone carbonyl group not uncommon for histidine-ligated cytochromes and predicted by Fourier transform infrared spectroscopy (Berthomieu et al. 1992). Furthermore, as mentioned above, there are π-stacking interactions with adjacent aromatic residues (Fig. 8.8). Therefore, any rotational motion of the imidazole rings is coupled to the surrounding protein and likely hindered. A possibility that remains is that the protein is rotated together with the imidazole rings. This would imply a change of the crossing angle of the two TMHs of PsbE and PsbF or a bending of the helices. A conformational change including whole TMHs could be a means to communicate changes at the donor side to the heme site.

An interesting detail is that Trp F20 points with the NH-group of its indole ring towards the heme macrocycle (Fig. 8.8). This arrangement suggests a kind of $N-H\cdots\pi$ interaction that could contribute to heme binding. The influence of this type of interaction on the redox potential is presently unknown.

4. Nonplanarity of the Heme

It is known for some time that a nonplanar distortion of the heme tetrapyrrole macrocycle alters the redox properties (Barkigia et al. 1988; Shelnutt et al. 1998). In a systematic study on site-directed mutants of the heme nitric oxide/oxygen binding (H-NOX) protein from *Thermoanaerobacter tengcongensis*, interactions of the heme with nonpolar side chains were modified and hence the distortion of the heme manipulated and confirmed by X-ray crystallography (Olea et al. 2008, 2010). The measured redox potential shows a clear correlation with heme distortion: The flatter the tetrapyrrole macrocycle, the smaller the redox potential. The explanation is that heme distortion leads to less electron density at the heme iron due to a perturbation of the metal site away from an octahedral geometry. By analogy, we can conclude that the nonplanar distortion of the heme in cyt b_{559} contributes to an increase of the redox potential. The question remains

open, whether the different redox potential forms have a different degree of nonplanarity of the heme.

5. Binding of Quinone-Like Molecules

The existence of a possible binding site for quinone-like molecules (i.e., PQ, PQH_2, herbicides) close to cyt b_{559} (see Sect. II.C.1) implies that binding of these molecules could lead to structural perturbations of the heme site affecting the redox properties. Indeed, Kaminskaya et al. (2007) reported evidence for a strong influence of herbicides on the redox potentials of the various forms, but a weaker influence on the amount of each form (Table 8.1). A straightforward interpretation is that binding of such a molecule close to cyt b_{559} would not cause a switch between redox potential forms, but merely modulate the potential of the pre-existing form, i.e., mainly that of the HP form. Depending on the specifity of the binding site for PQ or PQH_2, it is possible that the redox potential of the HP form is thus modulated by the redox state of the quinone pool in the thylakoid membrane. The physiological significance of such a modulation remains obscure.

6. Presence of Lipids, Detergent, and PsbY

Cyt b_{559} interacts with surrounding lipids and PsbY. Clearly, upon perturbation of the thylakoid membrane during the preparation of membrane fragments, core complexes or RCs, the lipids might be extracted or replaced with detergent molecules, and PsbY might be lost. Quantifying the consequences of these changes for the redox potential is difficult, but the data available (e.g., Table 8.1) suggest that overall the disruption of the membrane leads to a destabilization of the HP form. It would be desirable to analyse the lipid/detergent and protein subunit content in the various samples in greater detail in the future in order to establish a possible connection between redox potential shifts and these components.

IV. Function of Cytochrome b_{559}

In the literature, one frequently encounters sentences like the following: "Despite extensive research, the role of cyt b_{559} is still unclear." This statement remains true even though we have nowadays high-resolution crystal structures of PSIIcc at our disposal. Notwithstanding, numerous hypotheses concerning the function of cyt b_{559} have been proposed. In the following, we discuss those that are important in view of the recent literature.

A. Side-Path Electron Transfer and Photoprotective Role

The photon-triggered electron transfer in the RC ultimately leads to the oxidation of a special chlorophyll (Chl) a pigment and thus the formation of a strong oxidant that is able to take electrons from water by virtue of the WOC and the redox-active tyrosine Y_Z (Fig. 8.10). This strong oxidant is traditionally referred to as "P680$^+$", but is very likely $P_{D1}^{\bullet+}$ (Renger and Schlodder 2011). If the native electron transfer from the WOC is impaired, $P_{D1}^{\bullet+}$ could be accumulated and may cause damage by oxidizing other components of PSIIcc. This is referred to as donor side photoinhibition (Vass 2012). It has been proposed that cyt b_{559} acts as an electron donor to $P_{D1}^{\bullet+}$ and thus provides protection against donor side photoinhibition (Barber and De Las Rivas 1993; Thompson and Brudvig 1988). Meanwhile, there is plenty of evidence for a side-path electron transfer (Faller et al. 2005), in which $P_{D1}^{\bullet+}$ gets electrons from cyt b_{559} via the intermediate Car_{D2}, probably sharing electrons with $Chlz_{D2}$ (blue arrows in Fig. 8.10). However, other pigments might be involved as well. Recent progress in characterizing secondary electron transfer has been reviewed (Shinopoulos and Brudvig 2012). We note that the crystallographically detected PQ in the Q_C site (Guskov et al. 2009) is located close to Car_{D2} (see Figs. 8.1 and 8.4), and one may speculate that it acts as mediator in
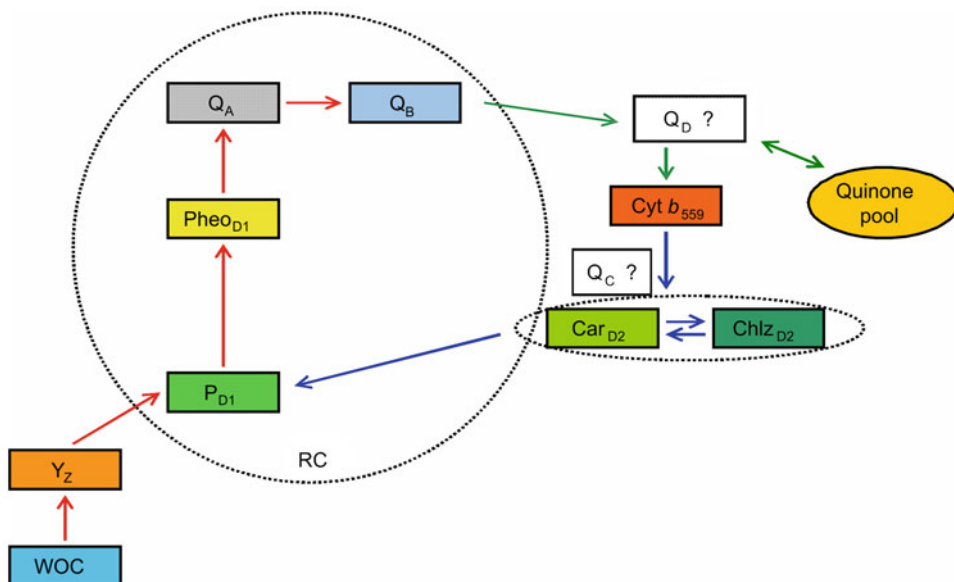
Fig. 8.10. Scheme of PSIIcc-cofactors involved in primary (*red arrows*), secondary (*blue arrows*), and putative (*green arrows*) electron transfer processes. For the real spatial arrangement of cofactors, see Fig. 8.1a. Q_C is the crystallographically detected PQ (Guskov et al. 2009), while Q_D indicates a hypothetical PQH_2 binding site close to cyt b_{559} (Kaminskaya and Shuvalov 2013).

side-path electron transfer. Furthermore, cyt b_{559} needs electrons to fulfill its protective role. A likely source of electrons is either the RC itself via the Q_B site or the quinone pool (green arrows in Fig. 8.10). In both cases, a hypothetical quinol binding site close to cyt b_{559} such as the proposed Q_D site (see above) could be important for efficient electron delivery. However, there is a problem with this photoprotective role of cyt b_{559}: The electron donation to $P_{D1}^{\bullet+}$ from the side path is extremely inefficient and slow. In particular, it is not able to compete with $P_{D1}^{\bullet+}Q_A^{\bullet-}$ charge recombination. Therefore, it has been argued that such a protection mechanism is actually invalid (Faller et al. 2005).

B. "Save-the-Carotene" Model

Another source of photodamage is the formation of Chl triplet states either through inter-system crossing from Chl singlet states or through radical pair recombination (Vass 2012). The Chl triplets can act as sensitizer to produce the harmful singlet oxygen.

Carotenoids serve a photoprotective role by quenching the Chl triplets or the singlet oxygen (Pogson et al. 2005). It has been proposed that the purpose of Car_{D2} is actually to quench singlet oxygen generated in the RC from Chl triplets (Faller et al. 2005). The peculiar positioning of Car_{D2} relative to the RC is a compromise between being close enough to serve as effective quencher and being not too close to avoid oxidation by $P_{D1}^{\bullet+}$. Nonetheless, Car_{D2} is oxidized by $P_{D1}^{\bullet+}$ at a low quantum yield. The role of the side path electron transfer from cyt b_{559} would then be to repair the Car_{D2} quencher by reduction, i.e., to save the carotene (Cardona et al. 2012; Faller et al. 2005).

C. Plastoquinol Oxidase and Oxygen Reductase

There is a second type of photoinhibition referred to as acceptor side photoinhibition (Vass 2012). It occurs, when the quinone pool is reduced and can no longer deliver PQ to the Q_B site. This leads to the stabi-

lization of $Q_A^{\bullet-}$ with the major consequence of enhanced triplet formation by radical pair recombination. It was proposed that PQH_2 oxidation by cyt b_{559} serves as a protective mechanism against acceptor side photoinhibition (Barber and De Las Rivas 1993). Indeed, there is evidence that PQH_2 can reduce cyt b_{559} as discussed above in the context of the putative Q_D site. In addition, it has been proposed that the dark oxidation of PQH_2 by cyt b_{559} results in the formation of superoxide, $O_2^{\bullet-}$ (Bondarava et al. 2003; Kruk and Strzalka 1999), where electron transfer from the heme to O_2 requires the LP form of cyt b_{559}. Independent evidence for an oxygen reductase activity of the LP form exists as reviewed by Pospisil (2011). Since $O_2^{\bullet-}$ is a harmful reactive oxygen species (ROS), the question arises, how detoxification can be accomplished.

D. Superoxide Reductase and Oxidase

Based on the observation that the photooxidation of the HP form of cyt b_{559} is prevented by exogenous $O_2^{\bullet-}$ scavengers (Tiwari and Pospisil 2009), it was proposed that cyt b_{559} acts as a superoxide reductase, whereby a protonated form of superoxide, the hydroperoxyl radical $HO_2^{\bullet}$, binds to the heme iron. The result of this reaction would be the formation of H_2O_2 and a transformation of cyt b_{559} to the IP form (Pospisil 2011). The H_2O_2 might be eliminated by catalase. The oxidized IP form of cyt b_{559} in turn was proposed to act as superoxide oxidase, transforming $O_2^{\bullet-}$ into O_2 while being switched back into the HP form. Thus, a possible role of cyt b_{559} is to remove ROS.

E. Role in PSIIcc Assembly

There is biochemical evidence that cyt b_{559} is required for the assembly of PSIIcc in the thylakoid membrane (Komenda et al. 2004, 2008; Morais et al. 1998; Müller and Eichacker 1999; Nixon et al. 2010; Tae and Cramer 1992). It has been proposed that cyt b_{559} functions as a nucleus for the assembly of PSIIcc that is under redox control (Morais et al. 1998). In this context, the ability of cyt b_{559} to bind redox-active molecules such as PQ/PQH_2, probably in conjunction with the D2 protein as discussed above, could be of importance. We would like to stress that during assembly of PSIIcc (Nixon et al. 2010), many subcomplexes occur that cannot oxidize water, but are able to perform light-induced charge separation in the RC, eventually with a non-functional acceptor side. This photochemistry is prone to the formation of harmful side products such as ROS. It is quite possible that cyt b_{559} puts into effect its protective role in these subcomplexes rather than in the fully assembled PSIIcc. The question remains, whether the corresponding electron transfer processes are more efficient under these conditions.

V. Conclusions and Outlook

It is fair to say that we are still far away from understanding the function of cyt b_{559}. As usual, the crystal structures do not give immediately answers to all questions, but rather form a basis, whereupon future work can be build. When surveying the literature, we got the impression that research on cyt b_{559} is not as systematic as it should be. Many labs use different organisms and different types of preparations under various buffer conditions (see Table 8.1), and it is hard to draw a unified picture based upon this material. We also note that our description may have gaps, and we apologize for any omission. From the viewpoint of structural biology and related theoretical biophysics, it is clear that more work must be done to better characterize cyt b_{559} in crystallizable samples such as dimeric PSIIcc from *T. elongatus* in order to understand what the available crystal structures represent. Eventually, it may be possible to crystallize PSIIcc in another state or with another form of cyt b_{559}. This kind of research will likely shed more light on the "enigma".

Acknowledgments

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Structure-Function of the Cytochrome b_6f Lipoprotein Complex

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Summary

The cytochrome b_6f complex (*f*, derived from "leaf" in Latin, "folium," or French, "feuille", see D. S. Bendall, this volume) is responsible for the proton-coupled electron transfer reactions that link the two light-trapping photosystem reaction centers in the electron transport chain of oxygenic photosynthesis, and contains the rate-limiting step of the entire electron transport chain. The understanding of the functions of the intra-membrane cytochrome b_6f complex in its role as the central electron transport complex in the photosynthetic electron transport chain has greatly increased in recent years because of the solving of the atomic structure of the complex, as well as its extrinsic electron transfer domains solved separately, by X-ray crystallographic analysis. The electron transfer and proton translocation functions of the cytochrome b_6f complex are discussed in a context that focuses on function in the context of structure. The structure considerations are based on the recently determined high resolution (2.50 Å) crystal structure (PDB, protein data base, accession 4OGQ). The complex consists of a 280 kDa hetero-oligomeric intra-membrane lipo-protein complex containing, per monomer, 13 trans-membrane α-helices and seven prosthetic groups, the latter consisting of five hemes, one chlorophyll *a* molecule and one β-carotene. In addition, each monomer contains 23 lipid binding sites, the function of which crosses into a completely new area of structure-function, both for the b_6f complex and for the entire broader field of membrane proteins. Considering energy-transducing membrane protein complexes, the b_6f complex, along with other hetero-oligomeric electron transfer membrane proteins provides a unique perspective in the broad field of membrane protein structure-function that encompasses transport and channel proteins, and extends beyond photosynthesis and bioenergetics to biomedicine. The additional perspective arises from the fact that the function of the cytochrome complexes, along with those of other redox proteins, can be assayed quantitatively through various high resolution spectroscopies.

 A major theme in the present article is the comparison of structure and function of the b_6f and the cytochrome bc_1 complex, which is located in the mitochondrial respiratory chain and in purple photosynthetic bacteria. One purpose of the comparison is to illustrate that although the two complexes are phylogenetically related, and there are many similarities in structure-function, there are many differences. An analysis and comparison of structures and functions implies that all charge mechanisms determined for bc_1 should not apply without exception or modification to the b_6f complex.

Abbreviations: AFM – Atomic force microscopy; b_p, b_n – *b*-type hemes on electrochemically positive, negative sides of membrane; C. reinhardtii – Chlamydomonas reinhardtii; CET – Cyclic electron transport; chl – Chlorophyll-*a*; Cyt – Cytochrome; EM – Electron microscopy; E_{m7} – Mid-point oxidation-reduction potential at pH 7; EPR – Electron paramagnetic resonance; ETC – Electron transport chain; Fd – Ferredoxin; FNR – Ferredoxin-NADP$^+$ reductase; ISP – Rieske iron-sulfur protein; k – Boltzmann's constant; LET – Linear electron transport; LHCII – Light-harvesting chlorophyll protein-

I. Introduction

The cytochrome b_6f complex of oxygenic photosynthesis is discussed from three perspectives: (i) mechanisms of electron/proton transfer that underlie its energy-transducing function, derived from biochemical, genetic, and crystallographic structure analysis; (ii) comparison of the structure and mechanisms in the cyt b_6f complex with those operating in the bc_1 complex of mitochondria and purple anoxygenic photosynthetic bacteria; and (iii) in a brief overview, structure properties of the bc complexes in the context of other hetero-oligomeric membrane protein structures that function differently. The present chapter inevitably includes some key aspects of recent previous reviews from ourselves and others (Crofts and Berry 1998; Berry et al. 2000, 2013; Crofts 2004a; Osyczka et al. 2005; Baniulis et al. 2008; Cramer et al. 2008, 2010, 2011; Crofts et al. 2008; Mulkidjanian 2010; Nitschke et al. 2010; Bernat and Roegner 2011; Berry and Huang 2011; Hasan and Cramer 2012, 2014; Kallas 2012; Dibrova et al. 2013; Hasan et al. 2013c; Osyczka 2013; ten Brink et al. 2013; Kao and Hunte 2014; Tikhonov 2014). In a number of instances, however, an updated perspective is presented based on recent structure data, which are utilized to discuss some controversial questions of function.

Crystal structures for cytochrome bc complexes (b_6f and bc_1) have been obtained for: (i) Cyt b_6f complex from the green alga, *C. reinhardtii* (Stroebel et al. 2003) and cyanobacteria (Kurisu et al. 2003; Yan et al.

2006; Yamashita et al. 2007; Baniulis et al. 2009; Hasan et al. 2013b; Hasan and Cramer 2014); (ii) for the Cyt bc_1 complex (a) from several mitochondrial sources, reviewed recently by (Berry et al. 2013; Kao and Hunte 2014), with the best resolution, 1.9 Å, obtained for the yeast complex (Solmaz and Hunte 2008) and (b) from anoxygenic photosynthetic bacteria (Esser et al. 2004, 2008, 2015). We also refer the readers to Chap. 1 in this volume by D. S. Bendall for the origin of heme protein notation, and Chaps. 10 and 11 by A. Esser et al. and L. Huang and E. Berry, respectively, for discussions of the structure of the bacterial Cyt bc_1 complex.

II. Historical Perspective

The historical development of concepts governing the mechanisms of membrane energization and function of the cytochrome bc complex in proton translocation is based initially on studies of the cytochrome bc_1 complex in mitochondria and purple photosynthetic bacteria (Chance and Williams 1956; Dutton and Jackson 1972; Wikström and Berden 1972; Mitchell 1976, 1979; Zhu et al. 1982; Trumpower and Gennis 1994; Berry et al. 2000, 2013; Hunte 2001; Crofts 2004a, b; Lanciano et al. 2013). A consequence is that it has often been assumed that models and concepts developed for the bc_1 complex apply in all detail to the b_6f complex, and that concepts of structure-function developed for the bc_1 complex can be completely applied to the b_6f complex. We suggest that this practice in the literature can be confusing, particularly in the teaching of the subject. Regarding notation, the ubiquitous use of 'i' and "o," to describe the two sides of the cytochrome complex and the membrane, and the sidedness of the two b hemes that span the membrane can cause confusion in the teaching of the subject. Thus, with respect to the direction of vectorial proton translocation, the inside ("i"), the matrix domain, of the mitochondrion, and the cytoplasmic domain of gram-negative including photosynthetic bacteria, corresponds to the

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II; M. laminosus – Mastigocladus laminosus; Ndh – NADH dehydrogenase; p n-side – Electrochemically positive, negative sides of membranes; PC – Plastocyanin; PDB – Protein data bank; PET – Photosynthetic electron transfer; PEWY – Proline-glutamate-tryptophan-tyrosine; PGRL1 PGRL5 – Proton gradient regulator 1 and 5; PQ-9 – Plastoquinone-9; PSI II – Photosystem I, II; PTOX – Plastoquinol terminal oxidase; Q_p, Q_n – Quinone binding site on p-, n-side of membrane; ROS – Reactive oxygen species; subIV – Subunit IV; TDS – Tridecyl-stigmatellin; TMH – Trans-membrane helix; $\Delta\tilde{\mu}_H^+$ – Trans-membrane difference of proton electrochemical potential

outside ("o") of the chloroplast thylakoid membrane. The definition of 'i' and 'o' as the sides corresponding to proton 'input' and 'output' arose from definitions of Peter Mitchell, who conceived the central role of the trans-membrane proton electrochemical potential gradient, $\Delta\tilde{}\mu_H^+$, in membrane energy transduction (Mitchell 1966, 1979). However, the more obvious meaning of 'i' and 'o' to the uninitiated not accustomed to the colloquial language of the field, is "inside" and "outside." It is suggested that a more conceptually useful notation is "n" and "p", referring to the thermodynamically defined electrochemically negative and positive sides of the membrane. This notation is used below to describe the orientation of the redox and protein components that function in the linear and cyclic electron transport pathways of oxygenic photosynthesis (Fig. 9.1a, b).

III. Cytochrome b_6f and bc_1 Complexes; General Aspects

The position of the cytochrome bc_1 and b_6f (together bc) complexes in the phylogenetic tree and their evolution is discussed elsewhere (Widger et al. 1984; Schutz et al. 2000; Nitschke et al. 2010; Hasan and Cramer 2012; Dibrova et al. 2013). The crystal structures of the mitochondrial and bacterial bc_1 complexes contain 11 and 3 polypeptide subunits, respectively, compared to eight in the Cyt b_6f complex from cyanobacteria (Kurisu et al. 2003; Hasan and Cramer 2014) and the green alga, *C. reinhardtii* (Stroebel et al. 2003). This subunit count refers to the tightly bound subunits that remain in the complex after the purification and crystallization procedures, and do not include weakly bound peripheral subunits that may be important for function but are lost during the process of purification and crystallization. The molecular weights, pI values, and coding origin, nuclear or organelle (Schwenkert et al. 2007), for the eight subunits of the b_6f complex are summarized (Table 9.1). Core

subunits containing key redox prosthetic groups with a high degree of conservation between b_6f and bc_1 complexes are: (i) cytochrome b in the bc_1 complex, which is equivalent in many structure-function aspects to cyt b and subunit IV in the b_6f complex (Widger et al. 1984); and (ii) the high potential ($E_{m7} = +300$ mV) iron-sulfur [2Fe-2S] protein, often called the "Rieske" iron-sulfur protein because of the origin of its discovery (Rieske et al. 1964a, b). The protein subunit containing the covalently bound c-type cytochrome, cyt f and c_1, respectively, is not conserved between b_6f and bc_1 complexes.

IV. Structure-Function of the Cyt b_6f Complex

A. Problems in Crystallization

Earlier studies on purification of the b_6f complex (Nelson and Neumann 1972; Hurt and Hauska 1981) provided a foundation for its eventual crystallization and determination of a high resolution crystal structure (Kurisu et al. 2003, 2004; Stroebel et al. 2003; Yan et al. 2006; Yamashita et al. 2007; Baniulis et al. 2009; Hasan et al. 2013b), presently 2.50 Å (Hasan and Cramer 2014). High resolution structures of a C-terminal soluble domain of the 250 residue extrinsic C-terminal domain of cytochrome f (Martinez et al. 1992, 1994, 1996), and of the N-terminal soluble domain of the Rieske protein (Carrell et al. 1997), were obtained prior to their determination in the intact cytochrome complex. In retrospect, two reasons for the delay in crystallization of the intact cyanobacterial b_6f complex were: (i) a lack of appreciation for the critical role of internal lipid in the native structure of the cytochrome complex (Zhang et al. 2003; Hasan et al. 2011, 2013a; Hasan and Cramer 2014). The problem was ultimately solved through the addition of synthetic lipid at a stoichiometry of ten lipid molecules/cyt f to the purified complex (Kurisu et al. 2003;
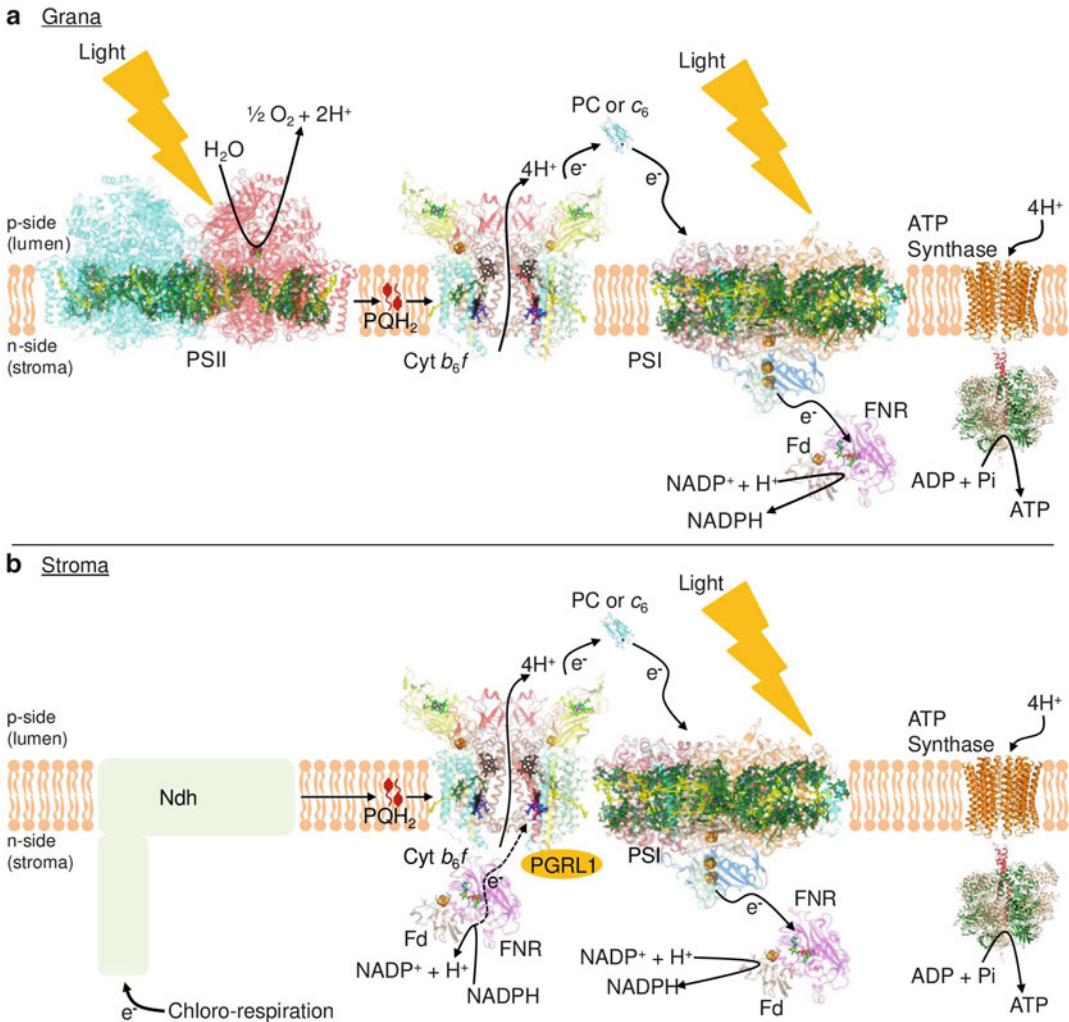
Fig. 9.1. Non-cyclic ("linear") (**a**) and cyclic (**b**) electron transport pathways for oxygenic photosynthetic electron transfer and proton translocation in a format emphasizing the membrane protein structures of the three major hetero-oligomeric membrane protein complexes which, along with plasto-hydroquinol/quinone (PQH_2/PQ), define the electron transport pathways: (a) photosystem II reaction center complex, PSII (Umena et al. 2011), 20 subunits, MW $\approx$ 350 kDa; PDB 3WU2; (b) cyt b_6f complex, 8 subunits, dimer MW = 250 kDa (PDB 4OGQ, 2.5 Å crystal structure; (c) PS I reaction center (RC) complex (Jordan et al. 2001), trimer MW $\approx$ 1 MDa (PDB 1JB0), is connected to the ferredoxin-dependent "cyclic pathway" (Johnson 2011; Joliot and Johnson 2011), which channels electrons back to the PQ pool via "ferredoxin – PQ reductase" whose identity is debated (see below). Electrons are transferred from cyt f to the P700 special pair via plastocyanin (PC, Cu^+/Cu^{2+} redox prosthetic group) or a c-type cytochrome (Fe^{2+}/Fe^{3+}). Light absorption by PSI RC results in 10^{-12} s (psec) electron transfer from an RC excited state to a ferredoxin-FNR complex (Kurisu et al. 2001) that reduces $NADP^+$.

Zhang et al. 2003). (ii) Monomerization of the detergent-extracted complex, resulting in inactive and un-crystallizable material, was partly attributed to the action of a native, as yet unidentified (Baniulis et al. 2009), protease present in the unicellular cyanobacteria *Synechocystis, Synechococcus,* and thermophilic *T. elongatus* (Zhang and Cramer 2005) that were initially used as sources of the complex (Zhang and Cramer

Table 9.1. Fundamental parameters of subunits of cytochrome b_6f complex.

Subunit	MW (kDa)	pI
Cyt f[a]	30.9	4.7
Cyt b[a]	24.7	8.5
ISP[a]	19.3	5.3
SubIV[a]	17.5	7.8
Pet G[a]	3.9	8.1
Pet L[a]	3.2	9.9
Pet M[a]	3.5	4.3
Pet N[a]	3.3	9.5
FNR[b]	35.4	6.3
PetO[c]	15.1	9.6
PetP[d]	7.4	8.2

[a]From cyanobacterium *Nostoc* sp. PCC 7120
[b]PetH; Resolved on SDS-PAGE of spinach prep; MW in *A. Thaliana*
[c]PetO, *C. reinhardtii*
[d]PetP, *A. platensi*

2005). The proteolysis itself may not have caused the monomerization, but rather facilitated the weakening of inter-monomer hydrophobic interactions. Monomerization associated with the delipidation and loss of the single chlorophyll and the small PetL subunit, has been observed in the complex derived from the alga *C. reinhardtii* (Breyton et al. 1997). The problem of proteolysis and monomerization of the complex was found to be less severe in filamentous cyanobacteria (Yamashita et al. 2007; Baniulis et al. 2009), although the quality of the crystals initially prepared remained relatively poor. This was ultimately realized, as mentioned above, to be a consequence of delipidation of the complex in the purification protocol dedicated to purity of the protein moiety of the complex (Zhang et al. 2003). Properties of the extensive lipid population inside the complex are discussed below (Sect. V). Electron transfer activity, which is an indicator of the quality of the preparation, and consequently its ability to crystallize, is greatly reduced in monomeric b_6f complex. Dimeric crystallizable b_6f complex was ultimately isolated from fila-mentous cyanobacteria (Kurisu et al. 2003; Baniulis et al. 2009), in which the problem of monomerization upon extraction was significantly smaller. A dimeric complex has recently been obtained from the thermophilic cyanobacterium *T. elongatus* (Rexroth et al. 2014).

B. General Structure Features of the Cyt b_6c Complex

The crystallized complex is a symmetric (C2 symmetry) dimer that consists of eight trans-membrane polypeptide subunits in plants, green algae, and cyanobacteria (Kurisu et al. 2003). The analogous Cyt bc_1 complex consists of 11 subunits in the yeast, PDB 3CX5 (Hunte et al. 2000) and bovine, PDB 1QCK, 1BE3 (Iwata et al. 1998) complex, and three in the complex from the photosynthetic bacterium, *Rps. sphaeroides* (Esser et al. 2008). In all cases, the dimeric structure of the complex is required for a physiologically significant electron transfer activity, 250–400 electrons transferred (cyt f-sec)$^{-1}$ (Pierre et al. 1995; Zhang et al. 2001; Rexroth et al. 2014). This is presumably because of (i) the defined inter-monomer quinol entry pathway and (ii) increased stability of the dimer (Fig. 9.2a) in a hydrophobic membrane environment. Proximity of the two monomers is also necessary for inter-monomer cross-over in rapid trans-membrane electron transfer (Swierczek et al. 2010; Khalfaoui-Hassani et al. 2012).

A ribbon diagram of the dimeric b_6f complex (PDB 2ZT9, 4H44, 4OGQ) derived from crystal analysis of the filamentous cyanobacterium *Nostoc sp.* PCC 7120 (Baniulis et al. 2009; Hasan et al. 2013b; Hasan and Cramer 2014), is shown in two views differing by a 180° rotation in order to display more clearly the arrangement of the 8 subunits that span the membrane with 13 TMH per monomer containing 7 prosthetic groups, including 5 with redox function consisting of 4 hemes and the [2Fe-2S] cluster, 1 chlorophyll a (Huang et al. 1994) and one β-carotene (Zhang et al. 1999) (Fig. 9.2a, b; Table 9.1).

C. Unique Chlorophyll a and β-Carotene

The presence of the single copy of the lipidic chlorophyll *a* (Huang et al. 1994; Pierre et al. 1997; Dashdorj et al. 2005) and β-carotene (Zhang et al. 1999) in each monomer of the complex, first determined in the absence of structure data, initially seems enigmatic. As shown in the first structures, the β-carotene is separated by 14 Å from the chlorophyll (Kurisu et al. 2003; Stroebel et al. 2003). Therefore, on the basis of the physics alone the β-carotene cannot quench a chlorophyll excited triplet state (Kim et al. 2005a). A mechanism of long-range transfer through an internal O_2 chain has been considered (Kim et al. 2005b). Recent inferences about the functions of the two pigments are: (i) the relevant group on the chlorophyll is not the tetra-pyrrole ring, but the elongate flexible phytyl chain that gates the passage of plastoquinone(ol) through the portal connecting its p-side binding site with the inter-monomer cavity and bulk membrane (Hasan et al. 2014a). (ii) Regarding the β-carotene, it protrudes by approximately 11 A from the complex between the TMH of Pet G and that of Pet M (Fig. 9.2b), and thus is in a position analogous to that of cardiolipin in the cytochrome bc_1 complex, which seems to function in the mitochondrial respiratory chain as a glue between the major electron transport complexes (Wenz et al. 2009). It is therefore suggested that the β-carotene functions as a 'latch' that connects the b_6f complex to the PSI reaction center, forming a super-complex (Iwai et al. 2010), analogous to that described for the mitochondrial respiratory chain (Althoff et al. 2011).

D. Trans-Membrane Hemes, b_n and b_p

Of the eight subunits in the monomer, all but two, the heme binding cyt *b* subunit and subunit IV (subIV), are monotopic (1 membrane span). For later discussion, it is noted that the two polytopic subunits, the 24 kDa cyt b_6 subunit with 4 TMH (labeled A-D), and the 18 kDa suIV with 3 TMH (E-G),

respectively, share major sequence (Widger et al. 1984) and structure identity with the 42 kDa cyt *b* major subunit of the bc_1 complex that has eight TMH, i.e., one more TMH, the 'H' helix, than the b_6f complex. The evolutionary origin of the latter difference between the b_6f sand bc_1 complexes is a subject of discussion (Mulkidjanian 2010; Nitschke et al. 2010; Hasan and Cramer 2012). The cyt *b* subunit also contains a short surface helix, formed by the N-terminal 28–29 amino acids, which is associated with the n-side membrane surface. The four TMH of the cyt b_6 subunit are connected by loops at the p- and n-side membrane interfaces. SubIV (∼17 kDa) has three TMH (E–G) that form a p-side saddle around the four helix bundle of cyt b_6. Helix E of SuIV is located in proximity to the A- and B-helices of cyt b_6 while the F and G-TMH span the four helix bundle, close, respectively, to the C, D and B-TMH (Fig. 9.3). The C-terminus of the E-TMH is separated from the N-terminus of the F-TMH by a distance of ∼40 Å, which is bridged by the p-side ef-loop. This seven TMH assembly forms the conserved core of the b_6f complex, which is bounded in each monomer by the four subunit hydrophobic picket fence.

The arrangement of the two pairs of trans-membrane *b*-hemes, b_p and b_n, with inter-heme edge-edge and center-center (Fe-Fe) distances (in parentheses) is shown (Fig. 9.2c), along with the heterogeneity of the inter-heme dielectric constants (Hasan et al. 2014b), and the complex of heme b_n with a skeleton of the covalently bound *c*-type heme c_n (Stroebel et al. 2003; Alric et al. 2005; Zatsman et al. 2006; Baymann et al. 2007; Twigg et al. 2009). The heme b_n-c_n complex, a unique feature of the b_6f compared to the bc_1 complexes, in which the close proximity of the two hemes results in sharing of the 3d electronic shells is displayed in a g = 12 EPR signal (Zatsman et al. 2006; Baymann et al. 2007), is shown in more detail (Fig. 9.2d). This proximity is considered relevant to the possibility of a two electron transfer mechanism at the Q_n site.
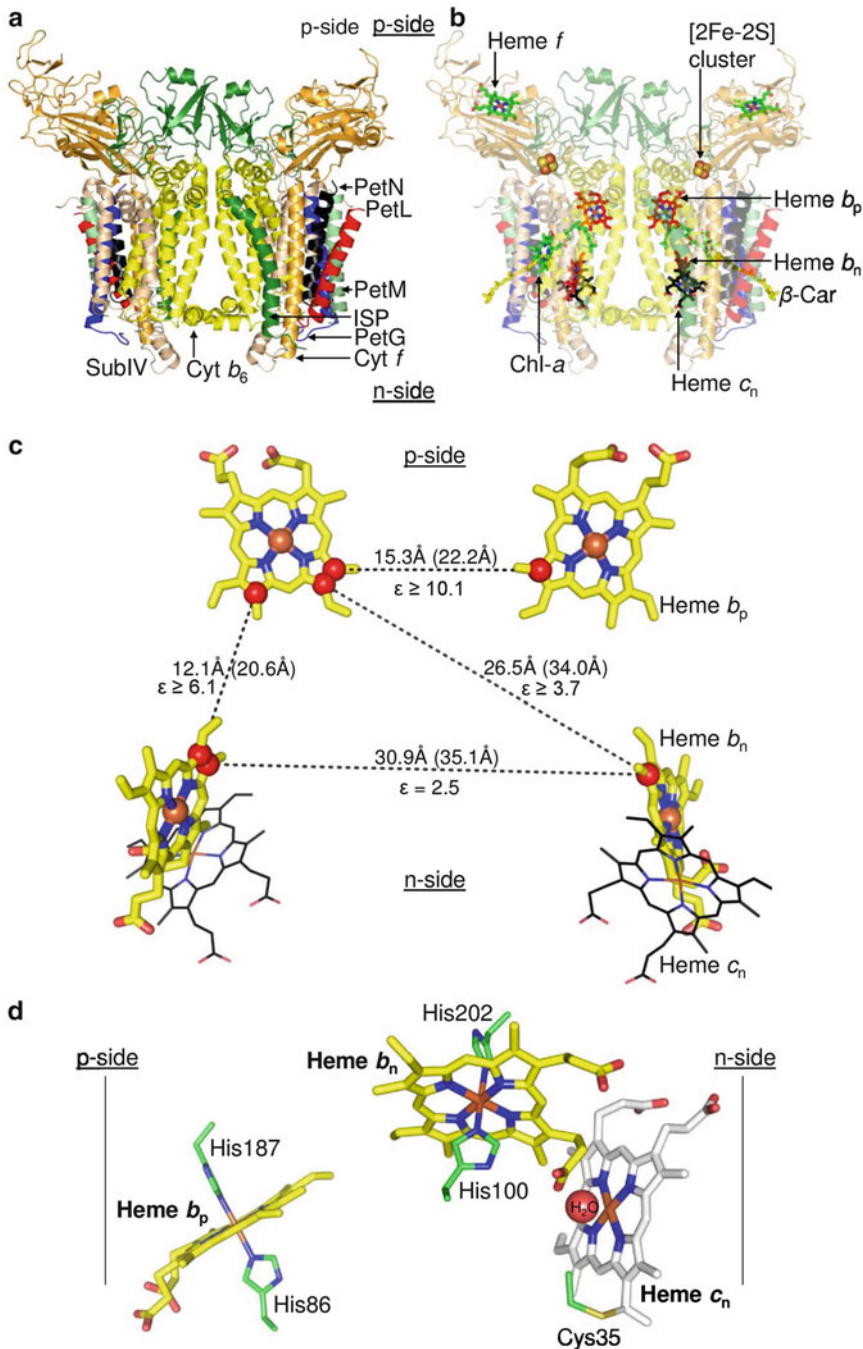
Fig. 9.2. The dimeric cytochrome b_6f complex. (**a**) Polypeptide subunits from the cyanobacterium *Nostoc* sp. PCC 7120 (PDB ID 4OGQ) are shown in *ribbon* format- cyt b_6 (*yellow*), subIV (*light brown*), cyt f (*orange*), ISP (*dark green*), PetL (*red*), PetM (*light green*), PetG (*blue*), PetN (*black*). (**b**) Prosthetic groups of b_6f complex. Trans-membrane hemes b_p, b_n (*red/blue*), and c_n (*black/blue*) are shown as sticks. On the p-side, heme f (*green/blue*) is shown as sticks, the [2Fe-2S] cluster as spheres (*brown/yellow*), and the Chl-*a* (*green/blue*) and β-car (*yellow*) as sticks. (**c**) Heme-heme distances within the trans-membrane domain of the complex (PDB ID 4OGQ). Distances shown as *black dashes*. Heme edge-edge, and center-center (Fe-Fe) distances are shown, respectively, outside and inside parentheses (selected atoms shown as spheres).

1. Hemes, b_n and b_p, Oxidation-Reduction Potentials

Determination of the oxidation-reduction potentials of these hemes in the bc_1 complex are consistent in showing a separation of 100–150 mV between the midpoint redox potentials of the two hemes, which are thus often labeled b_h and b_l (high and low potential hemes). For the two hemes in the b_6f complex, there is disagreement as to whether the mid-point potentials of the two hemes are (Kramer and Crofts 1994) or are not (Girvin 1985; Furbacher et al. 1989; Rich et al. 1991) separable in a redox titration of thylakoid membranes. A simulation shows that a mid-point potential difference of 50 mV or less of a one electron titration could not be resolved
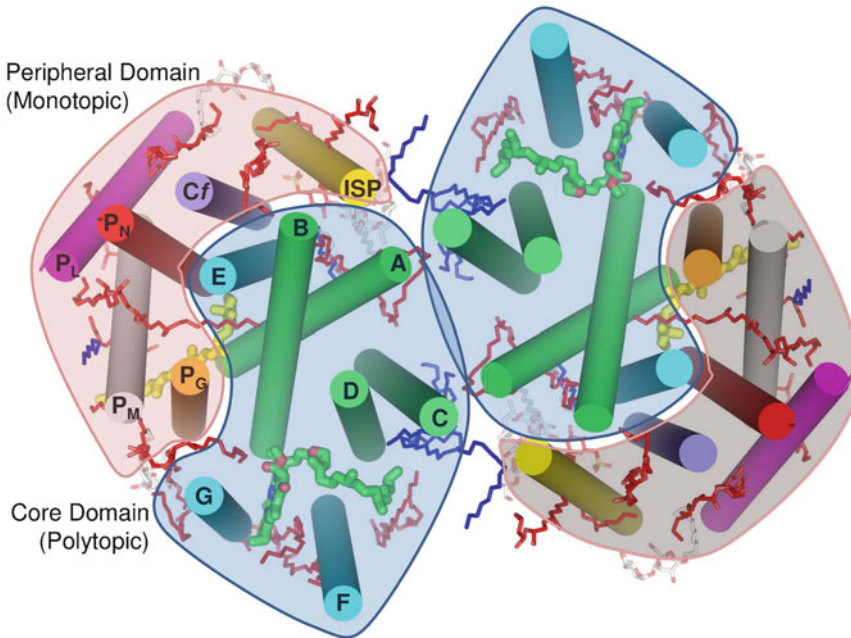
Fig. 9.3. Domain structure of b_6f complex (PDB ID 4OGQ). Central hydrophobic core consisting of polytopic subunits cyt b_6 and subunit IV is organized as a symmetric dimer, each monomer further divided into a core polytopic domain (*light blue*) and peripheral domain (*pink*). Polytopic domain consists of cyt b_6 (TMH "A-D"; *green*) and subunit IV ("E-F"; *cyan*). The peripheral domain consists of single helix subunits- cyt f ("Cf"; *blue*), ISP (*yellow*), PetG ("P_G"; *light orange*), PetL ("P_L"; *magenta*), PetM ("P_M"; *gray*) and PetN ("P_N"; *red*). Lipids, *red sticks*; detergent UMQ, *red/white*; acyl chains, *blue sticks*. Chl-a and β-car, *green/blue/red* and *yellow*. The cyt b_6/subunit IV core is conserved in bc complexes from mitochondria, chloroplasts, and photosynthetic bacteria (Widger et al. 1984). p and n sides of the complex possess negative and positive surface potentials (Cramer et al. 2006), respectively, which presumably have a role, as yet undefined, in the organization of peripherally bound subunits, which are not retained in the crystals (Sect. IV.H). *Left* and *right-side panels* show view from electrochemically positive and negative sides of membrane.

Fig. 9.2. (continued) The dielectric constant, εij, between each pair of hemes, $\varepsilon_{n1,p1}$, $\varepsilon_{n1,p2}$, $\varepsilon_{n2,p1}$, etc. (Hasan et al. 2014b) is shown. (**d**) Trans-membrane heme ligation in the cytochrome b_6f complex. The central Fe atom of heme b_p and b_n is axially ligated, respectively, by residues His86/His187 and His100/His202 which bridge the B and D TMH of the cyt b subunit, Heme c_n is covalently attached to the protein via Cys35. Heme c_n is unique as it lacks an amino acid axial ligand, and the central Fe-atom is penta-coordinated. The sole ligand of heme c_n, is provided by H_2O or OH^- on the heme b_n side. Fe of heme c_n separated by 4.0 Å from a propionate oxygen of heme b_n, which results in electronic coupling, a high spin g = 12 EPR signal (Zatsman et al. 2006; Baymann et al. 2007), and an oxidase-like reaction with nitric oxide (Twigg et al. 2009). Electrochemically positive, negative sides of membrane labeled p, n.

experimentally. For isolated complex, the midpoint potentials of the 2 b hemes have been found to be separated by 90–95 mV (Alric et al. 2005; Nelson et al. 2005).

2. Reducibility of b Hemes; Heterogeneity of Internal Dielectric Constants

Excitonically split circular dichroism (CD) spectra of the bc_1 (Palmer and Degli Esposti 1994) and b_6f complex (Schoepp et al. 2000; Hasan et al. 2014b) arise from interactions of the reduced hemes. Determination for isolated complex of the time course of heme reduction and the onset of the split CD spectra shows that the heme pair that is preferentially reduced is the intra-monomer pair, b_p and b_n. This result contrasts with that which is expected. If heme b_n, whatever its exact redox potential, has a more positive potential in isolated b_6f complex than heme b_p, the two hemes b_n should be preferentially reduced under equilibrium conditions. The straight-forward explanation is that the protein medium between hemes b_p and b_n is more polarizable, i.e., has a higher dielectric constant, than the medium between the two hemes b_n (Hasan et al. 2014b), implying that the dielectric constant in the complex is heterogeneous and anisotropic (Fig. 9.2c).

E. The High Potential [2Fe-2S] Rieske Iron-Sulfur Protein (ISP)

Because the degree of sequence conservation between the Rieske proteins in chloroplasts and mitochondria is greater than between the cytochrome c_1 and f subunits of the complex, for which sequence identity is negligible except for the heme binding motif (Martinez et al. 1996), it has been proposed that a better nomenclature for these bc complexes would be the "b – FeS complex" (Nitschke et al. 2010). To avoid confusion, however, in referencing, the notation "b_6f" will be retained in the present discussion. As discussed below, whatever title is given to these complexes, although there is a significant commonality in evolutionary origin and many similarities in structure/function, it is important to realize that there are major differences in both structure and function between the respiratory and photosynthetic bc_1 complexes on the one hand, and the photosynthetic b_6f complex on the other. It is noted that the electron transport pathway in the anoxygenic photosynthetic bacteria is discussed as a purely cyclic (CET) pathway (Petty and Dutton 1976; Bowyer and Crofts 1981; Cramer and Crofts 1981; Gennis et al. 1993; Berry et al. 2000), in which there is no energy-coupled diversion of electrons from the cyclic loop. In contrast to these bacteria, and to mitochondria that function through a linear chain, the electron transport pathway associated with oxygenic photosynthesis contains both linear (LET) and cyclic (CET) electron transport chains.

1. ISP Electron Transfer; a p-Side Conformation/Mobility Problem

A structure-function enigma is that the distance of closest approach of the cytochrome f heme to the 2Fe-2S cluster in the b_6f complex is 28 Å, which is much too large to support physiologically meaningful msec electron transfer rates (Moser et al. 1992; Gray and Winkler 1996). Then, how does the Rieske FeS protein transfer electrons to cytochrome f? A major gap in understanding the p-side electron transfer reactions for the b_6f complex results from the absence of structure data that would allow kinetically competent electron transfer from the [2Fe-2S] cluster to the heme of cytochrome f. Given the documented conformation change determined from crystal structure data for the avian mitochondrial bc_1 complex (Zhang et al. 1998; Huang and Berry 2015), electron transfer from the [2Fe-2S] cluster to the cyt f heme must involve a rotational-translational conformational change in the ISP (Hasan et al. 2013a). It has not yet been possible, however, to obtain similar structure-based data for the b_6f complex. An inhibitory effect on cytochrome f reduction of increased ambient viscosity, achieved in the presence of added glycerol (Heimann et al. 2000),

implies a requirement for motion of the extrinsic domain of the ISP. Extensive site-directed mutagenesis implies that the "hinge" segment of the ISP that connects the [2Fe-2S] cluster-bearing domain in one monomer of the b_6f complex with its TMH in the second monomer is more flexible than in the similar domain of the mitochondrial bc_1 complex (Yan and Cramer 2003; de Vitry et al. 2004). This flexibility may not apply in situ to all degrees of freedom (Soriano et al. 2002).

Crystallographic evidence for a significant conformation change of the ISP extrinsic domain has been inferred from the disorder of the ISP extrinsic domain observed in crystals of the b_6f complex formed in the presence of anionic lipid, compared to a largely ordered ISP structure observed in the b_6f complex supplemented with neutral lipids. A conformation change associated with the disorder is attributed to electrostatic interactions arising from the presence of anionic lipids (Hasan et al. 2013a), which are known to have a central role in the structure of the mitochondrial cyt bc_1 complex (Arnarez et al. 2013) and the respiratory chain (Pfeiffer et al. 2003). Crystal structure evidence for movement of the [2Fe-2S] cluster in the b_6f complex, comparable to that documented in the mitochondrial bc_1 complex (Zhang et al. 1998), which would provide structure-based documentation for the predicted domain movement of the extrinsic domain of the ISP, and document the mechanism of electron transfer from the ISP to the heme of cytochrome f, is presently missing.

F. Differences Between b_6f and bc_1 Complexes

1. Comparison of Cytochromes f and c_1

In contrast to the conserved nature of the cytochrome b subunit, for the cytochorme c_1 and f subunits, except for their single TMH that binds the subunit to the complex and the membrane, the structures are different in the b_6f and bc_1 complexes (Fig. 9.5), the only conserved sequence segment is the Cys-X-Y-Cys-His motif, responsible for

covalent binding of the c-type heme. The p-side heme binding domain has a completely different secondary structure, α and β, respectively in b_6f and bc1 (Martinez et al. 1992, 1994, 1996). The cyt c_1 and cyt f subunits of the bc_1 complex, each bound to the complex through one TMH (Fig. 9.4a, b), differ markedly in the structures of the p-side domain that bind the c-type heme. The secondary structure of the protein housing the c_1 and f hemes on the p-side of the complex is predominantly β and α, respectively (Fig. 9.4c, d). All three of the p-side electron transfer proteins that are part of the b_6f complex, or interact with it, the Rieske [2Fe-2S] protein, cyt f, and plastocyanin, that mediate electron transfer between the quionol hydrogen donor and the P700 PSI reaction center are predominantly in the β-conformation. Cyt f and cyt c_1 each have a relatively high potential, $+370$ mV and $+260$ mV, respectively, sufficient to provide an oxidant for the ISP, and each contains a covalently bound heme, and is bound to the complex through a single TMH orthogonal to the membrane plane. Otherwise, the structures are completely different, cyt f and c_1, respectively, having β sheet and α-helical secondary structure. Along with the dissimilarity in structure, the evolutionary origin of cyt f is unclear (Martinez et al. 1994).

G. The Four Small Peripheral Subunits, Pet G, L, M, and N; a Late Addition in Evolution?

A unique lattice of four short (3.3–4.1 kDa), single TMH "hydrophobic sticks," denoted as PetG, PetL, PetM, and PetN (Table 9.1), provides a hydrophobic lattice, or "picket fence," around each monomer, a structure which is unique among all known integral membrane protein structures. Of these four subunits, PetM (Schwenkert et al. 2007), along with the Rieske ISP, are the only subunits among the 13 in the complex that are nuclear-encoded. Specific functions of these four subunits are not defined, although PetL interacts sterically with the TMH of the
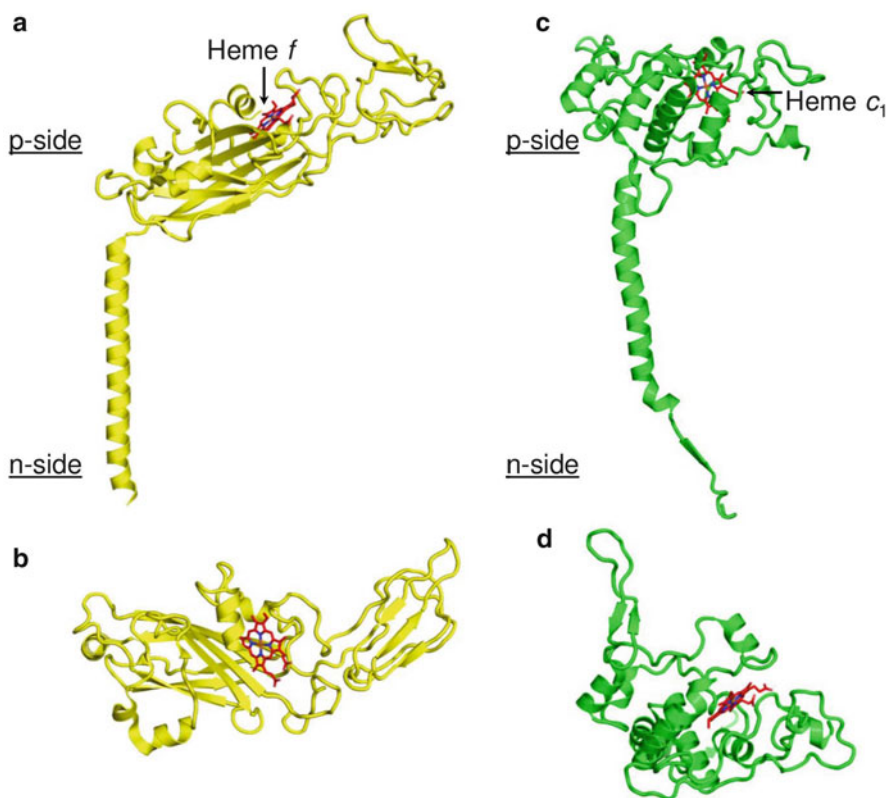
Fig. 9.4. Terminal electron acceptors of cytochrome *bc* complexes, cyt *f* of the b_6f complex (Martinez et al. 1992, 1994, 1996) and cyt c_1. (**a**, **b**) Cyt *f* subunit of b_6f complex attached to the complex and membrane through one TMH. The cyt *f* subunit binds a high potential *c*-type heme ("heme *f*", shown as *red/blue sticks*) in the p-side predominantly β-sheet extrinsic domain (panel **b**). (**c**, **d**) The cyt c_1 subunit of the bc_1 complex is also bound to the complex through one TMH (**c**). In contrast to cyt *f*, the p-side extrinsic domain of cyt c_1, in which the c-type heme is covalently bound (*red/blue sticks*), is predominantly α-helical (panel **d**). **b**, **d** panels rotated 90° around horizontal axis relative to presentation in **a**, **b**, respectively.

Rieske ISP and may provide a constraint on the membrane-spanning orientation of the latter. The position of much of the lipid in the complex between the Pet picket fence and the conserved cyt *b*-suIV core of the complex suggests a "boundary lipid" function and that the "picket fence" may have been added to the core of the complex relatively late in the evolution of the complex.

H. Peripheral Subunits Not Seen in the b_6f Crystal Structure

These subunits are PetP (Rexroth et al. 2001), PetO (Hamel et al. 2000), PetH (FNR) (Zhang et al. 2001) and Stt7 (Rochaix 2014). The FNR unit is seen in SDS-PAGE of purified b_6f complex isolated from spinach, usually at an estimated stoichiometry <1 per monomer (Zhang et al. 2001), and in the "super-complex" obtained from *C. reinhardtii* that includes b_6f and the photosystem I reaction center (Iwai et al. 2010). The inability to crystallize the complex from spinach b_6f complex is attributed at least partly to incomplete presence of the petH subunit in the b_6f complex (Zhang et al. 2001), which would result from its concentration in the stromal compartment of the thylakoid membrane (Joliot et al. 2004).

I. Distribution of Aromatic Residues in the Trans-Membrane Domain

Aromatic residues, Phe, Tyr, and Trp, differ in physical properties due to the absence (Phe) or presence (Tyr and Trp) of polar atoms within the aromatic side chain. The relatively polar Tyr and Trp residues are located preferentially proximal to the lipid-water interface of membrane proteins (Yau et al. 1998; Granseth et al. 2005), where they can interact with the polar head-groups of lipids (Killian and von Heijne 2000). In addition, binding of the phospholipids requires a niche that consists of aromatic residues as well as basic amino acids to neutralize the anionic lipid head-group phosphate (Palsdottir and Hunte 2004b). In this regard, protein complexes associated with the thylakoid membrane, including the b_6f complex, interact with the unique phosphate-free, neutral galactolipids (Gounaris et al. 1986). The absence of an anionic phosphate group eliminates the need for a basic residue for intra-membrane lipid binding. The recent advances in crystallographic studies of lipid-protein interactions of the b_6f complex in a synthetic neutral lipid environment has revealed a heterogeneous, bi-planar distribution of aromatic residues within the hydrophobic trans-membrane domain of the b_6f dimer (Fig. 9.5).

Despite the difference in lipid composition in thylakoid membranes, i.e., the dominant presence of galactolipids, the distribution of Tyr and Trp near the lipid-water interface, first noted in the crystal structure of the photosynthetic reaction center, is conserved in the cytochrome b_6f complex (Fig. 9.5), where Tyr-Trp residues lie within an inter-action distance of the lipid carbonyl groups and polar head groups. The more hydrophobic Phe residues are embedded closer to the membrane center, distal from the lipid-water interface.

J. Inhibitors; Differences Between the bc Complexes

Commonly used quinone-analogue Inhibitors that bind with sub-μmolar to μmolar affinity to the p- and n-side quinone-binding niche, are: (a) for the bc_1 complex, stigmatellin and antimycin A, respectively, in the p- and n-side quinone binding niches. Antimycin is known to reside with a high degree of preference at the n-side quinone binding site (Huang et al. 2005), and binds tightly enough that it induces trans-membrane conformational changes that are detected by g-value shifts of the EPR signal of the p-side Rieske 2Fe-2S complex (Cooley et al. 2009; Sarewicz et al. 2009; Cooley 2010). (b) In the b_6f complex, a major problem in studies on electron transport pathways is that there is no well defined high affinity n-side quinone analog inhibitor: (i) association of antimycin A with the Q_n site is equivocal, as discussed below in the context of cyclic electric transport (Sect. VI.B). (ii) NQNO, whose presence results in an increased amplitude of light-induced heme b (assumed to be heme bn) reduction (Joliot and Joliot 1988; Furbacher et al. 1989) is also a marginally effective (5–10 μM) n-side inhibitor of linear electron transport (Jones and Whitmarsh 1988; Rich et al. 1991; Baniulis et al. 2013), which may result from a second binding site of the inhibitor at the Qp site. (iii) the preferential side of action of stigmatellin inhibition, n vs. p, is reversed, relative to bc_1, as stigmatellin is known to be ineffective in p-side inhibition (Hope and Valente 1996), and is found bound at the n-side quinone binding niche in a crystal structure (Hasan et al. 2014a). (iv) Tridecyl-stigmatellin, with a less branched structure, is seen in crystal structures to bind at both Qp (Kurisu et al. 2003; Stroebel et al. 2003) and Qn sites (Yamashita et al. 2007). (v) The compound MOA-stilbene (Rich et al. 1992) has been proposed to be an n-side inhibitor although
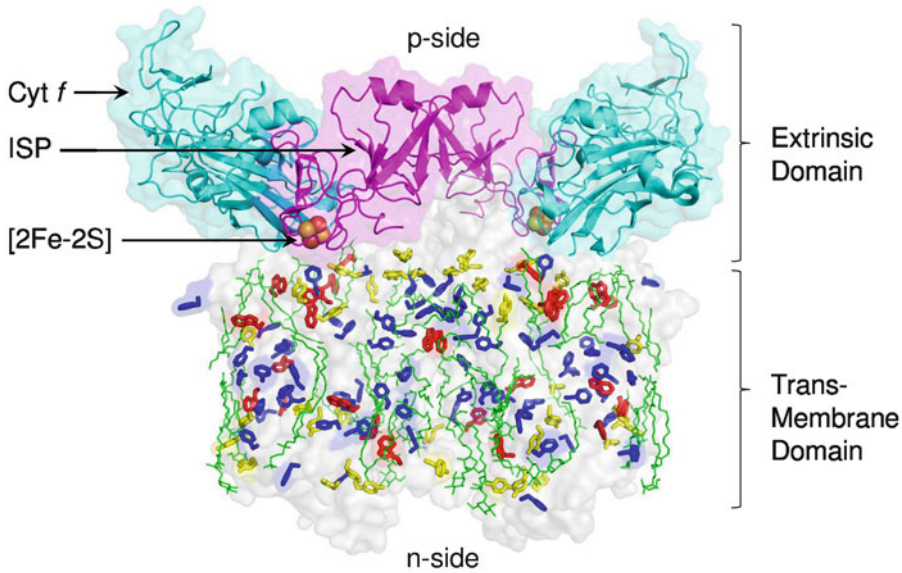
Fig. 9.5. Distribution of aromatic residues in the hydrophobic trans-membrane domain of the cyt b_6f dimeric complex (PDB 4OGQ). Phenylalanine (Phe, *blue sticks*) is distributed predominantly within the core of the trans-membrane domain, while tyrosine (Tyr, tyrosine, *yellow sticks*) and tryptophan (Trp, *red sticks*) are mainly restricted to the periphery, proximal to the lipid-water interface. Many lipids and detergent molecules are crystallographically ordered and are shown as *green sticks*. On the p-side, cyt f (*cyan*) and ISP (*pink*) extrinsic domains are shown as ribbons, and the [2Fe-2S] cluster associated with the ISP extrinsic domain as *brown/yellow spheres*. It has been shown recently that the primary determinant of dimer stability is inter-monomer interactions between tryptophan residues and not the inter-monomer connection between the ISP extrinsic domain and its trans-membrane α-helix (Agarwal et al. 2015).

is relatively unstudied; (vi) DBMIB is un-questionably a Qp –site inhibitor (Bohme et al. 1971; Rich et al. 1991), although it may utilize two p-side binding sites (Roberts and Kramer 2001; Roberts et al. 2004; Yan et al. 2006).

V. Lipids in the b_6f Lipoprotein Complex

"Boundary lipids" define a domain structure. Because the conserved cyt b_6-SU IV core of the complex is separated by a lipid layer from the "picket fence" (Fig. 9.3), it is implied, as noted above, that the "picket fence," was added to the core structure at a later stage in the evolution of the b_6f complex, per-haps to facilitate interaction with the reaction center complexes. As noted above, the β-carotene, which protrudes from the surface of the picket fence, may act as a "latch"

to facilitate super-complex formation with the PSI reaction center complex (Iwai et al. 2010).

A. Lipid Content; Anionic Lipid, an Assembly Factor

The high lipid content, at least 23 lipid binding sites per monomer (Hasan and Cramer 2014) (Fig. 9.6), defines b_6f as a lipoprotein complex with an intimate role of lipids in its structure, function, and assembly. Translocon-based models (e.g., Egea and Stroud 2010), of membrane protein assembly have not yet considered the additional problems associated with hetero-oligomeric membrane lipoproteins and lipid specificity, known in the case of the mitochondrial bc_1 complex and super-complex (Althoff et al. 2011) to include a requirement for the di-anionic lipid, cardolipin, as an assembly factor (Pfeiffer
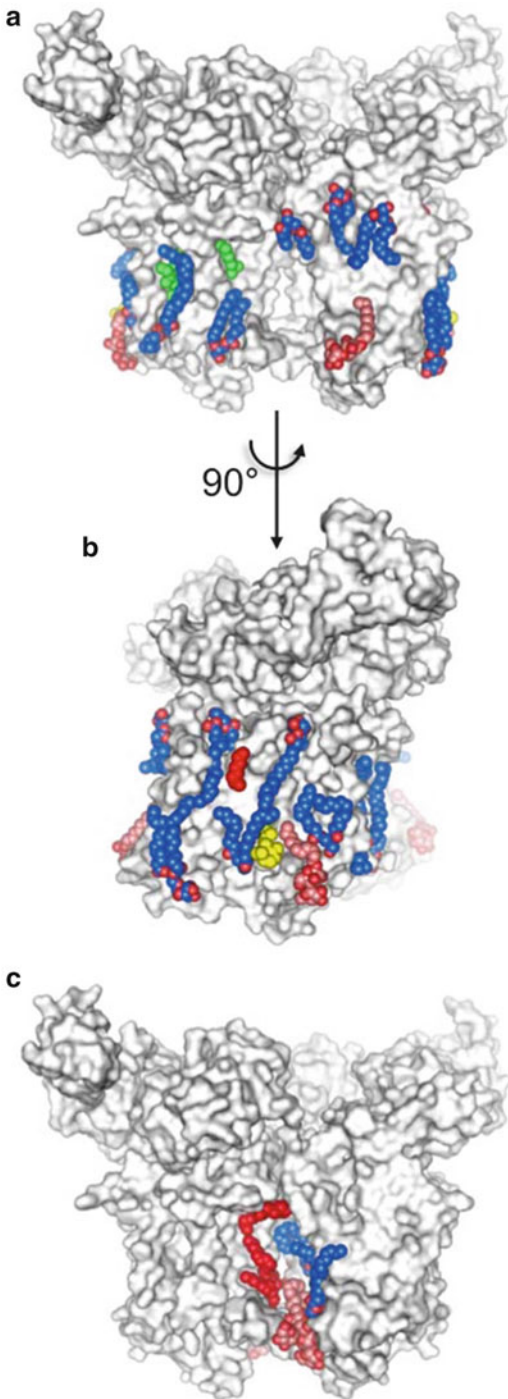
et al. 2003). The anionic lipids in the b_6f complex are sulfolipid (sulfoquinovosyl-diacylglycerol) and phosphatidyl-glycerol (Hasan et al. 2011; Arnarez et al. 2013; Hasan and Cramer 2014), which may act "as assembly factors."

VI. Pathways of Cyclic Electron Transport (CET)

A. Cyclic Electron Transport (CET) and the n-Side of the b_6f Complex

[cf., Chaps. 22 and 23 by Finazzi et al. and Minagawa in this volume]. Some details of the cyclic electron transport (CET) pathway from reduced ferredoxin to quinol in the main chain (Fig. 9.1b) are controversial. Independently of these complications, there is an energetic necessity for an additional contribution to the $\triangle\tilde{\mu}_H^+$ needed for ATP synthesis generated in a CET pathway. As much as two-thirds of the proton gradient used for trans-membrane free energy storage in oxygenic photosynthesis is generated by the b_6f complex. Considering the 4 electrons needed to form the 2 NADPH molecules required for fixation of one CO_2 by the Calvin-Benson mechanism, as many as 3 H^+ can be translocated across the membrane for every electron transferred through the chain, i.e., $H^+/e = 1$ for water splitting and as many as 2 for the b_6f complex. Thus, per 4 electrons transferred, 12 H^+ can be translocated to the electrochemically positive (p) side of the membrane and thus made available for discharge through the Fo c-ring of the ATP synthase, which would ideally be sufficient if the c-ring contained ≤ 12 subunits, as has been proposed (Jiang et al. 2001). Based, however, on data obtained subsequently by

Fig. 9.6. Lipids in the cytochrome b_6f complex (PDB 4OGQ). (**a**, **b**) Peripheral lipidic sites associated with the b_6f complex shown in two orthogonal views. Lipids shown as *blue*/*red spheres*, alkyl chains as *red spheres*, and detergent molecules as *pink*/*red spheres*. Pigments Chl-*a* and β-car (carotene) are shown as

Fig. 9.6. (continued) *green*, *yellow spheres*. (**c**) Lipidic sites within the inter-monomer cavity. Protein subunits are marked but not colored. Lipids within the inter-monomer cavity are implicated in modulation of dielectric properties that affect electron storage and transfer (Hasan et al. 2014b).

atomic force microscopy (AFM), 12 H^+ are insufficient to drive sufficient ATP synthesis in oxygenic photosynthesis, resulting in a mechanistic problem. In more detail, the AFM visualization of the ATPase c-subunit stoichiometry defines 14 H^+ conducting c-ring subunits, implying an H^+/ATP ratio of 4.7 (14/3) in the ATP synthase associated with spinach chloroplasts (Seelert et al. 2000, 2003). Thus, there is a problem of an insufficiency of protons ($12–14 = −2$). Furthermore, an H^+/ATP ratio of 5 is implied for the cyanobacterium, *Spirulina platensis*, in which 15 c-subunits were seen (Pogoryelov et al. 2005). The deficiency ($12–15 = −3$) between the maximum number of protons generated by the linear electron transport chain and those needed for ATP synthesis in one rotation of the *c*-ring would be even greater.

The requirement for the free energy derived from the proton electro-chemical potential gradient, $\triangle\tilde{\mu}_H{}^+$, is a substantially more formidable requirement than exists in bovine mitochondria, where the c-ring contains only 8 subunits (Watt et al. 2010), through which only 8 H^+ need be translocated for the synthesis of three ATP per complete revolution of the c-ring. The variability of the size of the F1 ATPase ring is a fascinating problem in the evolution of the structure of the energy transducing molecular apparatus.

B. The Proton Shortfall; Cyclic Electron Transport

The ATP/2e ratio measured in thylakoid membranes is 1.25 and 1.6 under conditions of LET and CET using methyl viologen and ferredoxin, respectively, as the electron acceptor to define non-cyclic (LET) and PSI cyclic (CET) electron transport (Hosler and Yocum 1987). The recently determined value of the H^+/ATP stoichiometry in plant chloroplasts is 4.7 (Petersen et al. 2012). Assuming that each complete rotation of the proton translocating c-ring results in the synthesis of 3 ATP (Ferguson 2010; Watt et al. 2010), the maximum efficiency

(ATP/2e) of ATP synthesis would be 1.3 (6 H^+/2e divided by 4.7 H+/ATP) or 1.2 (6 H^+/2e//5 H+/ATP) via LET coupled to water oxidation and a Q cycle operating at full efficiency, i.e., H^+/e = 2, in the b_6f complex (Mitchell 1975a), yielding an H^+/2e ratio = 6 for the LET chain. *n. b.*, an experimental nuance is that the experimental determination of the pH change associated with a full H^+/e ratio = 2 in the Q cycle mechanism requires addition of an ionophore to collapse the $\triangle\tilde{\mu}_H{}^+$ (Fowler and Kok 1976; Graan and Ort 1983; Willms et al. 1987; Berry and Rumberg 1999). The value of 1.25 measured for the ATP/2e stoichiometry in LET mode is appreciably smaller than the value of 1.5 needed to account for the ATP/NADPH stoichiometry. Thus, in addition to the LET pathway including the Q cycle driven through the b_6f complex (Fig. 9.1a), an additional pathway coupled to proton pumping is required is required to generate a $\triangle\tilde{\mu}_H{}^+$ sufficient for synthesis of ATP with the known efficiency. A solution to this problem is utilization of the pathway of photosystem I- ferredoxin-dependent cyclic electron transport, i.e., CET (Joliot and Joliot 2005, 2006; Breyton et al. 2006; Fisher and Kramer 2014), (Fig. 9.1b). Thus, LET alone cannot generate a $\triangle\tilde{\mu}_H{}^+$ sufficient to account for the measured maximum efficiency of ATP synthesis. CET can be viewed as a modulator of the rate of ATP synthesis, which is an increasing function of the ambient light intensity (Kramer et al. 2004).

By what detailed mechanism, and with what efficiency, the cyclic pathway is coordinated to the main chain or non-cyclic pathway is not well established. It has been estimated that in newly illuminated previously dark-adapted leaves of *Arabidopsis*, cyclic electron flow can operate in dark-adapted leaves at a rate, approximately 130 s^{-1} (Joliot et al. 2004) which is comparable to the rate-limiting step of LET, and that as much as 50 % of the light-dependent ATP synthesis can be derived from the cyclic pathway (Joliot and Joliot 2006). However, there is not a consensus on the pathway and redox

proteins involved in the connection of the physiological low potential electron donor, ferredoxin, to the main chain. Based on inhibition of ferredoxin-mediated cyclic phosphorylation by antimycin A (Tagawa et al. 1963; Arnon et al. 1967), a classical inhibitor of the mitochondrial bc_1 complex that blocks the oxidation of heme b_n (Chance and Williams 1956; von Jagow and Link 1986), it was proposed that the electron connection of ferredoxin with the main electron transport chain occurred via the cytochrome b_6f complex (Slovacek et al. 1979; Slovacek and Hind 1980). This result was, however, not confirmed (Moss and Bendall 1984; Bendall and Manasse 1995). The interpretation of the latter result was that cyt b_6 is not involved in the pathway of CET and led to different proposals for the identity of the ferredoxin-plastoquinone reductase (see below). To explain the two different results for the effect of antimycin A on heme b_6 reduction, it can be noted that 12 different isomers of "antimycin" are found in its Internet listing at the NCBI PubChem, and that different antimycin isomers with different specificity may have been used in the two studies on the effect of antimycin on the amplitude of the flash-induced reduction of heme b_n. It has also been noted that the binding of antimycin A at the n- side of the b_6f complex must differ from that in the bc_1 complex, and is likely to be of lower affinity, as a consequence of partial occupancy of the Q_n binding site (Yamashita et al. 2007; Cramer et al. 2011) by the unique heme c_n (Stroebel et al. 2003; Alric et al. 2005; Zatsman et al. 2006; Baymann et al. 2007; Twigg et al. 2009) of the antimycin binding site in the bc_1 complex.

C. Protein and Redox Components in the CET Pathway

Information on the identity of additional components involved in CET-dependent electron transport is contained in the isolated PSI reaction center "super-complex" (Iwai et al. 2010) which, in addition to PSI, contains the b_6f complex, FNR, and the integral membrane protein PGRL1 that is implicated in CET (Shikanai 2007; Hertle et al. 2013). Participation of FNR with b_6f complex in the cyclic pathway in plant/algal thylakoid membranes was suggested by the association of FNR with isolated spinach b_6f complex (Zhang et al. 2001). However, neither the Trol nor the Tic62 proteins that are known to be involved in the bonding of FNR to the thylakoid membrane (Mulo 2011) have been detected in the b_6f complex (Zhang et al. 2001). The less than stoichiometric binding of FNR to the b_6f complex (Zhang et al. 2001) would be consistent with the proposal that when FNR functions in CET, it binds only to the fraction of the b_6f population that resides in the non-appressed membrane fraction in the laterally inhomogeneous membrane (Anderson 2002), where CET is concentrated (Joliot and Joliot 2005) (Fig. 9.1b). An isolated complex containing both FNR and the b_6f complex implies a connection between the two, suggesting that the n-side of the cytochrome complex provides entry for electrons donated by ferredoxin to the main chain (G. Kurisu, this volume, Chap. 12), and that a ferredoxin-plastoquinone reductase provides the connection necessary for CET between the low potential redox components of the PSI reaction center and the high potential redox centers of the LET chain. A ubiquitous role of FNR in a cyclic pathway, however, is unlikely as it is not found in the cyanobacterial b_6f complex (Kurisu et al. 2003; Zhang et al. 2003; Yamashita et al. 2007; Baniulis et al. 2009, 2011; Hasan et al. 2013b; Hasan and Cramer 2014; Rexroth et al. 2014), nor in b_6f complex isolated from *C. reinhardtii* (Pierre et al. 1995; Stroebel et al. 2003), although it is possible that it is loosely bound and lost in purification or crystallization (cf., Sect. IV.H).

D. Other Candidates for the Ferredoxin-Plastoquinone Reductase (FQR)

Alternative proposals for the identity of this reductase, which are necessary for cyanobac-

teria that do not have a bound FNR, are shown (Fig. 9.1).

1. A Role for NADPH Dehydrogenase (Ndh)

Ndh, or Ndh-1 in cyanobacteria (Battchikova et al. 2011) in the cyclic pathway was suggested by: (i) studies on chloroplast mutants in the model plant, *Arabidopsis thaliana* (Livingston et al. 2010); (ii) reduction of the plastoquinone pool in tobacco leaves by a chloroplast Ndh complex whose rate was not documented to be kinetically competent (Sazanov et al. 1998), and (iii) an isolated 550 kDa Ndh complex suggested to function as a respiratory complex dispensable for plant growth under optimum conditions (Burrows et al. 1998). The characterization of this complex from cyanobacteria, particularly by electron microscopy. has been facilitated by a fluorescence tagging approach (Birungi et al. 2010). Although the latter study is a significant advance, it has not yet been possible to characterize a physiologically significant redox activity of the Ndh complex coupled to reaction with a known component in the LET.

2. PTOX

An NADH dehydrogenase may also function in a "chlororespiration" system terminated by a plastoquinol terminal oxidase (PTOX) that functions when the quinone pool is over-reduced. The biochemical identity of the PTOX is unknown (McDonald et al. 2011), and the only known redox component in the ETC that has properties resembling an oxidase is the novel heme c_n in the b_6f complex, discussed below, which has been shown through EPR spectral analysis to bind nitric oxide (Twigg et al. 2009).

3. PGRL Components

A 35.7 kDa (pl = 5.2) protein, "PGRL1," present at unknown stoichiometry in the PSI super-complex *C. reinhardtii* (Iwai et al. 2010), and at a stoichiometry comparable to,

although smaller (0.5:1), that of the Rieske [2Fe-2S] protein in *Arabidopsis thaliana,* has been inferred to be the ferredoxin-quinone reductase (Hertle et al. 2013). The absence of the protein results in a partial decrease of the chlorophyll fluorescence yield that results from reduction of the quinone pool. The redox function is believed to be derived from the six cysteine residues in the protein, perhaps in conjunction with iron that is present in the preparation. PQRL1 is also inferred to be able to bind to the b_6f complex (Hertle et al. 2013), although it has not been detected in mass spectroscopic analysis of isolated b_6f complex (Whitelegge et al. 2002). It may be appropriate to include PGRL1 to the list of proteins listed above in Sect. IV.H that are weakly bound to the b_6f complex. In addition, It is noted: (a) the fluorescence yield effects ascribed to PGRL1 are perhaps not quantitative indicators of an obligatory function in the ETC, as they involve changes of a factor of 2–3 on a time scale of 10 s, approximately a factor of 1000 times slower than the rate-limiting step of electron transport in this region of the ETC; (b) the rate of P700 reduction, a standard kinetically competent assay for cyclic electron transport, in the presence and absence of the peptide has not been reported. (c) Details of the chemical nature and quantitative properties of the hexaCys-Fe redox moiety in PQRL1, proposed to serve as the redox group responsible for FQR activity, are not presently available.

VII. Functions of the b_6f Complex in the Linear Electron Transport (LET) Pathway; the Q Cycle

Plastoquinone (PQ) is reduced by the PSII reaction center to PQH_2 concomitant with H^+ uptake, and is the second source, besides H_2O, of H^+ translocated to the p-side of the membrane to form the ΔpH component of the $\Delta\tilde{\mu}_H{}^+$. PQH_2 enters an intra-membrane pool of 5–10 PQ/PQH_2 per b_6f (Stiehl and Witt 1969; Graan and Ort 1984). Oxidation of PQH_2 from this pool by the b_6f

complex provides the second site in the LET, and a second mechanism, for generation of the $\Delta\tilde{\mu}_{H}^{+}$. This second mechanism, centered on the functions of the b_6f complex, is the "Q cycle" (Fig. 9.7a).

The historical development and evolution of the Q cycle model from its original formulation based on studies of the mitochondrial respiratory chain by Mitchell (1975a, b), and many experimental and conceptual problems associated with it, has been discussed recently in a perspective involving both bc_1 and b_6f complexes (Mulkidjanian 2010). The major points in the latter critique that apply to data obtained for the function of the b_6f complex are: (a) the initial suggestion of Mitchell that the quinone bound at the n-side of the complex could be reduced enzymatically (Mitchell 1975a, b); (b) historically, elucidation of the Q-cycle mechanism operating in bc_1 complexes was facilitated by utilization of the photosynthetic α-proteobacteria, *Rhodobacter sphaeroides* and *Rhodobacter capsulatus* (Crofts and Wraight 1983); (c) the relatively short electron transfer distance, 10–13 Å between the two b_p hemes, summarized for known structures of cytochrome *bc* complexes (Cramer et al. 2011) should allow electron transfer between the two monomers of the complex (Soriano et al. 1999; Sarewicz et al. 2010; Swierczek et al. 2010; Khalfaoui-Hassani et al. 2012). (d) An enigma associated with the Q cycle mechanism of trans-membrane electron transfer across the two hemes b_p and b_n is that it has not been possible to obtain an additive photo- (i.e., flash) induced reduction in situ of the two hemes from either the bc_1 (Klishin et al. 2002) or b_6f (Furbacher et al. 1989) complexes. (e) There are additional experimental problems associated with the analysis of the redox events involving the trans-membrane hemes in the b_6f complex: the wavelength maxima (λ_m) of the absorbance difference (reduced minus oxidized) spectra of hemes b_p and b_n are spectrophotometrically indistinguishable ($\Delta\lambda_m \leq 1$ nm) at the measuring light band-widths usually employed. The consequence

is that it is not possible to experimentally determine which heme, b_n or b_p, undergoes photo-reduction. (f) As discussed above (Sect. IV.J), there is no n-side chemical inhibitor of cyt *b* redox reactions in the b_6f complex that acts with the efficacy of antimycin A on heme b_n in the bc_1 complex.

A. Complexity of the b_6f Complex; Some Inconsistencies

With seven redox centers, four hemes (f, b_p, b_n, and c_n), plastoquinone/ol at two binding sites, and the [2Fe-2S] ISP, present in each monomer of the b_6f complex that are involved in redox and proton transfer reaction associated with a Q cycle, the associated redox and proton transfer reactions are inevitably complex. The number of disparities (e.g., Girvin and Cramer 1984; Rich 1988; Furbacher et al. 1989; Joliot and Joliot 1994) or variations (Ponamarev and Cramer 1998; Heimann et al. 2000) observed in the anticipated charge transfer parameters of the proposed Q cycle is significant. Many have been discussed previously (Mulkidjanian 2010). Some of the inconsistencies with the predictions of the Q cycle (Girvin and Cramer 1984) involve the slow electrochromic band shift associated with trans- or intra- membrane change transfer (Joliot and Delosme 1974; Rich 1988).

B. Alternative Q Cycle Model

Here, a modified alternative Q cycle model is suggested in the context of some of the perceived inconsistences in the canonical Q cycle model described above. To accomplish this goal, the participation of the cyclic pathway described above (Sect. VI) is included in the model to supply the second electron to the n-side plastoquinone. This avoids the necessity for turnover of two quinols on the p-side of the complex: thus, (i) plastoquinol, formed in the PSII reaction center by electrons donated from water and protons obtained from the n-(stromal) side of the membrane (Fig. 9.1a), enters the b_6f complex through the inter-
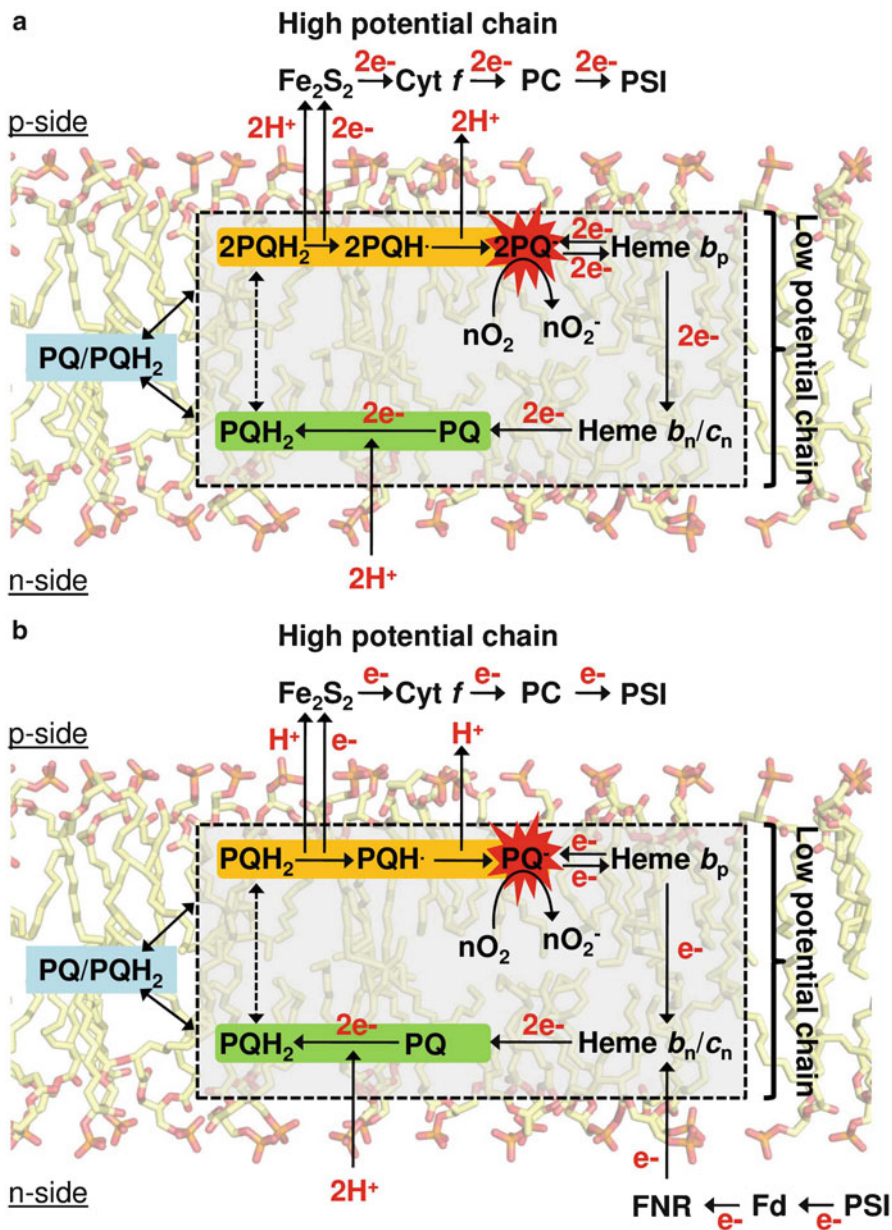
Fig. 9.7. Electron-proton transfer pathways in the cytochrome b_6f complex (Hasan et al. 2013b). Trans-membrane core represented as light *gray dashed box*, and lipid bilayer as sticks in the background. p-side plastoquinol deprotonation/oxidation (Q_p) site, n-side plastoquinone reduction/protonation site (Q_n) shown, respectively, as *yellow* and *green boxes*. Intra-membrane plastoquinone (PQ/PQH_2) pool highlighted as *blue box*. (**a**) p-side one electron oxidative/deprotonation reactions: PQH_2 bound within Q_p-site proximal to 2Fe-2S cluster transfers H^+ to His129 of the ISP and oxidation to neutral semi-plastoquinone ($PQH^\bullet$). Anionic semi-plastoquinone ($PQ^{\bullet-}$) (in *red*) generated either by deprotonation of the neutral semi-plastoquinone ($PQH^\bullet$) via the conserved PEWY (Widger et al. 1984) domain (Fig. 9.8). Anionic semi-quinone reduction of molecular oxygen to superoxide ($O_2^{\bullet-}$) (Baniulis et al. 2013). The prefix "n" in front of molecular oxygen (O_2) and superoxide (O_2^-) in the reaction scheme is a "scale factor" that describes the stoichiometry of superoxide generation within the cytochrome complex, which is 0.02 relative to the LET rate through the cytochrome complex, and approximately 20-fold larger than in the bc_1 complex (Baniulis et al. 2013). The prefix "2" associated with the trans-membrane electron transfer, denotes the stoichiometry of the p-side quinol oxidation reactions required for the n-side quinone reaction. (**b**) An alternative mechanism utilizes the cyclic pathway, in which one of the two electrons required for n-side reduction of plastoquinone is provided by the CET pathway from the PSI complex to the b_6f complex via ferredoxin and, at least in the plant system, FNR, and other possible cofactors.

monomer cavity of the dimeric complex (Fig. 9.2a, b) and quinone entry-exit portal (Hasan et al. 2014a), donates electrons and protons to the p-side [2Fe-2S] cluster of the b_6f complex (Hasan et al. 2014a). (ii) In the canonical Q-cycle mechanism (Mitchell 1975a; Joliot and Joliot 1985, 1988; Kramer and Crofts 1994; Cape et al. 2006) PQH_2 oxidation by the b_6f complex occurs through a split pathway involving high (2Fe-2S $\rightarrow$ cyt f $\rightarrow$ PC or cyt c $\rightarrow$ P700) and low (heme $b_p \rightarrow$ heme $b_n \rightarrow PQ_n$) potential ET chains (Fig. 9.7a). This results in a theoretically maximum H^+/e ratio $= 2$ contributed by the b_6f complex and, as discussed above (Sects. VI.A and VI.B), a total H^+/e ratio of 3 when the contribution from water splitting is included. Pathways of H^+ transfer on the p- and n-sides of the complex are described in Fig. 9.7a.

The Q cycle pathway described in Fig. 9.7b is, in two respects, an alternative to the conventional model shown in Fig. 9.7a, and is based on fundamental differences between the properties of the b_6f and bc_1 complex. The bc_1 complex differs by having: (a) no cyclic pathway in the mitochondrial respiratory chain operating from the high potential end of the LET; (b) no low potential reductant in the photosynthetic bacteria such as ferredoxin or heme c_n. The b_6f complex is distinguished by a unique tight coupling and sharing of electrons between hemes b_n and c_n on the n-side of the membrane, which results in the g $= 12$ EPR signal (Zatsman et al. 2006). Thus, for the n-side of the complex, it is proposed: (i) the heme couple b_n-c_n reduces quinone via a two electron mechanism that avoids the problem of quinol production via one electron transfer of a reactive semiquinone intermediate; (ii) one of the electrons that reduces PQ_n in the plant/algal Q cycle system can be donated by ferredoxin. This mechanism would explain why no more than one b heme in the b_6f complex can be rapidly reduced by multiple closely spaced short duration light flashes (Furbacher et al. 1989).

The mechanism for the canonical Q cycle described in Fig. 9.7a involves oxidation of one p-side plastoquinol molecule which provides one electron to the high potential chain, for transfer to the PSI reaction center complex. Oxidation of two p-side plastoquinol molecules is required to completely reduce one n-side plastoquinone to plastoquinol. The individual deprotonation and oxidation steps during the p-side redox reactions of plastoquinol, respectively, involve the transfer of only one proton and electron. Two electrons can be stored in the n-side heme b_n/c_n couple, to perform two closely spaced plastoquinone (PQ) reduction-protonation reactions, which would preclude/decrease semi-plastoquinone formation on the n-side. A mechanism alternative to that in the bc_1 complex, shown in Fig. 9.7b, is provided by utilization of the PSI cyclic pathway, in which one of the two electrons required for n-side reduction of plastoquinone would be provided by the CET pathway, from the reducing side of the PSI reaction center via ferredoxin to the b_6f complex. Under such conditions, the requirement of p-side plastoquinol moleculesis decreased from two to one for the complete n-side reduction of plastoquinone (PQ) to plastoquinol (PQH_2). This would remove the requirement in the conventional Q cycle model for a mechanism to coordinate the delivery of two quinol molecules in a msec time interval to the Qp site.

VIII. Quinone-Mediated H^+ Transfer Pathways; n-Side to Quinone Binding Site: Heme c_n; p-Side PEWY Exit Pathway with Invaginated H_2O Pocket

A. n-Side Entry Pathway to Heme c_n Quinone Binding Site

This domain consists of heme b_n, the unique heme c_n, whose close proximity to heme b_n (4 Å between the c_n heme Fe and propionate oxygen of heme b_n) is reflected in a unique g $= 12$ EPR signal (Zatsman et al. 2006; Baymann et al. 2007), and the quinone binding defined by these

prosthetic groups. The H^+ uptake pathway from the electrochemically negative (n) aqueous phase to the n-side quinone binding site of the complex has been defined in the 2.70-Å crystal structure (PDB 4H44; (Hasan et al. 2013b)), and in structures with bound quinone analog inhibitors, inferred from crystal structures with the quinone analogue inhibitors tridecyl-stigmatellin and 2-nonyl-4-hydroxyquinoline N-oxide, with a resolutionof 3.07 Å and 3.25 Å, respectively (Yamashita et al. 2007). The simplest n-side proton transfer pathway, which does not contain any as yet resolved proton transferring water molecules, extends from the n-side bulk aqueous phase via Asp20 to Arg207 (Arg 214 in Synechococcus PCC 7002) in the cyt b subunit to quinone bound axially to the heme c_n. This is a unique structure aspect of the b_6f compared to the bc_1 complex (Fig. 9.8a), and provides a function for the previously enigmatic heme c_n, whose presence in the b_6f complex is a unique property relative to b_6f. Additional possible n-side H_2O-containing H^+ – transfer pathways to the heme c_n quinone binding site have been proposed (Hasan et al. 2013b).

B. p-Side PEWY-Dependent Exit Pathway

The p-side H^+ transfer pathway in the b_6f complex (Fig. 9.8b) is structurally and conceptually similar to that previously described for the bc_1 complex in mitochondria (Solmaz and Hunte 2008; Berry et al. 2013; Kao and Hunte 2014) and purple photosynthetic bacteria (Esser et al. 2004, 2008). Information on details of the p-side H^+ transfer pathway were obtained from crystal structures of the b_6f complex obtained in the absence and presence of quinone-analog inhibitors from: (a) filamentous cyanobacteria, *M. laminosus* (Kurisu et al. 2003; Yamashita et al. 2007; Baniulis et al. 2009; Hasan et al. 2013b); PDB 1VF5, 2E74, 2E75, 2E76 and Nostoc PCC 7120 (PDB 2ZT9, 4H44, 4H0L, 4H13,

and 4OGQ for the highest resolution attained, 2.50 Å); (b) the green alga, *C. reinhardtii* ((Stroebel et al. 2003), PDB 1Q90). The exit pathway for the first proton donated by plastoquinol $(PQH_2 \rightarrow PQH^- + H^+)$, based on the structure obtained with the p-side quinone analogue, tri-decyl-stigmatellin (TDS), PDB 2E76 (Yamashita et al. 2007), involves His129 of the Rieske ISP in the p-side binding site (Qp) of PQH_2. His129 is located within an H-bond distance from O4 of the chromone ring of TDS (Yamashita et al. 2007), and can provide a route for transfer of the first proton donated by plastoquinol (PQH_2) to the p-side bulk aqueous phase. The structures of the cyanobacterial complex obtained subsequently at higher resolution, up to 2.5 A (PDB 4OGQ), imply that, as inferred for the bc_1 complex (Solmaz and Hunte 2008), a different route to the p-side aqueous phase is taken by the second proton. The p-side EF loop of the bc_1 complex and subunit IV (b_6f) has a conserved sequence motif, PEWY, as noted in the initial description of the membrane topology of the cytochrome b subunit in energy transducing membranes (Widger et al. 1984). Crystal structures of the mitochondrial (Solmaz and Hunte 2008; Kao and Hunte 2014), and chromatophore (Esser et al. 2004, 2008) bc_1 complex with quinone analog inhibitors imply that Glu272 in the PEWY sequence undergoes rotation from a quinol-liganding position distal to heme b_p to a heme b_p-proximal position. The Glu-78 residue of the PEWY sequence in b_6f is implicated in a similar function, participating in the exit of the second H^+ from the Qp-site (Zito et al. 1998, 1999). A difference in the function of the Glu residue at this position is that in its *trans*-position distal to the quinone, it forms a salt bridge with Arg87 (Hasan et al. 2013b), and is thus inferred to lengthen the lifetime of the protonated plastosemiquinone (Hasan et al. 2013b), perhaps providing an explanation for the elevated level of super-oxide production relative to the mitochondrial bc_1 complex (Baniulis et al. 2013).

C. Intrusion of Water in the p-Side Exit Pathway

The side chain carboxylate of the conserved Glu78 in the b_6f complex faces a water channel (Fig. 9.8) lined by polar residues that is connected to the p-side bulk aqueous phase. Despite some similarities between the b_6f and the bc_1 complex in the transfer mechanism of the second proton to the aqueous phase, there is a major difference in the organization of the p-side water network. In the yeast bc_1 complex, an extended water channel from a heme b_p propionate oxygen extends via hydrogen bonds in a network of water molecules to the p-side aqueous phase (Hunte et al. 2000). In the b_6f complex, a short channel from Glu78 to a water-filled invagination in the complex is provided by a conserved polar residue, Glu3, from the
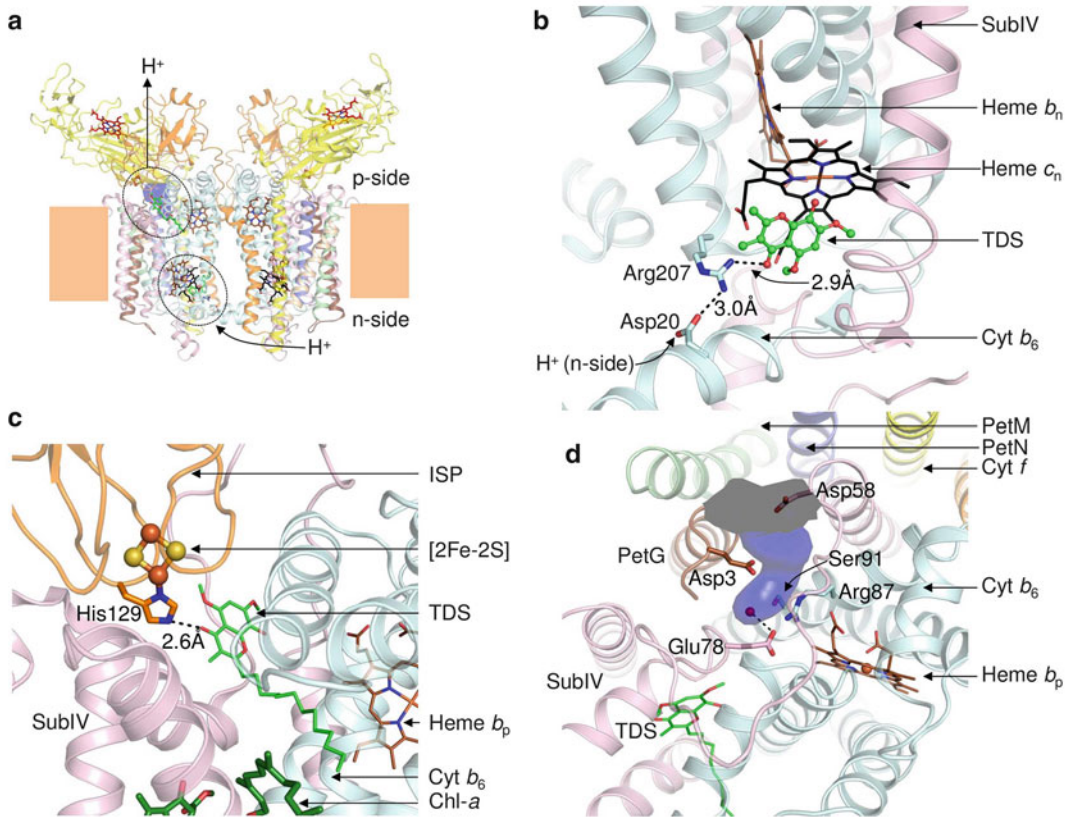
Fig. 9.8. Proton transfer pathways in the cytochrome b_6f complex. (**a**) Proton entry into the complex occurs on the electrochemically negative (n) side, and proton exit takes place from the electrochemically positive (p) side of the complex. The plane of the lipid bilayer is shown as *orange blocks*. (**b**) Proton entry has been suggested to involve the residues Asp20 and Arg207 of cyt b_6, which form a pathway for proton translocation from the n-side aqueous phase to the quinone reduction/protonation site, marked by the quinol analog TDS (*green/red ball* and *sticks*) (PDB ID 4H44). For simplicity, the TDS tail has been omitted. Proton exit on the p-side of the complex involves two distinct pathways. (**c**) The first proton released from the reduced quinol (QH_2) involves the ISP surface residue His129 (PDB ID 4H13). The position of the quinol is marked by the bound TDS (*green/red sticks*). (**d**) The second proton is extracted from the semiquinone by Glu78 of subunit IV (PDB ID 4H44). The position of the semiquinone is marked by the quinone analogue inhibitor, TDS, which has been superposed from PDB ID 4H13. The Glu78 side chain connects via a water molecule (water-316, PDB ID 4H44) to a hydrophilic cavity (*blue* surface), which provides a pathway for proton exit to the p-side bulk aqueous phase. The cavity is lined by Asp3 (PetG), Arg87 (cyt b_6), Ser91 (cyt b_6), and Asp58 (subIV).

peripheral PetG subunit in the b6f complex, for which there is no analogue in the bc1 complex. pKa calculations indicate that Glu3 ($pK \approx 4.6$), which borders the H_2O channel and is a conserved residue, is consistent with its participation in proton transfer during the p-side proton exit process (Hasan et al. 2013b). It is noted that pronounced water-filled invagination is a common feature of integral membrane transport proteins such as the *lac* permease (Smirnova et al. 2011).

IX. The Cytochrome b_6f Complex as a Hetero-Oligomeric Membrane Lipoprotein

In addition to its function in photosynthetic and membrane energy transduction, the structure studies on the cytochrome *bc* complexes provide information on fundamental properties of multi-subunit relatively complicated hetero-oligomeric membrane proteins (Althoff et al. 2011). Studies on protein-lipid interactions of the bc_1 (Palsdottir and Hunte 2004a; Wenz et al. 2009) and b_6f complexes l (Hasan et al. 2011, 2014b; Hasan and Cramer 2014) are of general interest in the membrane protein field. The b_6f complex has at least 23 lipid binding sites per monomer (see Sect. V, Fig. 9.6). In addition to conventional absorbance spectroscopy, the use other spectrophotometric approaches provided by the hemes to analyze the state of the complex (e.g., Hasan et al. 2014b) confers a unique handle for analysis of conformational changes of the complicated integral membrane lipoprotein complex.

Acknowledgments

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Structure-Function Studies of the Cytochrome bc_1 Complex of Anoxygenic Photosynthetic Purple Bacteria

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Summary

Photosynthetic non-oxygenic purple bacteria possess various energy-conserving machineries that allow them to grow either photosynthetically, anaerobically or aerobically. The photosynthetic and respiratory branches of the power system in purple bacteria are connected via the cytochrome bc_1 complex (cyt bc_1), which catalyzes electron transfer from quinol to cyt c and couples this reaction to proton translocation across the photosynthetic membrane, generating a trans-membrane proton gradient essential for various cellular activities including ATP synthesis. Crystal structures of wild-type and mutant cyt bc_1 complexes have been determined for the closely related photosynthetic bacteria *R. sphaeroides* and *R. capsulatus* in the presence of various inhibitors or substrates. These structures are remarkably similar to their mitochondrial counterparts but do feature insertions or deletions that are characteristic of the bacterial enzyme complexes. Thus, it is expected that bacterial cyt bc_1 complexes function in a manner similar to mitochondrial enzymes, justifying their use as model systems. The roles of insertions in bacterial cyt bc_1 function are discussed in relation to the subunit compositions of bacterial complexes and to mutagenesis studies. The mechanism of cyt bc_1 function is also discussed in the context of the architecture of quinol oxidation and quinone reduction sites, as revealed by inhibitor binding studies of both mitochondrial and bacterial bc_1 complexes.

I. Introduction

As part of the cellular respiratory chain and photosynthetic apparatus, cytochrome bc_1 complexes (cyt bc_1 or bc_1) translocate protons endergonically across the membrane and propel the proton movement by energetic electron transfer (ET) from ubiquinol (QH_2) to cyt c (cyt c_2). The activity of bc_1 thus contributes to the trans-membrane potential or proton motive force (pmf) that is vital to various cellular activities (Trumpower and Gennis 1994). As in all areas of research, much of our understanding of the mechanism that underlies the function of cyt bc_1 came from studies of many laboratories using various technical approaches. This paper, however, will focus on bc_1 function from a structural perspective, based on detailed 3-dimensional structures determined by X-ray crystallography. Over the past two decades, structures of bc_1 from different kingdoms of life have been determined to near atomic resolution, providing an opportunity to examine differences in structures and relate these differences to different aspects of the reaction mechanism as well as to functional variations of the evolutionarily related enzymes. Based on our recent work on the crystal structures of cyt bc_1 from the anoxygenic, photosynthetic purple bacterium *Rhodobacter sphaeroides* ($Rsbc_1$) (Esser et al. 2006, 2008a), we will provide a structural interpretation of bc_1 function and discuss functional implications of the features unique to bacterial bc_1 in comparison to their mitochondrial counterparts.

A. Anoxygenic Photosynthetic Purple Bacteria's Ability to Convert Light into Chemical Energy

Photosynthesis is arguably the most important reaction on planet earth, as it converts sunlight into chemical energy. Photosynthetic purple bacteria are believed to be one of the most primitive life forms on earth (Woese 1987). In contrast to oxygen-evolving bacteria such as cyanobacteria, anoxygenic photosynthetic bacteria, as the name indicates, are incapable of producing molecular dioxygen by oxidizing water and are distinct by having only one reaction center (RC) that resembles photosystem II of green plants.

In anoxygenic, facultative photosynthetic bacteria such as *Rhodobacter sphaeroides*, the presence of light and oxygen induces the development of the machineries for both photosynthetic and respiratory growth (Fig. 10.1). In fact, these bacteria can grow aerobically, anaerobically, or photosynthetically. Depending on growth conditions, respiratory and/or photosynthetic apparatus are selectively produced. Under continuous exposure to light, respiration is slowed or inhibited due to the well-known inhibitory build-up of membrane potential or possibly, by competition between the photosynthetic reaction center (RC) and the cyt c oxidase for reduced cyt c_2 (Oh and Kaplan 2001). Cyt bc_1 lies at the center of the two processes: phototropic and chemotropic electron transfer (ET). The bacterial photosynthetic apparatus consists of membrane-bound light harvesting complexes (LHCs), photosynthetic reaction centers (RCs), cyt bc_1 complexes and the soluble electron carrier cyt c_2. In the presence of oxygen, the machinery for cellular respiration is produced, including NADH and succinate dehydrogenases, cyt bc_1 complex, and various terminal oxidases. Most of the respiratory complexes contribute to the buildup of the pmf (Fig. 10.1).

Under light conditions and in the absence of molecular oxygen, all photosynthetic bacteria rely on a functioning cyt bc_1 complex for growth, making it essential for photosynthetic growth. To ensure an efficient collection of light energy, the purple bacteria usually contain two types of LHC: LH1 and LH2. After light absorption by the LHCs, the excitation energy is transferred to the RC, where a charge separation occurs between donor and acceptor molecules. The photo-oxidized primary electron donor, a dimer of bacteriochlorophyll (Bchl) molecules, is reduced by the high potential electron carrier
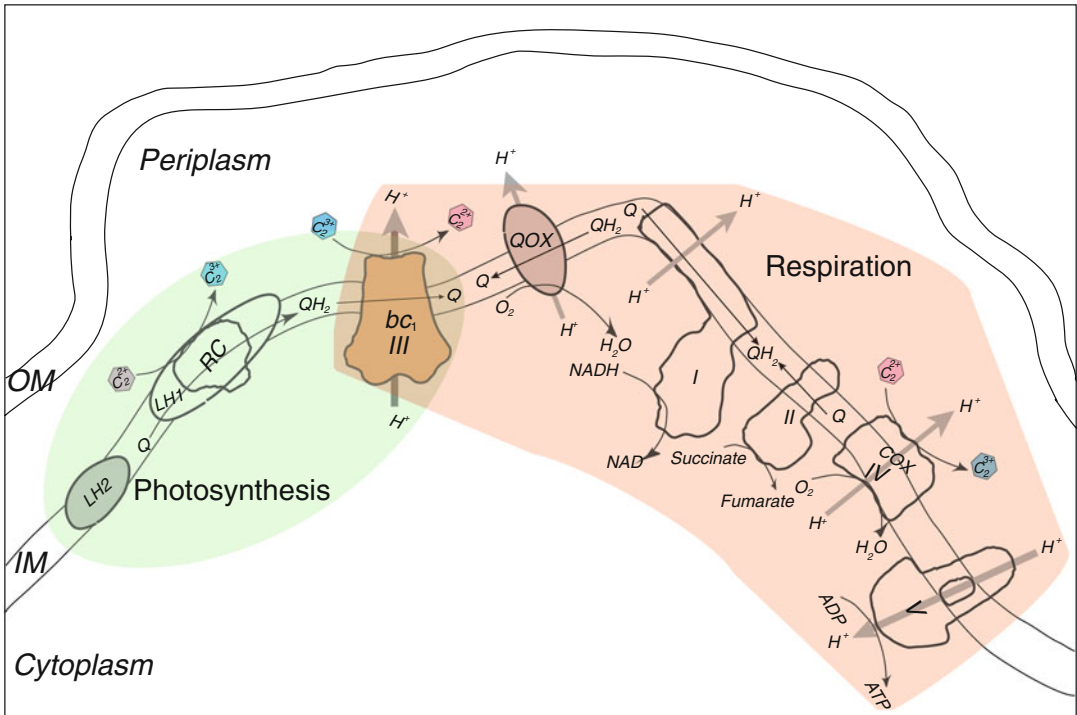
Fig. 10.1. Photosynthetic and respiratory apparatus in *R. sphaeroides.* The two branches of the energy-conserving membrane (chromatophore) are shown. The photosynthetic branch, shaded in *green*, consists of light harvesting complexes (LH1 and LH2), a reaction center (RC), and the cyt bc_1 complex (Complex III or III), where light energy is transduced by RC to reduce quinone (Q) into quinol (QH_2). The cyt bc_1 complex in turn passes electrons from QH_2 to cyt c_2 and couples the electron transfer reaction to protons pumping across the membrane. The respiratory branch, shaded in *red*, is composed of various dehydrogenases including NADH-quinol oxidoreductase (Complex I or I), succinate-quinol oxidoredctase (Complex II or II), and the cyt bc_1 complex, a number of oxidases such as cyt c oxidase (Complex IV, IV or COX) and quinol oxidases (QOX), and ATP synthase (Complex V or V). The cyt bc_1 complex is part of both branches.

cyt c_2, which is in turn reduced by the cyt bc_1 complex (Fig. 10.1). To complete the cyclic electron transfer, the bc_1 complex oxidizes the QH_2 and then reduces mobile cyt c_2 (Vermeglio and Joliot 1999).

When switched from photosynthetic growth to aerobic growth, expressions of the photosynthetic apparatus, as shown by RNA synthesis, are down regulated, while those for aerobic growth are up regulated. Interestingly, many respiratory complexes such as NADH-quinol oxidoreductase, cyt bc_1 complex, as well as a number of cyt c oxidases are also down regulated (Arai et al. 2008), suggesting a complex regulatory mechanism. Nevertheless, under aerobic conditions, electrons flow downhill energetically through various respiratory complexes, ending in molecular oxygen. This ET path is coupled to proton pumping across the membrane, contributing to the pmf.

Under anaerobic or photosynthetic growth conditions, bacteria are sensitive to both bc_1 inhibitors and mutations that render them non-functional. This is in sharp contrast to growth under aerobic conditions where respiration is supported by alternate oxidases in the absence of bc_1. The key difference to the alternate oxidase pathway is that cyt bc_1 couples the oxidation of ubiquinol more efficiently to proton translocation. As it turns out, it is this growth-mode switch feature that renders photosynthetic bacteria useful tools in the study of respiratory components.

B. Enzymatic Functions of Bacterial Cyt bc₁

1. Electron-Transfer-Coupled Proton Translocation

All cyt bc_1 complexes catalyze the same antimycin-sensitive ET reaction from the lipophilic substrate QH₂ to cyt *c* (cyt c_2 in *R. sphaeroides*), coupled with proton translocation across the membrane (Trumpower and Gennis 1994). This reaction contributes to the maintenance of a potential gradient across the cytoplasmic (mitochondrial in eukaryotes) membrane. As a result of this reaction, four protons are deposited on the positive side of the membrane and two molecules of cyt c_2 are reduced for every QH₂ molecule oxidized. This net stoichiometry of the ET reaction, not necessarily reflecting the underlying mechanism, is presented in the following equations, where QH₂ and Q represent lipid-soluble reduced and oxidized ubiquinone, respectively, c^{3+} and c^{2+} represent oxidized and reduced cyt *c*, and H^+_N and H^+_P represent protons on the negative and positive side of membrane.

At the Q_P site : $2QH_2 + 2c^{3+} \xrightarrow{bc_1} 2Q + 2c^{2+} + 4H_P^+ + 2e^-$

At the Q_N site : $Q + 2e^- + 2H_N^+ \xrightarrow{bc_1} QH_2$

The overall reaction : $QH_2 + 2c^{3+} + 2H_N^+ \xrightarrow{bc_1} Q + 2c^{2+} + 4H_P^+$
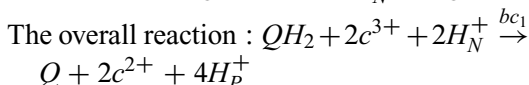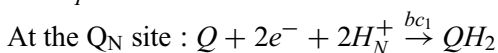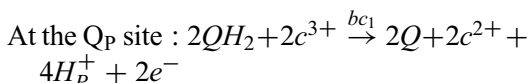
2. Superoxide Generation

Under normal respiration conditions, the ET from QH₂ to cyt *c* catalyzed by the bc_1 complex is accompanied by the production of a small amount of superoxide anions presumably through electron leakage to molecular oxygen, which increases dramatically when the ET within the bc_1 complex is blocked by specific bc_1 inhibitors such as antimycin A or when the ET chain becomes over reduced (Boveris and Chance 1973; Korshunov et al. 1997; Rottenberg et al.

2009). The proposed sites for superoxide anion generation under normal ET conditions are at the Q_P site and the low-potential cyt *b* heme (b_L heme). Mutations in the cyt *b* subunit result in not only impaired ET activity but also increased amounts of superoxide anions. The role played by the small amount of superoxide anions generated under physiological conditions is not clear but they are thought to be part of cellular signaling mechanisms (Muller et al. 2004; Staniek et al. 2002; Yee et al. 2014).

C. Composition of Cyt bc₁ Complexes from Photosynthetic Bacteria and Mitochondria

The cyt bc_1 complexes are multi-subunit, integral membrane protein complexes. Three subunits are essential for the ET-coupled proton translocation function: cyt *b*, cyt c_1, and Rieske iron-sulfur protein (ISP) and they are universally present in all bc_1 complexes. Any additional polypeptide components of a given bc_1 complex are called supernumerary subunits (Trumpower 1990; Yang and Trumpower 1988). Not participating in the catalytic function, these supernumerary subunits are believed to be involved in complex assembly and stability. Mitochondrial bc_1 complexes ($Mtbc_1$) have seven or eight supernumerary subunits, as in yeast or bovine bc_1 (Gao et al. 2002; Hunte et al. 2000), while bacterial bc_1 complexes do not have as many. For example, *R. sphaeroides* bc_1 ($Rsbc_1$) has only one additional subunit, but the closely related *R. capsulatus* ($Rcbc_1$) has none (Berry et al. 2004; Xia et al. 2008).

The cyt *b* subunit of $Mtbc_1$ is the only subunit encoded by the mitochondrial genome. It features two non-covalently bound heme moieties: a low potential (b_L, $E_m = -103$ mV for bovine bc_1 at pH 7.2) and a high potential (b_H, $E_m = + 125$ mV at pH 7.2) heme (Dutton et al. 1970). The cyt c_1 subunit is associated with cyt *b* by a C-terminal
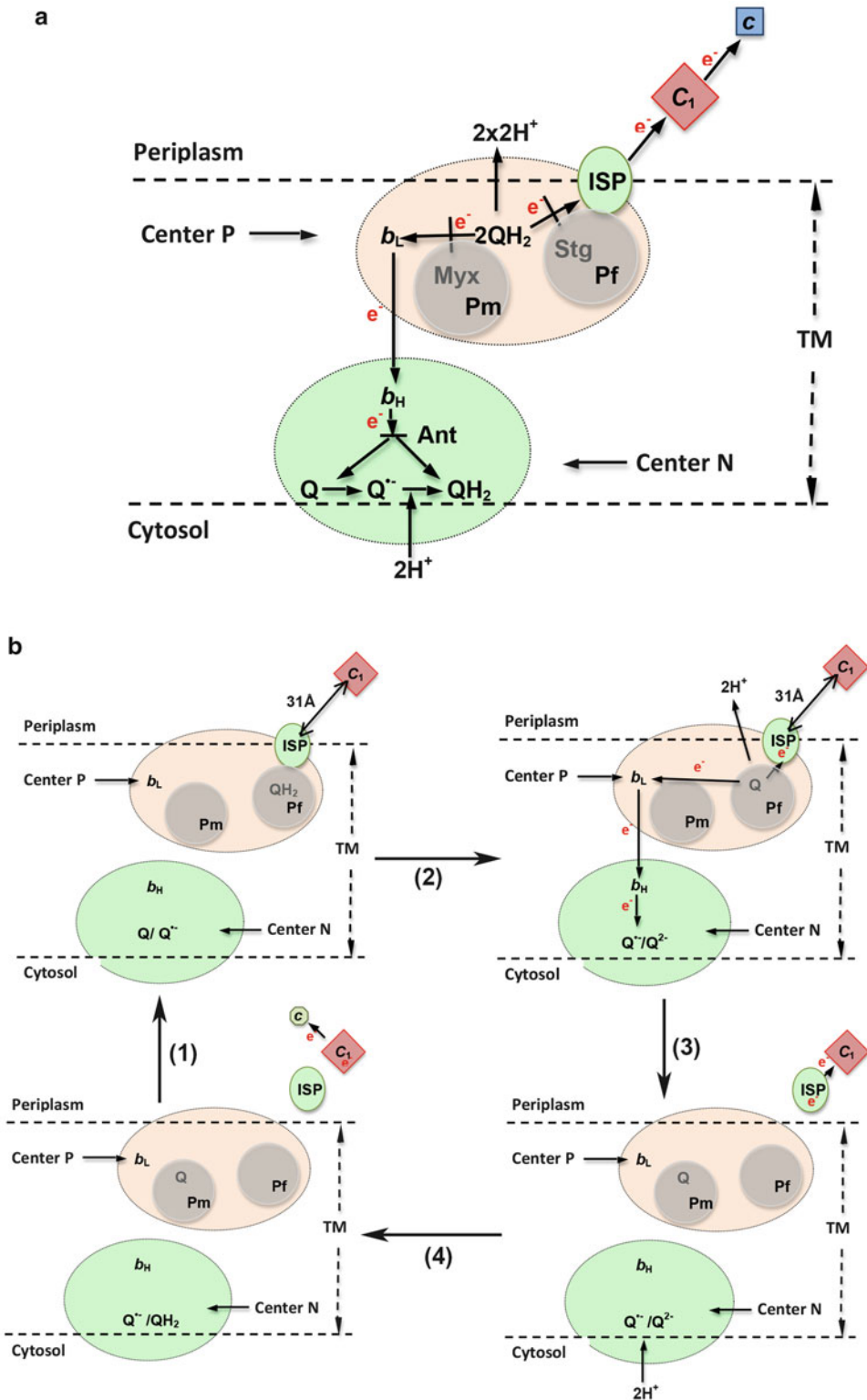
Fig. 10.2. The Q-cycle mechanism and bifurcated ET at the Q_P site. (**a**) The modified Q-cycle defines two active sites within the photosynthetic membrane. The center P (Q_P) is near the periplasm and center N (Q_N)

trans-membrane (TM) helix. Its N-terminal domain, which contains a covalently bound type-*c* heme, resides in the inter-membrane space (IMS) for mitochondrial bc_1 or in the periplasm for bacterial bc_1. Finally, the ISP is bound to the membrane protein via a single N-terminal TM helix. Its functional domain, carrying the 2Fe-2S cluster, is exposed to the aqueous phase and is a neighbor of cyt c_1.

II. The Mechanism of Electron Transfer (ET)-Coupled Proton Translocation Function of Cyt *bc*₁ Complexes

A. The Q-cycle Mechanism

The modified Q-cycle mechanism is based on Mitchell's original work (Mitchell 1972, 1976) and has found general acceptance on the basis of overwhelming biochemical, biophysical and structural evidence (Fig. 10.2a) (Crofts et al. 1983; Garland et al. 1975; Trumpower 1976). According to this mechanism, cyt bc_1, embedded in the lipid membrane bilayer, contains two distinct binding sites for both quinol (Q_P or quinol oxidation) and quinone (Q_N or quinone reduction) near the electrochemically positive and negative ('p' and 'n', respectively) sides of the membrane, respectively. Each site is characterized by the presence of a *b*-type heme (b_L or

b_H) necessary for the obligatory electronic coupling of the oxidation of quinol on the one side to the reduction of quinone on the other. In one complete cycle, two molecules of quinol are oxidized in succession and their four protons are released into the positive side. Simultaneously, one quinone molecule is reduced, requiring the uptake of two protons from the negative side. This means that in one half-cycle, a single electron enters the 2Fe-2S cluster of the ISP and is delivered to the final electron acceptor within bc_1, cyt c_1. At this point, a net imbalance of charge exists, as only one electron from quinol was removed to the p-side aqueous phase, but both of its protons enter the p-side (protons exit bc_1 on the positive side of the membrane). The remaining electron takes a transverse path to the n-side of the membrane through the cyt *b* hemes. It transiently reduces the proximal heme b_L, followed by the remote b_H and ends up on a quinone (Q) or quinone radical (Q•). Protons coming from the n-side aqueous phase of the membrane compensate the sequential arrival of negative charges at the Q_N site (Fig. 10.2a).

B. Proposed Mechanisms of the Bifurcated ET at the Quinol Oxidation Site and Proton Translocation

While the overall outline of the process is well accepted of the bc_1 complex (See article by Hason et al. in this volume for

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Fig. 10.2. (continued) is near the cytosol. In a complete Q cycle, two QH_2 molecules are consumed at the Q_P site and one QH_2 is generated at the Q_N site. The ET reaction at the Q_N site can be blocked by antimycin (Ant). At the Q_P site, the two electrons from a substrate QH_2 diverge, the first going to the high potential chain (ISP, c_1 and c) and the second going to the low potential chain (b_L, b_H and Q/Q•). Reactions at the Q_P site can be inhibited by stigmatellin (stg) or myxothiazol (myx). From crystallographic studies, stigmatellin fixes the conformation of ISP (P_f site), whereas myxothiazol mobilizes the ISP (P_m site). (**b**) The surface-affinity-modulated ISP conformation switch mechanism (SAMICS). The key feature of the mechanism is that when a substrate QH_2 enters the Q_P pocket, it binds to the P_f site first and fixes the conformation of ISP, creating a 31-Å gap between the ISP and cyt c_1. This gap prevents ET between ISP and cyt c_1. ET follows with the first electron entering the ISP, which is a one-electron carrier and remains fixed in place. The second electron moves to the b_L heme. Once the second electron arrives at the Q_N site, the product Q at the Q_P pocket is able to move to the P_m site, which liberates the ISP, allowing it to deliver the first electron to cyt c_1, and subsequently to cyt c. The reduced $Q^{2•}$ at the completion of the Q-cycle picks up two H^+ from the cytosol to form a QH_2.

discussion of Q cycle in b_6f complex), exactly how the separation or bifurcation of electrical charges at the Q_P site as well as how coupled proton flow is accomplished remain the subject of much debate and research. For the electron bifurcation, a number of models with varying degrees of acceptance and refinement have been proposed: (1) catalytic switch model (Brandt and von Jagow 1991), (2) double-occupancy Q_P site model (Ding et al. 1992), (3) proton-gated charge-transfer mechanism (Brandt 1996), (4) logic-gated fit mechanism (Rich 2004), (5) sequential model (Cape et al. 2007; Crofts and Wang 1989), (6) concerted model (Crofts et al. 1983; Zhu et al. 2007), (7) R-complex model (Berry and Huang 2003) and (8) surface-affinity modulated ISP conformation switch (SAMICS) (Fig. 10.2b) (Esser et al. 2006, 2008b; Xia et al. 2007, 2013).

Proton transport from the n-side of the membrane (matrix or cytosol) to the positively charged periplasm (IMS in mitochondria) is achieved within bc_1 with a stoichiometry of two protons per electron transferred. The two protons translocated are mechanistically non-identical. One is called 'scalar' and the other is 'vectorial' (Guerrieri and Nelson 1975; Leung and Hinkle 1975), reflecting the exit of protons on the positive side and uptake from the negative side, respectively. The proton exit must be coupled with ET, as cyt bc_1 activity is pH dependent with a maximum activity at pH 8.0 under steady state conditions for $Mtbc_1$ (Hsueh et al. 2010; Lin et al. 2006; Link et al. 1992; Prince and Dutton 1976; Ugulava and Crofts 1998). Two protonatable histidine residues bound to the 2Fe-2S cluster with pK 6.6 and 9.2, respectively, have been identified as responsible for the pH dependency (Brandt and Okun 1997). Not surprisingly, various proposals exist on how such coupling might take place. Similarly, a number of mechanisms for proton uptake from the negative side of the membrane have been proposed (Gao et al. 2003; Lange et al. 2001).

III. Comparison of Crystal Structures Between Bacterial and Mitochondrial Cyt bc_1 Complexes

A. Structural Studies of Cyt bc₁ Complexes

Prior to the availability of high-resolution crystal structures, mitochondrial cyt bc_1 complexes were studied by 2-D electron microscopy, which revealed the dimeric nature of the complexes (Akiba et al. 1996; Leonard et al. 1981). Crystal structures of mitochondrial cyt bc_1 complexes from bovine, chicken, and yeast revealed the subunit arrangement, the locations of the prosthetic groups, namely the hemes b_L, b_H, and c_1, the 2Fe-2S cluster and bound substrate ubiquinone. They also clearly delineated the locations for ubiquinol oxidation (Q_P) and ubiquinone reduction (Q_N) (Hunte et al. 2000; Iwata et al. 1998; Kim et al. 1998; Xia et al. 1997; Zhang et al. 1998b) (Fig. 10.3). These studies were followed by structure determinations of the related cyt b_6f complexes from the cyanobacteria *Mastigocladus laminosus* (Baniulis et al. 2009; Hasan et al. 2013; Kurisu et al. 2003; Yamashita et al. 2007) and from alga *Chlamydomonas reinhardtii* (Stroebel et al. 2003), revealing structural features unique to the b_6f complexes, most notably the presence of heme c_n at the Q_N site (Nogueira et al. 2013). More recently, the structures of cyt bc_1 from photosynthetic purple bacteria *R. capsulatus* ($Rcbc_1$) (Berry et al. 2004) and *R. sphaeroides* ($Rsbc_1$) (Esser et al. 2006, 2008a) as well as the soil bacterium *Paracoccus denitrificans* ($Pdbc_1$) (Kleinschroth et al. 2011) were reported (Table 10.1).

The bacterial forms of bc_1 from α-proteobacteria $Rcbc_1$ and $Rsbc_1$ have a long history of being model systems for the study of functional aspects of the enzyme, as they are more amenable to genetic manipulations than their mitochondrial counterparts. Because they are part of
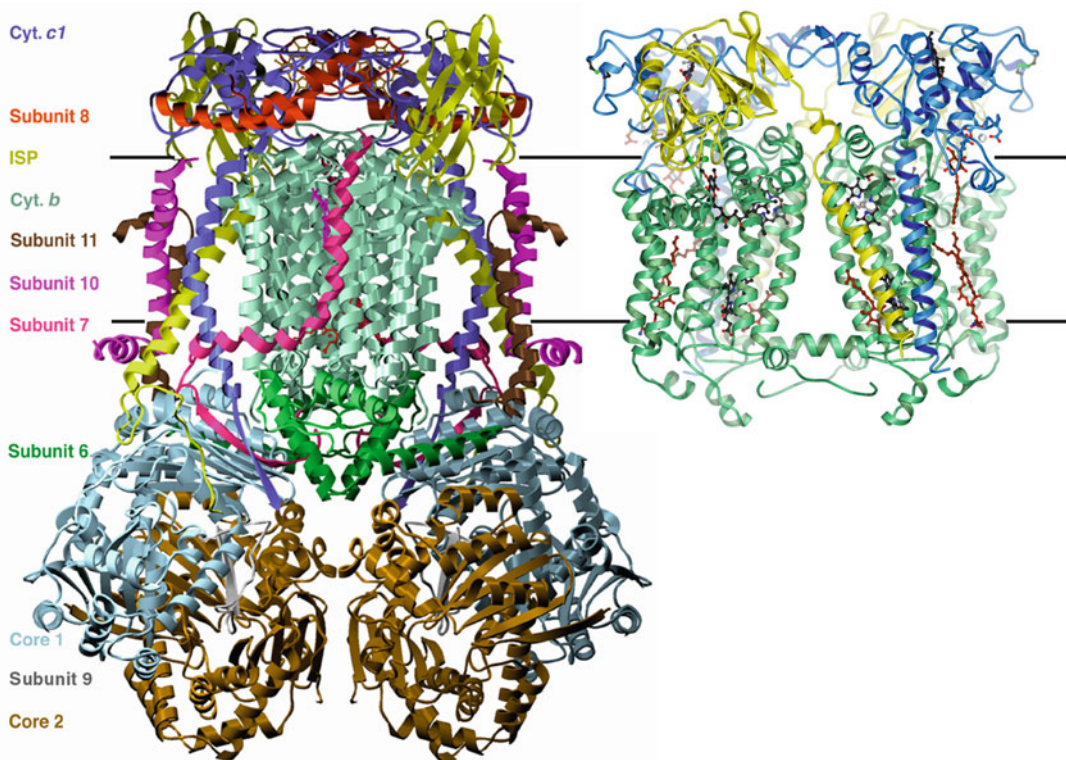
Fig. 10.3. Structures of cyt bc_1 from bovine mitochondria and photosynthetic bacterium *R. sphaeroides.* Dimeric mitochondrial bc_1 is shown on the *left* with labels for each individual subunit and bacterial bc_1 is shown on the *right*.

the photosynthetic apparatus (Fig. 10.1), the bacterial cyt bc_1 complexes can be studied less invasively in their native lipid environment (Mulkidjanian 2007). Even more effective using the bacterial bc_1 are the functional studies analyzing spontaneous revertants in response to point mutations introduced to impair or influence the function of bc_1 or the action of a bc_1 (Q_P or Q_N site) inhibitor. Furthermore, the relatively close genetic relationship of *Rhodobacterales* to *Rickettsiales* (Andersson et al. 1998), which are the precursors of mitochondria, allows one to extend mechanistic conclusions obtained from bacterial bc_1 to its mitochondrial analogues (Crofts and Wang 1989). Solved crystal structures of native, and if possible, mutant forms would help to ascertain that biochemically obtained results from mutational studies are based on the expected local changes and are not due to

unintended changes to the overall structure. This can be achieved much more easily in bacterial bc_1 than in their mitochondrial counterparts. Since the mitochondrion and with it the bc_1 enzyme arose from bacteria that underwent endosymbiotic incorporation into eukaryotic cells, a comparative analysis between evolutionarily derived and modified forms of bc_1 should shed significant light on the function of supernumerary subunits as well as the process of evolution in general.

Because of the advantages of bacterial cyt bc_1 complexes with respect to their structural simplicity, low cost and ease of use in genetic manipulations, high-resolution structures of bacterial enzymes were sought for many years in laboratories worldwide. A decade ago in 2004, the crystal structure of the bc_1 complex from *R. capsulatus* ($Rcbc_1$) (Berry et al. 2004) was reported at 3.8 Å resolution (Table 10.1). This was the first bc_1 structure

Table 10.1. Available crystal structures of cyt bc_1 from prokaryotic organisms.

Pdb ID	Species	Mutation	Q_P occupant	Q_N occupant	Res. (Å)	References
1ZRT	*R. capsulatus*	wt	Stigmatellin	None	3.8	Berry et al. (2004)
2FYN	*R. sphaeroides*	[b]S287R/[ISP]V135S	Stigmatellin	None	3.2	Esser et al. (2006)
2QJP	*R. sphaeroides*	wt	Stigmatellin	Antimycin A	2.6	Esser et al. (2008a)
2QJK	*R. sphaeroides*	[b]S287R/[ISP]V135S	Stigmatellin	Antimycin A	3.1	Esser et al. (2008a)
2QJY	*R. sphaeroides*	[b]S287R/[ISP]V135S	Stigmatellin	Ubiquinone	2.4	Esser et al. (2008a)
2YIU	*P. denitrificans*	wt	Stigmatellin	None	2.7	Kleinschroth et al. (2011)

from a prokaryotic organism and confirmed the overall similarity to mitochondrial bc_1; however, it lacked sufficient detail to reveal reliably the unique structural features of the bacterial form. Higher resolution structures of *R. sphaeroides* (*Rsbc_1*) could initially only be obtained when the stabilizing double mutations, S287R (cyt *b*)/V135S (ISP), were introduced. Crystallization succeeded in the presence of stigmatellin, which has been known to immobilize the extrinsic domain of the ISP subunit (Esser et al. 2006; Xia et al. 2008) (Fig. 10.3, Table 10.1). Crystal structures of wild-type and mutant *Rsbc_1* in complex with inhibitors and substrates were subsequently determined up to 2.4 Å resolution (Esser et al. 2008a) (Table 10.1). The purified *Rsbc_1* was crystallized in a number of different crystal forms and required the presence of histidine for stability of the dimer and monodispersity of the protein sample in detergent solutions (Xia et al. 2008).

B. Structural Differences between Mitochondrial and Bacterial bc₁

Although all bc_1 complexes catalyze the same reaction, conservation of protein sequences is usually limited to the active sites across different organisms, making it difficult to correlate the sequence differences with functional variations. The availability of a large number of bc_1 structures from different species allows one to perform structure-based sequence alignments. These alignments coupled to the large number of available sequences (for example, >10,000 for cyt *b*) from all kingdoms of life reveal a wealth of information encompassing commonalities, strict conservation, and the correct identification of insertions and corresponding deletions that are organism-specific environmental or functional adaptations.

To compare bacterial with mitochondrial cyt bc_1, it is necessary to find a consensus on available bacterial structures. While the two high-resolution structures of *Rsbc_1* and *Pdbc_1*, determined at 2.4 and 2.7 Å Bragg spacing, respectively, agree well with each other, the same cannot be said concerning the structure of *Rcbc_1*. In the latter case, unfortunately due to diffraction limitations, the structural features that are unique to this and many other prokaryotic enzymes could only be modeled as polyalanine segments (PDB: 1ZRT). A structure of *Rcbc_1* at higher resolution has not since been reported. In the following sections, we will use *Rsbc_1* and *Pdbc_1* as representatives of bacterial bc_1 for comparison with mitochondrial complexes.

1. Structural Differences Observed in the Cyt b Subunit

At first glance, a major difference between bacterial and mitochondrial bc_1 complexes is the lack of supernumerary subunits on the cytoplasmic side of the membrane in bacterial bc_1 (Fig. 10.3). A proposed function for these additional subunits is to shield the portion of the enzyme complex, which is embedded in the membrane bilayer against proton and or water leakage. Supporting this hypothesis is the fact that the sequence of *Rs* cyt *b* is 66 residues (≥15 %) longer than the bovine sequence (Fig. 10.4a). Furthermore,
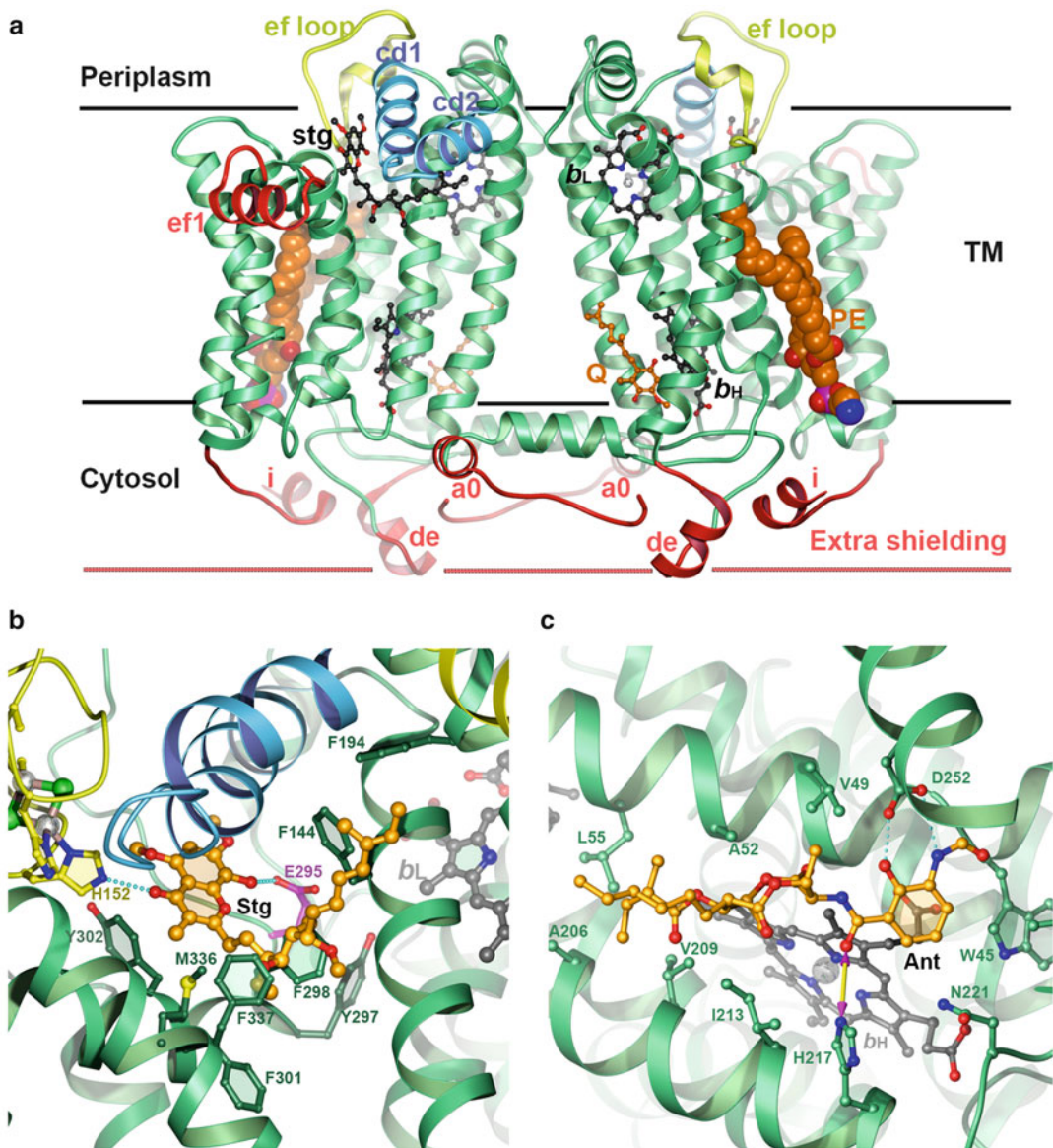
Fig. 10.4. Structures of bacterial cyt *b* and its inhibitor complexes. (**a**) Ribbon diagram of the dimeric cyt *b* of *Rsbc$_1$* is given. The insertions unique to the bacterial cyt *b* are highlighted in *red* and labeled, most of which are on the cytosolic side of the membrane, creating an extra layer of shielding. The cd1 and cd2 helices are shown in *blue*. The *yellow* elements belong to the ef loop. The heme moieties and bound Q$_P$-site stigmatellin are shown in stick models with carbon in *black*, oxygen *red* and nitrogen *blue*. The substrate ubiquinone bound to the Q$_N$-site is also shown as the stick model with carbon in *gold*. The bound lipid molecule is rendered as ball model with carbon also in *gold* color. (**b**) Close-up view of the quinol oxidation site with bound stigmatellin in the cyt *b* subunit of *Rsbc$_1$*. Structural elements from the cyt *b* subunit are colored *green* except for cd1 and cd2 helices, which are in *blue*. Structural elements from ISP are colored *yellow*. Residues contributing to the Q$_P$ environment are labeled and shown as the ball-and-stick models. (**c**) Close-up view of the antimycin A-binding site in the cyt *b* subunit of *Rsbc$_1$*.

the insertions and extensions are concentrated on the negative side of the membrane, suggesting that their function(s) were eventually taken over by the two large core subunits in $Mtbc_1$. In fact, bacterial cyt b has significant extensions at both its N-terminus (see helix a0) and at its C-terminus (helix i). In the crystal structures of all bacterial forms, the first ordered residue (as judged by electron density) for cyt b starts at position 3 and ends at residue 430. This suggests that the 15 remaining C-terminal residues are highly flexible and can in fact be deleted without affecting the function of the enzyme in *R. sphaeroides* (Liu et al. 2004). The extra helices a0, de and i enable cyt b to interact with lipid molecules molded as PE, as shown in Fig. 10.4a, apparently creating additional shielding for enhanced membrane impermeability.

Located near the positive side of the membrane, the insertion of 18 residues (310–327) provides an additional structural element in *R. sphaeroides* and *R. capsulatus* denoted as the ef1 helix (Fig. 10.4a). As seen in the high-resolution crystal structures of $Rsbc_1$ the N-terminal side of the ef1 helix interacts with lipid-like molecules (as deduced from the residual density that is not shown in Fig. 10.4a), which so far defied attempts to model them as lipids or possibly detergent molecules. However, it has been shown that deletion of residues 309–326 (ef1 helix) or as little as the point mutation S322A significantly impairs the function of bc_1 (Esser et al. 2006). Since mitochondrial cyt b does not have the ef1 helix, its direct involvement in catalysis or structural integrity can be ruled out. This protruding element may be needed to interact with membrane lipids, providing help to orient the complex in the membrane environment, as seen in Fig. 10.4a.

2. Structural Differences Observed in Cyt c_1

Among the three bacterial species with known 3-D coordinates, the sequence identities range from 59 % to 63 %. Comparing mitochondrial cyt c_1 sequences, one finds approximately the same degree of conservation (58 %). However, the sequence identity drops to 33–40 % between bacteria and mitochondria. Most notably, the bacterial cyt c_1 sequence suffers a loss of two major structural elements and a gain of two smaller insertions (Fig. 10.5a). In the following sections, *R. sphaeroides* sequence numbers are used, since the cyt c_1 structure of $Rsbc_1$ shows the highest degree of completeness and therefore lends itself most conveniently as the reference. First, the $C^{36}XXC^{39}H^{40}$ signature motif for c-type heme groups is perfectly conserved including the remote M185, which forms together with H40 the two axial ligands to the heme iron. R107 is also conserved; it forms a salt bridge with one of the propionate groups of the heme and is located on the surface of cyt c_1 that interacts transiently with the moving extrinsic domain of ISP (ISP-ED). A superposition of coordinates (Cα positions) reveals a very high degree of preservation of structural elements, and in particular the position of the important heme redox group as well as the C-terminal TM helix. Two insertions have occurred in mitochondrial cyt c_1 after position 76 and 94 (bovine sequence), both of which contribute to a closing-up of cyt c_1 including the establishment of a direct bridge between the two cyt c_1 units of the homo-dimeric bc_1 complex (Fig. 10.5b).

A prominent loop is inserted into the sequences of *R. sphaeroides* and *R. capsulatus* containing a stabilizing disulfide bridge C^{145}-C^{169}. Remarkably, the disulfide bridge is absent in *P. denitrificans* (Fig. 10.5a), which features instead a highly charged foreshortened loop. In fact, *P. denitrificans* cyt c_1 resembles mitochondrial cyt c_1 more closely with respect to the "missing" disulfide-linked loops. Mitochondrial cyt c_1, however, recruits subunits 7 and 8 to gain stability. Subunit 8 is a disulfide-linked helical hairpin guarding the perimeter of cyt c_1, which suggests a compensatory function for the loss of secondary structure in cyt c_1. Another highlighted feature of bacterial cyt c_1 is the largely helical insertion between
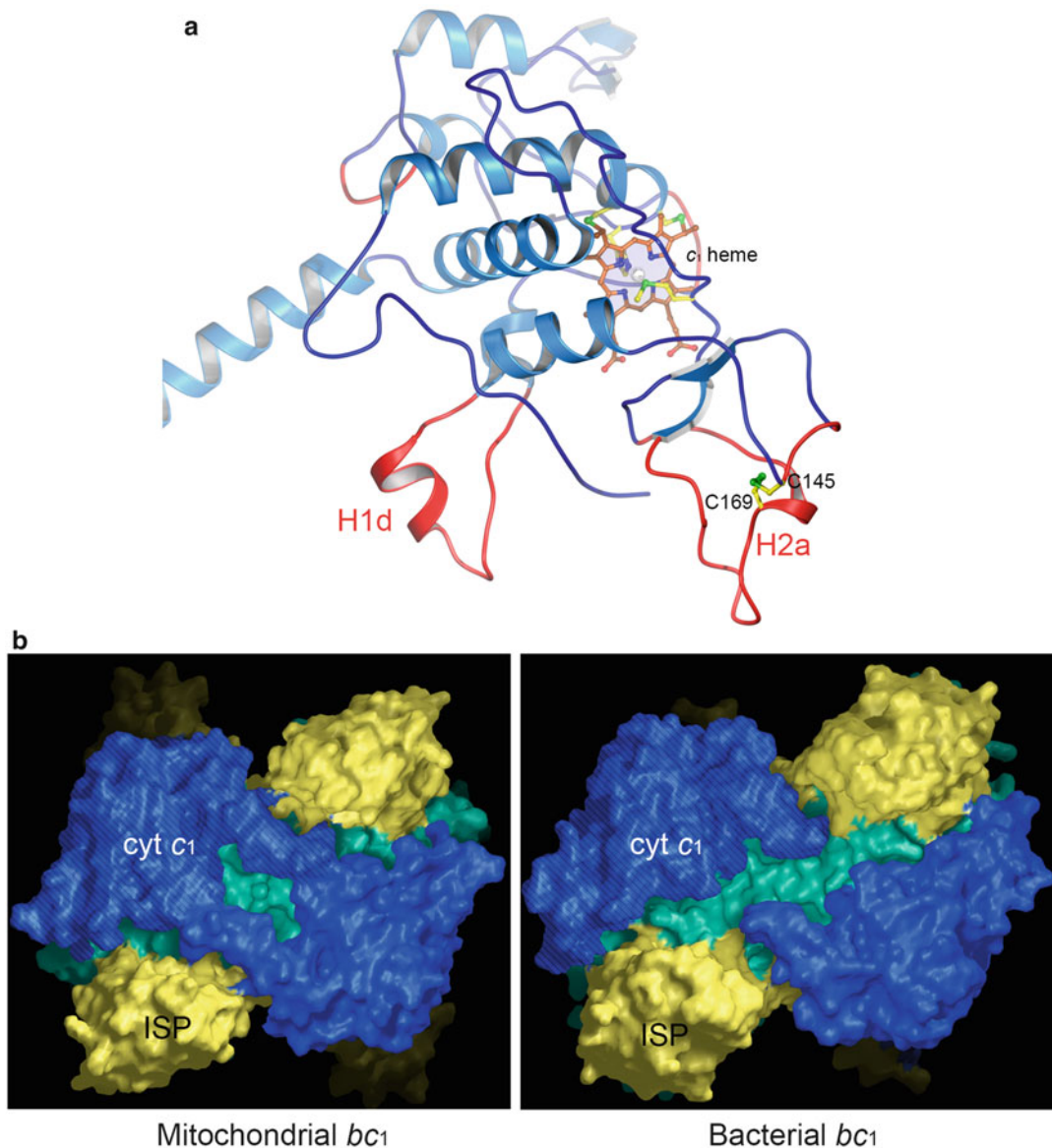
Fig. 10.5. Structural comparison of cyt c_1 between $Rsbc_1$ and $Btbc_1$. (**a**) Ribbon diagram of cyt c_1 of $Rsbc_1$ is shown with insertions highlighted in *red* and labeled. The C145 and C169 residues that form a disulfide bond are shown as stick models. The heme c_1 is also labeled. (**b**) Comparison of mitochondrial and bacterial cyt c_1 in the context of dimeric bc_1 showing the presence and absence of bridging, respectively, in the two symmetrically related structures of cyt c_1 (mitochondrial and bacterial).

residues 107 and 125 (Fig. 10.5a). This element, designated h1d helix, has been deleted from mitochondrial forms of cyt c_1 without any discernable compensation. The high degree of flexibility of this loop rendered it "invisible" in the structures of

R. capsulatus and *P. denitrificans* cyt c_1 but could be modeled with high temperature factors in $Rsbc_1$. Here the lateral displacement of the helix away from the globular cyt c_1 core revealed a cavity that appears to provide space for a lipid head group of

a phosphatidylethanolamine (PE). However, the high degree of disorder in this region prevented it from being included in the final model. While the exact nature of the function of this loop awaits further investigation, it may be another demonstration that the three core subunits of bacterial bc_1 not only fulfill their redox catalytic functions but also deal with proper partitioning of the complex into lipids, a task that appears to be shared to a large degree with supernumerary subunits in mitochondria.

A strontium ion (Sr^{2+}) from the crystallization additive has been shown to bind to the side chains of D8, E14 and E129 and the backbone carbonyl oxygen atom of V9 in *R. sphaeroides* cyt c_1. As the crystallization conditions of $Rcbc_1$ and $Pdbc_1$ did not contain equivalent metal ions, their respective, but otherwise perfectly conserved, residues remain free. While the Sr^{2+} ion is most likely not a physiological substrate, it was shown that the presence of Ca^{2+} ions can prevent the binding of subunit IV of $Rsbc_1$, possibly through blocking the acidic cluster of residues meant to bind a basic portion of $Rsbc_1$'s only supernumerary unit. The purpose of this site in $Rcbc_1$ and $Pdbc_1$, which are not known to require an additional subunit, remains unclear. In mitochondrial bc_1 this site is structurally but not electronically maintained, as the amino acids D8 and E14 have been replaced by neutral, polar or even basic ones. Only vestiges of the formerly acidic site remain. Residue E129 was replaced by D125 (bovine) or D189 (yeast), forming hydrogen bonds to the indole group of W12 (or W76), which replaced F11 in bacterial sequences.

3. Structural Differences Observed in the Iron-Sulfur-Protein Subunit

The ISP is anchored to the membrane via its N-terminal TM helix. The TM helix is connected to the extrinsic domain of ISP (ISP-ED) by a flexible linker, which enables ISP-ED to relay the electron from the QP site to cyt c_1. The ISP is well conserved among species but structure-based sequence
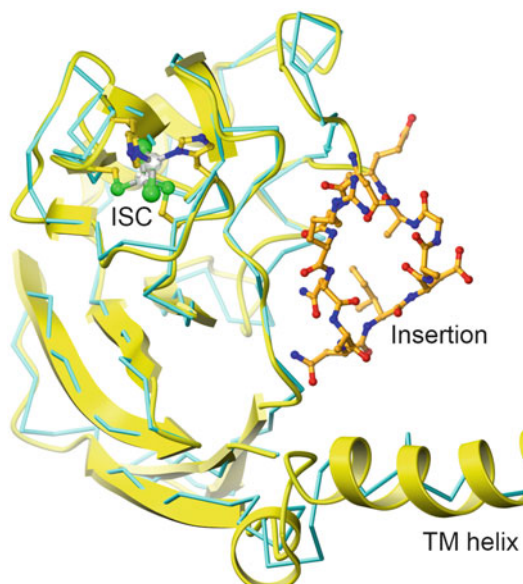
Fig. 10.6. Structure of bacterial ISP and comparison to its mitochondrial counterpart. The model of bacterial ISP is shown in a *yellow* ribbon diagram. Superimposed is a Cα-trace of the bovine ISP in *cyan*. Unique to the bacterial ISP is the globular-shaped insertion, which is illustrated as a ball-and-stick model.

alignments highlight one ∼12-residue long globular insertion in the bacterial ISP featuring three β-turns and an inverse γ-turn (Fig. 10.6). Despite the remoteness of this element from the iron-sulfur cluster (20–25 Å), an interruption in its hydrogen bonding network by more than one point mutation results in a failure to integrate this subunit into bc_1 (Xiao et al. 2004).

IV. Structural Studies of Cyt *bc*$_1$ with Bound Inhibitors

To gain a survival advantage, many bacterial and fungal species developed the ability to produce quinol or quinone binding site inhibitors, some of which are close chemical analogues of the substrates as well (Esser et al. 2014). Inhibitors targeting bc_1 have helped unravel the mechanism of electron flow including the elucidation of the phenomenon of oxidant-induced cyt *b* reduction in the presence of antimycin (Slater 1973;

Wikstrom and Berden 1972). Because complex III is not only present in a large number of diverse organisms but also structurally well conserved, cyt bc_1 complexes have been selected as the targets of modern pesticides and specific antibiotics in agriculture and medicine (Bueno et al. 2012; Esser et al. 2014).

A. Crystal Structures of Cyt bc₁ in Complex with Inhibitors

In contrast to the many published mitochondrial bc_1 structures from beef, chicken and yeast with a good cross-section of bound Q_P- (Berry and Huang 2011; Gao et al. 2002; Xia et al. 1997) and Q_N–site (Gao et al. 2003; Kim et al. 1998) inhibitors, crystal structures of bacterial complexes are still rare. At the Q_P site, the only inhibitor bound to bacterial bc_1 in all published structures is stigmatellin (Fig. 10.4b) (Berry et al. 2004; Esser et al. 2008a; Kleinschroth et al. 2011). This is not surprising, since crystallization depends on conformational stability or overall rigidity of the protein and stigmatellin effectively arrests the motion of ISP-ED. Serendipitously, many mitochondrial bc_1 crystals have insulated the IMS domains of cyt c_1 and ISP from crystal contacts by providing interactions exclusively from several supernumerary subunits. As far as Q_N site inhibitors are concerned, so far, only one crystal structure of wild-type $Rsbc_1$ with the stigmatellin at Q_P and antimycin at Q_N has been published (Fig. 10.4c) (Esser et al. 2008a) (Table 10.1). Despite the small number of inhibitor-complexed structures for bacterial bc_1, conclusions obtained from those studies using mitochondrial bc_1 are applicable to the bacterial enzymes.

B. Inhibitor Binding-Induced Conformation Switch in ISP-ED

The binding of inhibitors has a profound effect on the spectra of *b*-type hemes and on the redox potential of ISP (Link et al. 1993; von Jagow and Link 1986). Inhibitor binding was also recognized to be a function of the redox state of the enzyme (Brandt and von Jagow 1991). However, these phenomena were difficult to explain in the absence of structural information. In 1998, less than a year after the first bc_1 structure was published, Kim et al. reported crystallographic observations demonstrating the conformational switch of ISP-ED in response to binding of different types of bc_1 inhibitors to the Q_P site (Kim et al. 1998). One type of inhibitors, including stigmatellin, UHDBT, and famoxadone, led to fixation of ISP-ED conformation at the Q_P site of cyt *b* (*b*-site) and another type, including myxothiazol, azoxystrobin, and MOA stilbene, mobilized ISP-ED (Table 10.2). These two types of inhibitors were named P_f- and P_m-type inhibitors, respectively, according to their ability to fix or mobilize ISP-ED (Esser et al. 2004). High-resolution crystallographic data also showed that these two types of inhibitors bind to two different sites, the P_f- or P_m-sites, within the Q_P pocket (Fig. 10.2a), driving movement of the highly conserved cd1 helix in a bi-directional fashion (Esser et al. 2006) (Fig. 10.2a). Since the cd1 helix forms part of the ISP-ED binding surface, this bi-directional movement was hypothesized to modulate the binding affinity of ISP-ED, leading to a conformation switch (Esser et al. 2006; Xia et al. 2013).

C. Structural Basis of the Mechanism of Electron Bifurcation at the Q_P Site: The Surface-Affinity-Modulated ISP-ED Conformation Switch Mechanism

Elucidating the mechanism that enforces bifurcation of the electrons in an energy conserving process of bc_1 was not expected to be trivial, as evidenced by many proposed mechanisms (see Sect. II.B). In the first publication on the crystal structures of bovine bc_1 (Xia et al. 1997), the ISP-ED was found to be disordered in both native and inhibitor-bound (antimycin A or myxothiazol) structures, demonstrating its mobility. However, the 2Fe-2S cluster of ISP-ED left an unmistakable signal in the anomalous

Table 10.2. Conformation switch of the ISP-ED in the presence of different types of inhibitors.

Ligand at Q_P site	Space group	Inhibitor type	Normalized ISC anomalous peak	ISC-cyt c_1 distance (Å)	Estimated ET rate (s^{-1})	References
None	$I4_122$	–	0.45	27.7	2.1×10^{-5}	Kim et al. (1998)
Azoxystrobin	$I4_122$	Pm	0.36	27.7	2.1×10^{-5}	Esser et al. (2006)
MOAS	$I4_122$	Pm	0.23	27.7	2.1×10^{-5}	Esser et al. (2006)
Famoxadone	$I4_122$	Pf	1.02	27.7	2.1×10^{-5}	Esser et al. (2006)
Stigmatellin	$I4_122$	Pf	1.20	27.7	2.1×10^{-5}	Esser et al. (2006)
UHDBT	$I4_122$	Pf	0.96	27.7	2.1×10^{-5}	Esser et al. (2006)
None	$P6_5$	–	–	23.6	0.007	Iwata et al. (1998)
None	$P6_522$	–	–	7.8	2.9×10^7	Iwata et al. (1998)

difference Fourier map, placing it very far ($\sim$31 Å) away from the cyt c_1 heme iron. This distance is incompatible with the observed turnover rate of bc_1 reaction, as it would prohibit efficient ET between ISP and cyt c_1. Subsequent crystal structures showed that ISP-ED can be in different locations in different crystal forms (Table 10.2), leading to the proposal that ET between ISP and cyt c_1 can be accomplished by domain movement (Iwata et al. 1998; Zhang et al. 1998b). The discovery of the motion of ISP-ED through crystal structure analyses of mitochondrial bc_1 solved only part of the problem of electron bifurcation in that it enabled an understanding of the mechanism that relays electrons from the quinol oxidation site to the remote cyt c_1 electron acceptor. The mobility of ISP-ED also complicated the system by allowing the head domain of ISP to become a second substrate, binding adjacent to ubiquinol in the enzyme-substrate complex. Furthermore, despite much effort, the reaction product bound in an enzyme-product complex at the Q_P site has not been identified by crystallographic methods; the best approximations are complexes with inhibitors that closely resemble quinone.

The essence of the operation of bc_1 is separation of the electrons from substrate quinol, which is initiated after the binding of the substrate and the docking of the mobile ISP-ED at the Q_P site of cyt b, bringing the high-potential 2Fe-S2 cluster into close proximity to the substrate. Since direct structural details on several critical waypoints along the reaction coordinate are not yet available, researchers remain cautious not to over-interpret observations including thermodynamic data that would (at times strongly) suggest a certain sequence of events. Nevertheless, the experimental observations of ISP-ED conformation switch in the presence of two different types of inhibitors suggested a mechanism for the bifurcated ET at the Q_P site. As shown in Fig. 10.2b, ISP-ED must be in a fixed conformation when substrate QH_2 enters the Q_P pocket, occupying first the P_f-site. It remains fixed in place during the first electron transfer to the one-electron carrier ISP. At the same time, the two protons transfer to their respective receptors, the glutamate of the PEWY motif and a histidine ligand to 2Fe-2S. Because of the 31 Å separation between the 2Fe-2S and heme c_1, ET between these two redox partners will not occur for as long as the reduced ISP-ED remains fixed at this position. The resulting highly unstable Q$\bullet$ radical, is however electronically coupled with hemes b_L and b_H, allowing the second electron to reduce the Q_N site occupant. Once the 2nd electron reaches the Q_N site, the now fully oxidized quinone substrate Q moves to the P_m-site, releasing ISP-ED from the b-site to deliver the first electron to cyt c_1. This surface-affinity modulated ISP-ED conformation switch mechanism or SAMICS can be summarized with the following formulae in six steps:

Step 1: cyt b, QH$_2$ and ISP form a complex

$$b\,(H-L) + \ddot{Q}_P H_2^P + ISP \rightarrow b\,(H-L)$$
$$= \ddot{Q}_P H_2^P = ISP$$
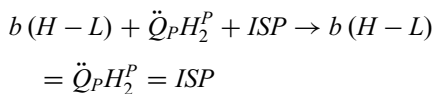
Step 2: QH$_2$ transfers a proton to ISP

$$b\,(H-L) + \ddot{Q}_P H_2^P + ISP \rightarrow b\,(H-L)$$
$$= \ddot{Q}_P H = ISP \bullet H^+$$
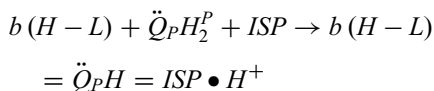
Step 3: QH transfers the first electron to ISP and another proton to cyt b

$$b\,(H-L) + \ddot{Q}_P H = ISP \bullet H^+$$
$$\rightarrow b\,(H-L) \bullet H = \dot{Q}_P H = \dot{ISP} \bullet H^+$$
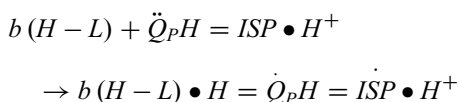
Step 4: Q radical transfers the remaining electron to b_L heme of cyt b

$$b\,(H-L) \bullet H = \dot{Q}_P = \dot{ISP} \bullet H^+$$
$$\rightarrow b\left(H - \overset{\circ}{L}\right) \bullet H^+ = Q_P = \dot{ISP} \bullet H^+$$
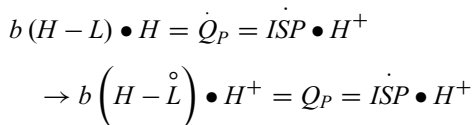
Step 5: b_L heme delivers the electron to b_H heme

$$b\left(H - \overset{\circ}{L}\right) \bullet H^+ = Q_P = \dot{ISP} \bullet H^+$$
$$\rightarrow b\left(\overset{\circ}{H} - L\right) \bullet H^+ = Q_P = \dot{ISP} \bullet H^+$$
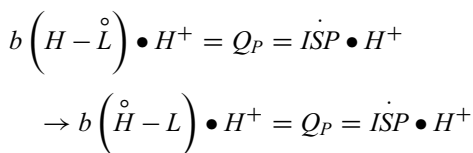
Step 6: ISP and Q dissociate from cyt b

$$b\left(\overset{\circ}{H} - L\right) \bullet H^+ = Q_P = \dot{ISP} \bullet H^+$$
$$\rightarrow b\left(\overset{\circ}{H} - L\right) + 2H^+ + Q_P + \dot{ISP}$$
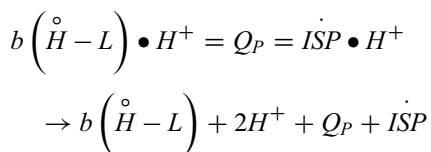
This hypothesis is consistent with a large body of biochemical, biophysical and genetic evidence: (1) it provides a functional explanation for the extraordinary sequence conservation of the cd1 helix. (2) A logical consequence of this hypothesis is that the reduction of b hemes precedes that of cyt c_1, as shown by the pre-steady state kinetic analysis (Bowyer and Crofts 1981; Gopta et al. 1998; Hansen et al. 2000; Zhu et al. 2007). (3) The strict inhibition of bc_1 by antimycin A, which binds exclusively to the remote Q$_N$ site (Gao et al. 2003), can be readily explained by SAMICS, whereas an unregulated, mobile ISP-ED would be incompatible with the observed antimycin inhibition.

V. Experimental Verification of the Bifurcated ET Mechanism

As mentioned above, cyt bc_1 is required for bacteria under photosynthetic growth conditions (Fig. 10.1). During the photosynthetic energy conversion process, electrons are shuttled back and forth between RCs and cyt bc_1 by cyt c_2 and quinol/quinone. Importantly, there is no exogenous electron donor or final acceptor in this cyclic ET pathway and light energy is converted to the pmf for ATP synthesis. However, the quinol oxidation activity catalyzed by bc_1 can be bypassed when photosynthetic bacteria are grown under aerobic conditions, which is an important feature that allows an impaired or defective bc_1 to be experimentally examined (Gennis et al. 1993; Thony-Meyer 1997).

In *R. sphaeroides*, the *fbc*FBC operon encoding the three core subunits of the cyt bc_1 complex is only 3.3 kb (Yun et al. 1990). A separate gene *fbc*Q encoding the subunit IV is approximately 278 kb away from the *fbc*FBC operon. Thus, the *R. sphaeroides* strain BC17 that has the *fbc*FBC and *fbc*Q operons removed from the chromosome cannot grow photosynthetically. It must grow aerobically. A bc_1 expression system that affords easy manipulation was created by inserting *fbc*FBC and *fbc*Q genes into the low copy number expression vector pRKD418 (Mather et al. 1995) and transforming it into BC17 (Yun et al. 1990). This system was further modified by inserting a hexahistidine tag into the C-terminus of cyt c_1 (Tian et al. 1998), permitting affinity purification of the bc_1 complexes from *R. sphaeroides* cells. A similar system that was developed for cyt bc_1

A. Mutagenesis in Cyt bc$_1$ Supports the Functional Importance of ISP-ED Movement

Mutations were introduced into cyt b and ISP of $Rsbc_1$, $Rcbc_1$ and also into $Scbc_1$ to determine whether the mobility of ISP-ED is a critical and vital aspect of the bc_1 function. The matter of a mobile ISP-ED arose immediately from the first structure determination of $Mtbc_1$ (Kim et al. 1998; Xia et al. 1997), which placed the 2Fe-2S cluster at a distance too far from its oxidation partner cyt c_1 for rapid electron transfer. With a globular head domain connected to a single TM through a flexible "neck" region, the ISP has all the structural features required to swivel between cyt b to cyt c_1 carrying one electron at a time. Mutations that modify the neck region of ISP to make it more rigid, more flexible, or mutations that alter the length (deletion or insertion mutants) clearly inhibit or even abolish the enzymatic activity of bc_1 (Darrouzet et al. 2000a, b; Obungu et al. 2000; Tian et al. 1998, 1999). To leave no doubt about the mobility of the ISP-ED, the Yu lab (Ma et al. 2008; Xiao et al. 2000) produced a double cysteine mutant of $Rsbc_1$. The mutation sites, one in cyt b and one in the ISP-ED, are spatially close so that a disulfide bridge could be formed under oxidizing conditions or broken under reduction or alkylation. Thus, manipulation of the disulfide bridge provided an effective and reversible on-off switch for the mobile ISP head domain (Xiao et al. 2000).

B. Mutagenesis in bc$_1$ Supports the Presence of a Control Mechanism for the ISP-ED Conformation Switch

On the basis of structural analysis of mitochondrial bc_1 in the presence of P$_f$- or P$_m$-type inhibitors, the SAMICS mechanism was proposed (Esser et al. 2006; Xia et al. 2013). A requirement of this mechanism is that the ISP-ED remains fixed at the Q$_P$ site or the b-position until the transfers of the second electron and protons from ubiquinol are completed, which effortlessly explains the high fidelity of the bifurcation of the electron pathway. This hypothesis also predicts that mutations in residues of the cyt b subunit lining the surface of the ISP-ED binding site would significantly alter the dynamics of ISP-ED movement, thus affecting bc_1 activity. The ISP-ED binding surface at the b-site consists of residues from the cd1 helix and EF loop with a total of 13 residues, five on the cd1 helix and eight on the EF loop, that have their side chains exposed to the binding surface (Fig. 10.7). The mutations introduced into these residues produced effects that agreed well with the prediction of altered ET kinetics between the ISP and cyt c_1 but these mutations rarely influence the substrate binding. For instance, mutations W142R/T/K/S (W141 in $Btbc_1$) were introduced to $Scbc_1$ (Bruel et al. 1995) and T160Y/S (T144 in $Btbc_1$) to $Rsbc_1$ (Mather et al. 1995), both on the cd1 helix, resulting in a dramatic reduction in bc_1 activity and altered cyt c_1 reduction kinetics; yet substrate binding was not affected. Similar observations were made to residues on the EF loop as in K329A of $R.$ $sphaeroides$ (K287 $Btbc_1$) (Esser et al. 2006), L286 and I292 (I268 $Btbc_1$) of $R.$ $sphaeroides$ (Rajagukguk et al. 2007), K329 of $R.$ $sphaeroides$ (Crofts et al. 1995), Y279 of yeast (Wenz et al. 2007), and Y302 in $R.$ $capsulatus$ (Lee et al. 2011).

A number of these mutants were purified and ET rates between ISP and cyt c_1 were measured by rapid photooxidation of a laser-excitable ruthenium cluster (Millett and Durham 2009). For example, residue I292 on the EF loop was mutated to I292L/M/V/E/R and the ET rate between ISP and c_1 dropped precipitously for all mutants, when compared to the wild-type bc_1. In particular, the I292A and I292M mutants behaved like bc_1 inhibited by the P$_f$ inhibitor famoxadone (Rajagukguk et al. 2007).

The current hypothesis also predicts that limiting the movement of the cd1 helix leads to a modification in the activity of the complex depending on the size of substitution, as

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in $R.$ $capsulatus$ has also been widely used (Gennis et al. 1993).
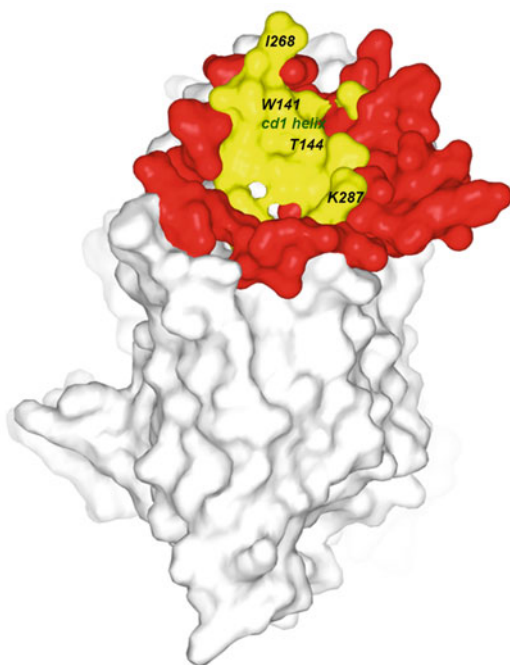
Fig. 10.7. The binding surface in the cyt b subunit for the ISP-ED. A van der Waals surface plot for the cyt b subunit is given in *gray* and the ISP-0 binding surface is a shallow depression shown in *red*. Residues that are in direct contact with ISP-ED when it is fixed at the b-position in the presence of P_f inhibitors like stigmatellin are shown in *yellow*. Those residues that have been mutated are labeled.

seen in S155 of *R. sphaeroides* (Tian et al. 1997) and in G158 of *R. capsulatus* (Ding et al. 1995).

C. The Balance of Inter- and Intra-Monomer ET of the Cyt bc_1 Complex

According to the SAMICS mechanism, before the ISP-ED is released to reduce cyt c_1, the second ET must take place. This demands a clear ET passage to the Q_N site. To avoid charge congestion, nature has apparently evolved a backup mechanism by making the coupled system larger. In the first dimeric cyt bc_1 structure, the distance between the two b_L hemes was determined to be as short as ~14 Å (Xia et al. 1997), which remains unchanged in the dimeric $Rsbc_1$ and

permits inter-monomer ET. Indeed, the ET rate between the two b_L hemes was estimated to be 0.025–0.25 ms (Osyczka et al. 2004). Communications between the monomers of the dimeric bc_1 was also demonstrated experimentally in *P. denitrificans* by deactivating the Q_P site of one monomer, which was fully active but was unable to stimulate the second site in response to antimycin binding at the Q_N site (Castellani et al. 2010). In a more elegant investigation of inter-monomer ET, a fully functional dimeric bc_1 with a covalently linked cyt b dimer was engineered (denoted B-B dimer vs. BB dimer for the wild type) (Swierczek et al. 2010). Using site-directed mutagenesis, each of the four redox sites within the cyt b dimer could be deactivated alone or in groups. The quinol oxidation site can be deactivated by a point mutation that prevents quinone binding through a G158W change. The fused, but otherwise unaltered cyt b dimer, assembled into a cyt bc_1 complex, performs catalysis at the same turnover rate (B-B dimer: 60.0 s^{-1}) as the wild-type bc_1 dimer (denoted BB dimer: 56.4 s^{-1}). Inactivating one entire branch by a double mutation reduces the performance to 60–70 %. Simultaneous deactivations of the Q_P site of one monomer and the Q_N site on the opposite side (denoted with $_WB$-B^N) indeed decreases the enzyme's activity to about 50 % of the wild type (Swierczek et al. 2010).

VI. The Mechanisms of Proton Uptake at the Q_N Site and Exit at the Q_P Site

A. Conformational Changes at the Q_N and Q_P Site for Possible Proton Uptake and Exit

In a complete catalytic cycle, a ubiquinone molecule at the Q_N site receives two electrons from the b_H heme and two protons from the negative side of the membrane; this process is specifically inhibited by antimycin A and NQNO. In a number of published crystal structures, the substrate ubiquinone,

with either two or six isoprenoid repeats, was modeled into the electron density in the Q_N site of the bc_1 complexes (Esser et al. 2008a; Gao et al. 2003; Hunte et al. 2000; Lange et al. 2001). Structures of bovine mitochondrial bc_1 in the presence of antimycin A_1 or NQNO were also reported (Fig. 10.4c). Interestingly, ubiquinone and antimycin A were found to occupy overlapping but different portions of the Q_N pocket. Mechanistically, proton uptake at the Q_N site was considered in the context of a lipid environment; two potential proton uptake pathways were proposed based on analysis of bound water molecules (Lange et al. 2001). By comparing bovine and yeast bc_1 structures with ubiquinone at the Q_N site, a mechanism based on conformational changes in residues H201, K227 and D228 and a ubiquinone protonation pathway via two water molecules was also proposed (Gao et al. 2003).

Proton exit at the Q_P site has been proposed to be coupled to the bifurcated ET events. The complexity of the reaction is compounded by the number of participants involving the cyt b subunit, the substrate QH_2, and the ISP-ED. Without dismissing differences of various mechanisms, it is generally agreed that the two protons of QH_2 are extracted and exported sequentially (Crofts et al. 2006). Lacking the crystal structures showing the binding of substrate QH_2 at the Q_P site, most recent discussions in the literature on the proton exit pathway were based on modeling of substrate binding using existing inhibitors such as stigmatellin (Fig. 10.4b), which is sandwiched between H161 of ISP and E271 of cyt b (bovine sequence). Thus, these two residues are primary candidates for moving protons out of the complex to the inter-membrane space or periplasm. Indeed, both residues were seen to undergo conformational changes upon bc_1 binding to different inhibitors (Esser et al. 2004; Gao et al. 2002). However, other structural elements, such as cd1 and cd2 helices, and residues mostly in the cyt b subunit, such as Y131 and Y287, undergo large conformational changes as well and the roles of these residues in

proton pumping are not known (Esser et al. 2004; Gao et al. 2002).

B. Mutational Studies Help to Define Proton Movement Pathways

Although the concept of ET-coupled proton translocation has been widely accepted, details describing the paths the protons take and how their movements are coupled to ET remain elusive. Because the bacterial cyt bc_1 complexes from *R. rubrum and R. capsulatus* consist of only three subunits and are able to carry out full proton transfer activity (Guner et al. 1991; Robertson et al. 1993), the proton translocation must be accomplished by the three essential subunits. The accessible surface of bacterial bc_1 complex is not completely closed off to the cytosolic side especially at the Q_N site so as to provide possible proton uptake pathways (Esser et al. 2008a). Because the proton movement at the Q_P site is proposed to couple to the electron bifurcation (Brandt 1996; Brandt and von Jagow 1991; Crofts et al. 2006; Link 1997; Rich 2004) and the cyt bc_1's surface facing the periplasm is completely sealed, proton exit to the periplasm must be accompanied by some sort of conformational change of the protein. One particular residue, E271 of bovine cyt b, is nearly universally conserved and was believed to be involved in proton pumping as a general base at the Q_P site. Indeed, in *R. sphaeroides* cyt b, the corresponding residue E295 was mutated to E295Q/G/D, which severely reduced bc_1 activity (Crofts et al. 1999). Kinetic analysis of these mutants suggested that the interactions between hydroxyl groups of quinol and side chains of E295 of cyt b and H152 of ISP are essential for proton pumping out of the enzyme. Some studies have supported the role of the iron-sulfur cluster (ISC) in proton exiting. Protons can enter and leave the bc_1 complex when the ISC is chemically or genetically destroyed (Gurung et al. 2005; Miki et al. 1991). Similarly, when the ISP head domain was removed by thermolysin digestion, a decrease in proton pumping activity and an increase in

proton leakage were observed (Gurung et al. 2005). Based on these results, the protons are thought to exit via the histidine ligand of the ISC and glutamate of the -PEWY- motif.

VII. Future Perspective

Although a full review of what has been accomplished in the structural study of cyt *bc*$_1$ from photosynthetic bacteria is beyond the scope of this manuscript, it would be incomplete without at least providing a brief outlook on future research efforts from a structural perspective. It has been more than one and half decades since the first crystal structure of cyt *bc*$_1$ complex debuted, and now over 50 entries of structures of *bc*$_1$ and related *b*$_6$*f* complexes are available in the Protein Data Bank for detailed scrutiny by researchers. Reliable structural information is now available for *bc*$_1$ from mitochondrial origins to bacterial species. Furthermore, structures of native and mutant *bc*$_1$ with a variety of inhibitors and substrates bound are beginning to shed light on details of the reaction mechanism. Despite the progress that has been made, the following areas deserve special attention from structural biologists.

A. The Mechanism of Superoxide Generation by Cyt *bc*$_1$

Reactive oxygen species (ROS) are well known contributors to oxidative stress (Andreyev et al. 2005; Barja 2004; Van Remmen and Richardson 2001), causing damage to DNA/RNA, proteins, carbohydrates, and lipids. Damage caused by ROS has been linked to a number of diseases, including cancer, inflammation and aging (Moro et al. 2005; Sadek et al. 2003; Shigenaga et al. 1994). As a progenitor of ROS, superoxide is generated by electron leakage from the oxidative phosphorylation pathway (Boveris et al. 1972) with a significant contribution from the cyt *bc*$_1$ complexes (McLennan and Degli Esposti 2000; Nohl and Jordan 1986; Turrens et al. 1985). More significantly, ROS generated by cyt *bc*$_1$ has more impact on the

cytosolic concentration of ROS than those generated by other respiratory complexes (Muller et al. 2004). Mechanistically, identifying the source of electron leakage has been a centerpiece of ROS research on the *bc*$_1$ complex. It is known that under normal catalytic conditions, very small amounts of ROS are generated by *bc*$_1$. But the amount increases dramatically when *bc*$_1$ is inhibited by antimycin A at the Q$_N$ site or when the membrane is fully energized (Andreyev et al. 2005; Quinlan et al. 2011; Zhang et al. 1998a).

It has been reported that the production of superoxide by *bc*$_1$ is reciprocally related to its ET activity. Bovine cyt *bc*$_1$ has 10 times higher ET activity than *Rsbc*$_1$ but produces superoxide only at one-tenth of that generated by the *Rsbc*$_1$ (Yin et al. 2009). The ΔIV-subunit mutant of *Rsbc*$_1$, in which the only supernumerary subunit is deleted, has lower ET activity but shows four times higher superoxide generation activity than that of the wild type (Tso et al. 2006; Yin et al. 2009). Mutations introduced to conserved residues often lead to changes in superoxide activity. For example, mutations introduced to Y302 of *R. capsulatus* cyt *b*, the equivalent of Y278 in human and Y279 in bovine cyt *b,* all have decreased ET activity and an increased rate of superoxide generation (Lee et al. 2011). Similarly, the M183L mutant in cyt *c*$_1$ of *Rcbc*$_1$ was unable to grow photosynthetically due to cyt *c*$_1$ inactivation (Gray et al. 1992), but a higher superoxide generation activity was observed (Borek et al. 2008).

Two possible sites for electron leakage have been considered: one is the ubisemiquinone radical (Q$^{\bullet}$) at the Q$_P$ site (Muller et al. 2002; Nohl and Jordan 1986; Turrens et al. 1985) and the other site involves the reversed electron flow to heme *b*$_L$ (Drose and Brandt 2008). The lack of detection of the Q$^{\bullet}$ radical under normal conditions at the Q$_P$ site obscures the Q$^{\bullet}$-mediated mechanism. The reduced *b*$_L$-mediated mechanism has gained support from the observation that superoxide production is maximal when the quinol pool is partially oxidized. This view is difficult

to reconcile with the observation that the $Rsbc_1$ mutant lacking heme b_L produces as much superoxide as the wild type (Yang et al. 2008).

Thus, the mechanism of superoxide generation in atomic detail remains obscure. Although a direct demonstration of trapped superoxide in the cyt bc_1 structure seems distant, indirect evidence may be within reach. Analogues of superoxide could be included in crystallization experiments to reveal potential binding sites for both wild-type and mutant bc_1 complexes. The readers are encouraged to read contributions by Baniulis et al., Osychka et al., and Kramer et al. in this volume on related topic.

B. Interactions of Cyt bc₁ with Other Protein or Non-protein Factors

As part of the cellular respiratory chain, the activity of the cyt bc_1 complex is regulated by redox potential and by the concentration of substrates. It has been known for some time that many factors regulate bc_1 activity, though the physiological significance of these effects was not fully appreciated. Molecules as small as ions and as large as entire proteins were found to interact with cyt bc_1, and seem to be able to regulate its activity at least in vitro. Ions or small molecules that are reported to interfere with bc_1 activity are Zn^{2+} (Skulachev et al. 1967), ATP (Lorusso et al. 1990), and dioxygen (Zhou et al. 2012). Recently, cyt bc_1 has been reported to form a supercomplex with other respiratory chain components (Schagger and Pfeiffer 2000) as well as to interact with matrix proteins such as malate dehydrogenase (Wang et al. 2010). Detailed interactions at sub-molecular resolution and their physiological roles in regulating bc_1 activities remain to be elucidated.

C. Structure-Based Drug Design to Overcome Widespread Resistance

The cyt bc_1 complex is a time-tested and highly effective target of numerous antibiotics and fungicides. Nearly all cyt bc_1 in-

hibitors act on either the Q_P or Q_N site. The emergence of drug resistance, however, has had devastating economic and social consequences. The primary cause of resistance to bc_1 inhibitors is target site mutations, creating a need for novel agents that act on alternative sites within the cyt bc_1 (Esser et al. 2014). The accumulation of crystallographic data of bc_1 complexes with and without inhibitors bound offers rich information on the inhibitor binding modes, conformational changes upon inhibitor binding of side chains in the active site and large-scale domain movements of the iron-sulfur protein subunit, providing the structural basis for rational drug design. The challenge before us is to make use of the existing knowledge and devise novel strategies that would allow the discovery of better agents for disease control in a more efficient manner.

Acknowledgments

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Chapter 11

Rieske Iron-Sulfur Protein Movement and Conformational Changes in Cytochrome *bc–bf* Complexes

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Summary

A detailed description is provided of the different positions observed for the Rieske iron-sulfur protein in different crystal structures of the bc_1 complex, and the conformational changes taking place in the "neck" or "linker" region to make those positions possible. Significant movement in the neck is limited to residues 69–71. The effect of neck mutations is interpreted in terms of their effect on the tension exerted by the neck on the extrinsic domain, rather than flexibility of the neck.

I. Introduction

The cytochrome bc_1 complex has proved to be a particularly productive target for crystallization, perhaps because of the large extrinsic domains on both sides of the membrane with the mitochondrial complex. Today there are ~40 structures of cyt bc_1, from 6 different organisms, in 11 different crystal forms, in the Protein Databank (PDB). There are

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Abbreviations: Cyt – Cytochrome; ED – Extrinsic domain; HHDBT UHDBT – 5-n-heptyl or 5-n-undecyl −6-hydroxy-4,7-dioxobenzothiazole "*bc* complex" is used as a generic term referring to the superfamily of enzymes including a cyt *b* homolog Rieske ISP, and c-type cytochrome including bc_1 b_6f, b_6c_1, and *b-cc* complexes; ISP – Iron sulfur protein; *Pc.d.*

– *Paracoccus denitrificans*; PDB – Protein Databank (www.pdb.org); Q_N – Quinol reduction site; Q_P – Quinol oxidation site of *bc–bf* complexes; *Rb.c. – Rhodobacter capsulatus*; *Rb.s. – Rhodobacter sphaeroides*; *T. thermophilus – Thermus thermophilus*; – Structures in the PDB are referred to by their 4-letter accession code

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12 structures of the b_6f complex from three different organisms, in two different crystal forms. Many of the crystal forms contain more than one monomer in the asymmetric unit, giving us multiple crystallographically independent structures from a single crystal.

Comparing these structures allows us to identify conformational changes. Some of these just reflect plasticity of the protein, as it is influenced by different crystal contacts in the different crystal forms. Other changes represent significant changes that may be related to the function of the enzyme. Describing these changes, and deducing their significance, if any, for the mechanism, is important for getting the most understanding from the structures.

The most significant changes observed in the bc_1 complex structures have involved different positions of the Rieske iron-sulfur protein. At the least these involve different positions of a tethered, soluble, electron carrier as it diffuses between electron donor and acceptor. However, as described below, it is likely that it involves a carefully orchestrated movement that is important for ensuring the strict bifurcation of electron transfer at the Q_P site, and thus the efficiency of energy conservation by the bc_1 complex. Herein we focus mainly on the detailed description of the conformational changes without overly emphasizing the possible interpretations, which have been covered in a number of reviews. In particular we provide a detailed description of conformational changes occurring in the "neck" of the iron-sulfur protein.

II. Requirement for Movement of the ISP Extrinsic Domain to Shuttle Electrons

The Rieske iron-sulfur protein (ISP) consists of a hydrophilic globular domain of approximately 120 residues which includes the Fe_2S_2 cluster (the ISP extrinsic domain or ED), anchored to the membrane by a single transmembrane helix (TMH). There is a short, flexible linker between the ISP-ED and the transmembrane helix, referred to as the

linker, neck, tether, or hinge; and the ED is mobile as indicated by different orientations in different crystal structures. As pointed out by Iwata et al. (1996), this arrangement suggests that the ISP may have arisen as a soluble periplasmic electron carrier that became anchored to the membrane and recruited into the complex to improve efficiency of electron transfer. Another advantage in the case of a bacterial complex could be in preventing loss through a leaky outer membrane.

The first evidence for mobility of the ED came from the poor order (high B-factor, unclear electron density) of this protein in crystal structures (Xia et al. 1997) and the observation of different positions in different structures (Iwata et al. 1998; Zhang et al. 1998). There seemed to be a mechanistic requirement for movement, since none of the positions for the cluster seen in crystals were close enough to both the presumed site of the electron donor at the Q_P site in cytochrome b and the electron acceptor, heme c_1, to allow electron transfer between them at a kinetically competent rate. Various mutations, both spontaneous and site-directed, have also provided evidence for the importance of mobility (Tian et al. 1999; Darrouzet et al. 2000a; Obungu et al. 2000; Xiao et al. 2000; Ghosh et al. 2001; Darrouzet and Daldal 2002; Rajagukguk et al. 2007; Ma et al. 2008).

III. Classification and Depiction of Different Positions of the ISP-ED

Different positions of the ISP-ED seen in the various mitochondrial bc_1 structures, in different crystal forms and with different inhibitors bound, have been reviewed (Berry and Huang 2011). The fixed, or b position, which is seen for example in all structures with stigmatellin bound, has the Rieske protein tightly docked in the binding crater on cytochrome b, with maximal buried surface area between cyt b and the ISP-ED and an H-bond from stigmatellin to cluster-ligand His161 of the ISP. This position is also seen in structures with H- or U- HDBT, atovaquone, or ascochlorin, which form

similar bonds to cluster-ligand histidine. And it is also seen, at lower occupancy, in the tetragonal crystals of bovine bc_1 with no inhibitor. We assume that electron transfer from ubiquinone to the ISP occurs in this position, with ubiquinol in the place of the inhibitor.

In the presence of MOA[1] inhibitors, the tetragonal crystals (1sqb, 1sqp, 1sqq) disengage the cluster-bearing end of the ISP-ED from the binding crater by about 4 Å. Since this state promoted by MOA inhibitors is known as the released state, we have called this the released position. But the ISP-ED is present here at low occupancy as judged by the cluster's Fe anomalous signal, and in fact the truly released ISP-ED are the majority that we don't see in these crystals because they occupy a large number of different positions in very low occupancy. This ensemble of invisible positions is assumed to include some that are competent for electron transfer to cyt c_1. Nonetheless, the position close to cyt b, that is modeled in the structures of these crystals in the presence of MOA inhibitors, is in fact "released" from its tight binding to cyt b as indicated by a decreased number of H-bonds and decreased buried surface area between the ISP-ED and cyt b, compared to the fixed position (Esser et al. 2006), concomitant with its 4 Å retraction of the cluster tip from the binding crater.

This released position is still too far (26 Å) from cyt c_1 for kinetically competent delivery of the electron. Other structures show the cluster within electron transfer (<14 Å) distance of heme c_1, in what has been called the c_1 or "distal" position(s). These are found in the chicken crystals, either with MOA inhibitors or with no inhibitor (Zhang et al. 1998), and in the $P6_522$ bovine crystals (Iwata et al. 1998).

Factors governing the different positions seen in crystals, and the relation between these positions and the mobility required by the reaction, are not clear. Mechanisms invoking gated motion (or position) of the ISP to enforce the bifurcated reaction at the Q_P site have been proposed (Link 1997; Brandt 1998; Esser et al. 2006), and it may be that

different inhibitors mimic different intermediates in the reaction cycle resulting in different positions of the ED.

We assume that electron transfer from ubiquinol occurs when the ISP is in the "fixed", or "b" position, H-bonded to ubiquinol at the Q_P site by cluster-ligand His161, much as it is H-bonded to stigmatellin, nHDBT, or atovaquone in various crystal structures. The second electron may be transferred to cyt b_L either subsequently (Link 1997; Junemann et al. 1998) or concertedly (Snyder et al. 2000), leaving the reduced ISP H-bonded to the oxidized ubiquinone in a product complex similar to that giving rise to the gx $=1.8$ epr signal observed when membranes containing endogenous ubiquinone are treated with ascorbate to reduce the ISP (Ding et al. 1992). This complex would dissociate with some rate/frequency. Alternatively, the semiquinone may dissociate after the first electron is transferred to the cluster, and move to a more proximal location from which the second electron is transferred to cyt b (Crofts et al. 1999; Victoria et al. 2013). In either case the reduced, protonated ISP is released, allowing it to transfer the electron to cyt c_1 and release one proton on the P side of the membrane.

While the static X-ray structures do not describe the actual path of movement, the positions that have been seen in various crystals can be assumed to be accessible and can be used to map out a possible path for the ISP-ED between its position docked at the Q_P site on cyt b and that with the cluster in proximity of heme c_1 where the electron is delivered. In this we are also guided by a "steered" molecular dynamics simulation of the movement reported by Izrailev et al. (1999).

Figure 11.1a illustrates in stereo the first part of the path of the ISP-ED from the fixed or b position to the c_1 position. This is the movement from the fixed position (brown trace) seen for example in the presence of stigmatellin, to the "released" position (green) seen in the tetragonal beef crystals in the presence of MOA inhibitors. This is a

a (left panel)
103 189
Rieske ISP
72
70
Variable helix
67
68
Loop 1
α-cd1
α-ef
Stig.
α-C
ISP TMH
Cyt b
positions:
Fixed, or "b"
Famoxadone
Released

a (right panel)
103 189
Rieske ISP
72
70
Variable helix
67
68
Loop 1
α-cd1
α-ef
Stig.
α-C
ISP TMH
Cyt b
positions:
Fixed, or "b"
Famoxadone
Released

b (left panel)
Rieske ISP
70
Variable helix
67
68
Loop 1
α-cd1
α-ef
Stig.
α-C
ISP TMH
Cyt b
Positions:
Released
c_1 (3L71)
c_1 (1be3)

b (right panel)
Rieske ISP
70
Variable helix
67
68
Loop 1
α-cd1
α-ef
Stig.
α-C
ISP TMH
Cyt b
Positions:
Released
c_1 (3L71)
c_1 (1be3)

c (left panel)
Rieske ISP
Heme c_1
ef loop
α-cd$_1$
α-ef
Stig.
α-C
69
66
ISP TMH
Cyt b
Positions:
Released
c_1 (3L71)
c_1 (1be3)

c (right panel)
Rieske ISP
Heme c_1
ef loop
α-cd$_1$
α-ef
Stig.
α-C
69
66
ISP TMH
Cyt b
Positions:
Released
c_1 (3L71)
c_1 (1be3)

see-saw or rotational motion, with the cluster end of the molecule moving away from its docking position in cyt b by about 4 Å while the opposite end, to which the linker is attached, moves toward the membrane. This allows the helix in the linker to grow, and take up the slack, as discussed in the next section. The Fe_2 atom of the cluster is modeled as a sphere of the same color as the respective trace, showing how far the cluster moves.

Also shown in Fig. 11.1a is an intermediate position seen with the famoxadone-like inhibitors, JG144 in this case (yellow trace). This class of inhibitors does not form an H-bond with the ISP but for reasons that are not well understood results in fixing the ISP near cyt b. The cluster-ligand His161 (which H-bonds the inhibitor in structures with stigmatellin) H-bonds Y279 of cyt b. Before it was ever observed in an actual x-ray structure, this H-bond was seen at one point along the simulated trajectory of steered molecular dynamics (Izrailev et al. 1999). This more distal H-bonding partner pushes His166 of the ISP, and cluster-bearing loop 2 to which it is attached, farther from cyt b. Loop 1 maintains essentially the same interactions with cyt b. This results in pivoting the ED about a point in loop 1 by about 10°, in turn resulting in movement of the opposite end (around residue 103 or 106) of the ISP by about 6 Å between the cyt b position and the famoxadone position, while Fe_2 of the cluster moves away from cyt b by a little over 1.5 Å.

Figure 11.1b and c illustrate the motion from the released position of the tetragonal crystals (green) to the "cyt c_1" position of the chicken crystals (blue) and the extreme cyt c_1 position of the hexagonal beef crystal 1be3 (magenta). Figure 11.1b is a side view with the cluster on our left and the linker on the right, in the same orientation as Fig. 11.1a. However, the motion is different from the seesaw motion in that figure, as here the rotation is about an axis that passes near the attachment point of the linker (approximately in the plane of the paper). It thus does not involve any significant change in length of the tether, as can be seen in Fig. 11.1b, while the cluster (large sphere) rotates upwards and away from the viewer. The hinge for this motion is mainly in residues 72 and 73, with flexion but very little translation of these residues. Figure 11.1c shows the same structures, but looking roughly down the rotation axis, with the cluster toward the viewer and the linker attachment in the rear. Notice how Fe_2 of the cluster swings upward and to the (viewer's) left to face the heme of cyt c_1 (The Fe_2 atom for the fixed and famoxadone positions are included in Fig. 11.1b and c for completeness).

Unlike mitochondrial cytochrome c and other "large" Class I cytochromes, which have a loop covering the heme propionates, cytochrome c_1 has the propionates exposed (Zhang et al. 1998). They are approached by the cluster-bearing tip of the ISP in the c_1 positions, and in the structure 1be3 there is a hydrogen bond between the cluster-ligand His166 and the "D" heme propionate. The heme is also exposed (as in other class I cytochromes), at the surface of the heme binding cleft, where the C pyrrole ring protrudes. This is where it interacts with its acceptor, cytochrome c (Broger et al. 1980; Stonehuerner et al. 1985; Hunte et al.

Fig. 11.1. Trajectory of the ISP-ED from the Q_P site to cyt c_1. Illustrated by stereo views of crystal structures with the ISP-ED in different positions. (**a**) In moving from the fixed position to the released position, the ED rotates clockwise lifting the Fe_2S_2 cluster (round ball at position of Fe_2) out of the Q_P site, while the variable helix in the linker takes up the slack by adding another turn. *Arrows* along the upper edge indicate the same residue in different positions. (**b**) Viewed from the same orientation as in (**a**), the ISP rotates about an axis roughly *horizontal* in the plane of the picture, turning the cluster to face cyt c_1 without affecting the variable helix. (**c**) shows the same motion as (**b**) viewed along the rotation axis to illustrate the path of the cluster. The structures used are 1sqx (stigmatellin), 2fyu (famoxadone), 1sqb (beef azoxystrobin), 3 L71 (chicken azoxystrobin), 1be3 (beef P6$_5$22) for fixed, famoxadone, released, c_1, and extreme c_1 positions. Stigmatellin from 1sqx is included in all three views to indicate the Q_P site.

2002). Thus rather than interacting with its donor and acceptor using the same surface, as the ISP does, and requiring movement to disengage from the donor before interacting with the acceptor, cyt c_1 can interact with both simultaneously to mediate electron transfer from the ISP to cyt c.

The ef-loop, which has been considered a barrier obstructing movement between the b and c_1 positions (Darrouzet and Daldal 2002) or a support stabilizing the c_1 position (Berry et al. 2013), has also been rendered in Fig. 11.1c. The effect of "mobility" mutants in the ef loop will be discussed below in relation to mutations in the neck region.

The ED is often referred to as the "head" of the Rieske ISP, with the linker as the "neck". To extend this analogy we can describe these two steps of the motion in cartoon form as first (Fig. 11.1a) lifting the head to raise the snout out of the binding crater where it receives the electron from the Q_P site, and then (Fig. 11.1c) turning the head (twisting the neck) to face heme c_1, where the electron will be deposited.

It can be assumed that the Rieske ISP of the b6f complex also undergoes movement to deliver electrons from the QP site to cytochrome f, as again the distance is too great to be traversed by direct electron transfer (Kurisu et al. 2003). Also, the rate of electron transfer from the QP site to cyt f is sensitive to viscosity (Yan and Cramer 2003). Despite large differences in the linker region of the ISP as compared with the bc1 complex (discussed in the next section), similar positions of the ISP have been observed.

The three b_6f structures with the stigmatellin analog TDS (1Q90, 2E76, 4H13) have the ED tightly docked in its binding crater with cluster ligand His129 H-bonded to the inhibitor, as in the fixed or "b" position of the bc_1 complex. The Fe_2S_2 cluster and cluster-bearing loops in these structures superimpose well with mitochondrial bc_1 structures in the presence of stigmatellin, when the structures are superimposed based on cytochrome b.

Structures without inhibitor (1vf5, 2e74,2e75, 2d2c, 2zt9, 4h0L, 4 h44, 4ogq)

have the cluster a little farther from cyt b, as in the released position of the tetragonal beef crystals. Specifically, the Fe_2 atom of the cluster in the structures with an empty Q_P site is 3.5–4.0 Å from its position in 4H13 (with TDS). There are no structures as yet illustrating the position in which the b_6f ISP transfers an electron to cytochrome f. However a recent structure (4i7z) of the cyanobacterial complex treated with anionic phospholipids has a disordered ISP-ED, which is interpreted as meaning that it is fully released from cytochrome b and distributed over a large number of distal positions with low occupancy in any one position (Hasan et al. 2013).

The electron acceptor from the b_6f ISP, cytochrome f, bears little similarity to cytochrome c_1 other than presence of a c-type heme and a C-terminal membrane anchor. Superposition of cytochromes bc_1 and b_6f by the transmembrane helices of cyt b/SU_{IV} places the heme iron of cyt f 18 Å from that of cyt c_1, but both c-type hemes are about the same distance from the Fe_2S_2 cluster docked in the b position. While the heme of cyt c_1 faces the ISP edge-on with its propionates, that of cyt f is flat-on with its axial ligand His26 (His25 in *Chlamydomonas*) directed toward the ISP.

Rather than being inserted into a binding cleft perpendicular to the protein surface, the heme of cyt f is lying nearly flat on the surface opposite the ISP, partly covered by the N-terminal helix and the other axial ligand Tyr1. The axial ligand, His26, is buried under the heme as seen from this side but is near the surface on the side facing the ISP. It has been estimated that a rotation of the ISPED by 25°, with 14 Å movement of the cluster, would bring the cluster into a favorable position for electron transfer to the heme of cyt f via Leu27 and His 26 (Kurisu et al. 2003).

Plastocyanin is believed to interact with the hydrophobic patch around Tyr1 (Ubbink et al. 1998; Diaz-Moreno et al. 2005). Thus it is likely that cyt f, like cyt c_1, has separate binding sites for its electron donor and acceptor allowing it to interact with both simultaneously, the heme acting as an electron wire between the ISP and plastocyanin.

IV. Conformational Changes in the ISP Hinge Region

There is no indication of conformational flexibility in the transmembrane helix anchor of the ISP. Assuming for now (but see next section) that the extrinsic domain moves as a rigid body, the mobility of that body thus results from conformational changes in the short linker region, connecting it to the transmembrane helix. As described in the previous section, in bc_1 complexes this region not only provides a flexible hinge but also takes up slack by adding a turn to the variable helix as the ED rotates. A possible function for this helix in "spring-loading" the ED to facilitate leaving the b position has been discussed (Berry et al. 2000, 2013; Crofts et al. 2002).

Having accurate atomic models for the bc_1 complex with the ISP in a wide range of positions allows us to pinpoint the region of flexibility in the neck and describe the conformational changes taking place there. Here we use the sequence numbering of the mature vertebrate ISP. By convention the yeast protein uses the numbering of the apoprotein, which adds 20 to the number of each residue relative to the vertebrate sequences. Proteobacterial (*Rhodobacter* and *Paracoccus*) proteins have shorter N-termini even than the mature mitochondrial proteins, and the residue numbers in the conserved neck region are smaller by 25 (Rb.) or 20 (Pc) than the corresponding vertebrate residues. Table 11.1 provides the vertical alignment of sequences for which structures are available.

The transmembrane helix ends at about residue 61 in the vertebrate numbering. The stretch between 61 and 66, although appearing unstructured when the subunit is depicted by itself, is packed into a dense structure with cytochrome b from both dimers, called the "clamp" or "vise" region, and does not vary significantly in the different structures. Figure 11.2a shows these contacts in a stereo ribbon diagram. The vise consists of the conserved di-glycine at 167,168 in cyt b of the monomer with which the extrinsic domain interacts, and

Table 11.1. Vertical sequence alignment of residues in and around the ISP neck.

SS[a]	Beef	Chick	Yeast	Rb.s	Rb.c	Pc.d
HH	S56	T56	E76	W32	W32	W36
HH	Q57	Q57	T77	P33	P33	T37
HH	F58	F58	F78	L34	L34	L38
HH	V59	I59	I79	I35	I35	V39
HH	S60	S60	S80	N36	N36	N40
HH	S61	S61	S81	Q37	Q37	Q41
– –	M62	L62	M82	M38	M38	M42
– –	S63	S63	T83	N39	N39	N43
– –	A64	A64	A84	P40-S[b]	A40	P44
– –	S65	S65	T85	S41 |	S41	S45
GH	A66	A66	A86L[c]	A42-SΔ[d]	A42	A46
GH	D67	D67	D87	D43 |Δ	D43	D47
GH	V68	V68	V88	V44-SΔ	V44P[e]	V48
– H	L69	L69	L89	Q45 |	K45P	Q49
– T	A70	A70	A90	A46-S	A46P	A50
– T	M71	L71	M91	L47	M47P	L51
– T	S72	S72	A92	A48	A48P	A52
– –	K73	K73	K93	S49	S49P	S53
EE	I74	I74	V94	I50	I50	I54
EE	*E75*	*E75*	*E95*	*F51*	*F51*	*Q55*

[a]SS: Secondary structure assignment by DSSP algorithm (Kabsch and Sander 1983). The first column gives the assignment (H α-helix, G 3_{10} helix, T turn, E sheet, – random coil) for structure 1PPJ, in the "b" position, while the second is for structure 3 L70 in the "c_1" position
[b]-S: Cys mutants of Tian et al. (1999)
[c]L: A86L mutation of Obungu et al. (2000)
[d]Δ: residues deleted in ΔADV mutant of Tian et al. (1998)
[e]P: residues mutated to Pro or Gly in the 6-Pro, 6-Gly mutants of Daldal and coworkers (Darrouzet et al. 2000a)

residues 73–75 (at the turn before the start of helix αB) of the other monomer. Van-der-Waals contacts and H-bonds hold this stretch immobile during the movement of the ED. Given this entrapment in the cyt b dimer interface, it may seem surprising that the ISP can be removed by fairly gentle treatment, and reconstituted into ISP-depleted complex to restore activity, but this has been demonstrated by a number of groups (Trumpower and Edwards 1979; Trumpower et al. 1980; Hovmoller et al. 1981; Engel et al. 1983; Shimomura et al. 1984; Gonzalez-Halphen et al. 1991; Valkova-Valchanova et al. 1998).

a

Strand β1

L71

D67

αD

αab

αA

αB

K52

ISP

Cyt b

b

Strand β1

L71

D67

S65

K52

L62

c

74
73

72

"c"

"released"

"fixed"
or "b"

Fmx
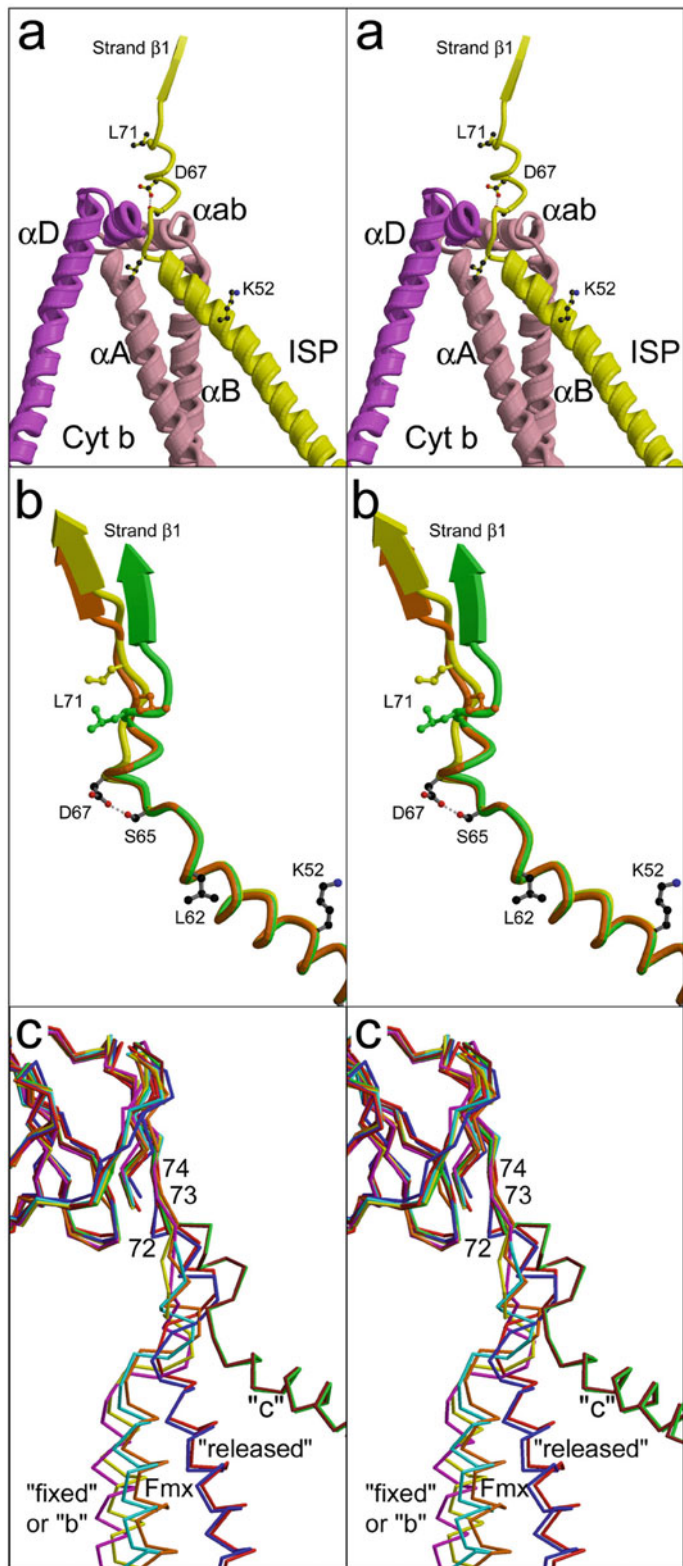
In Fig. 11.2a there appears to be a gap through which the neck could pop out of the vise. Alternatively, the ISP-depleted complex may reversibly dissociate into monomers (Musatov and Robinson 1994) that can bind the ISP on reassembly. That reconstitution does in fact involve specific reinsertion of the transmembrane helix is supported by the fact that a transmembrane fragment inhibits (Gonzalez-Halphen et al. 1991), and by the failure of the soluble fragment to reconstitute activity (Valkova-Valchanova et al. 1998).

An H-bond between the side chains of conserved pair Ser(Thr)65 and Asp67 holds residues 65, 66, and 67 in a non-helical loop which also does not vary between structures. It may also hold residues 66 and 67 in a conformation to seed formation of a helix, as these two residues are shown by secondary structure analysis to be part of the helix in either the condensed or stretched conformation of the neck.

Figure 11.2b shows a superposition of the ISP backbones from chicken crystals in the presence of azoxystrobin, famoxadone, and stigmatellin, with the superposition based on the transmembrane helices of cyt *b*. Comparing the three structures, the first residue that shows significant movement is 69. The motion consists of stretching the neck by partially unwinding a variable helix involving residues 66–71. In the presence of azoxystrobin, when the cluster is away from the Q_P site in the c position, the neck is shortest, with α-helical H-bonds reaching as far as residue 71. In the presence of stigmatellin, when the cluster-bearing tip of the ISP is deep in the binding crater and binding to stigmatellin in the Q_P site, the neck is maximally extended. In this position only one 3_{10} H-bond remains (between residues 65 and 68),

as noted for the yeast structure with stigmatellin (Hunte et al. 2000). Residues 69–71 have been pulled out of the helix by stretching.

Essentially all the flexibility of the neck is exhibited by residues 69–72, with everything from residue 73 on moving as a rigid body with the extrinsic domain. This can be shown by superimposing the ISP from different structures based on the extrinsic domain as in Fig. 11.2c. Residue 74 is the beginning of the first strand of beta sheet 1, and 72–73 form H-bonds with Trp91 and Arg92 of the globular domain, both in the structure of the soluble fragment 1rie (Iwata et al. 1996) and in the bc_1 complex. Stretching of the helix occurs between the "fixed" and "released" positions, while the much larger movement of the ED between the released and c_1 positions is accommodated by rotation about bonds in the range 72–73. This implies that the full energy of formation of the helix contributes to moving the cluster from its bound "*b*" position to the position in the released state of the tetragonal crystals (a distance of only 4 Å).

The stretching and "melting" of the helix is quantitatively documented in Table 11.2, which classifies the available mitochondrial bc_1 structures into (i) fixed, (ii) famoxadone, (iii) released, or (iv) c_1 positions. The length of the neck, measured as distance from $C^α$ of residue 65 to $C^α$ of residue 74, is provided in the first numerical column. The range is from 17 Å in the released or c_1 position to 22–24 Å in the *b* position. Among the structures with the *b*-position, the length varies from 21.5 to 24.5, probably due to different crystal contacts. Of these the longest necks are in the chicken crystals, which have no significant crystal contacts on the ISPED.

Fig. 11.2. Details of the linker segment of the bc_1 Rieske ISP. (**a**) *Ribbon diagram* showing how the ISP is clamped between the two monomers of cyt *b*. One side of the clamp consists of the conserved di-glycine turn at 167, 168 (between helix cd$_2$ and D) in cyt *b* of the monomer with which the extrinsic domain interacts, and residues 73–75 (at the turn before the start of helix αB) of the other cyt *b* monomer. (**b**) linker region of the ISP of chicken bc_1 in three different positions superimposed based on cyt *b*. *Yellow*, position (3h1h). *Brown*, famoxadone (3 L74). *Green*, c_1 position (3 L71). side chains of K52, L62, S65, and D67 are shown for 3 L71, and L71 is shown for each. (**c**) Superposition of ISP based on the ED. Residues from 73 on move as a rigid body with the ED.

Table 11.2. ISP "Neck" length and H-bond distances in structures with different ISP ED positions.

	C^α–C^α	N->O								
PDBID	65–74	68–65*	69–65	69–66*	70–66	70–67*	71–67	71–68*	72–68	72–69*
Position: stigmatellin										
3h1i	24.48	3.82								
3h1j	24.24	3.34	4.41							
1 pp9	21.41	3.00	3.42	3.38	3.59	3.47	–	–	–	–
1ppj	21.78	2.91	3.49	3.59	3.93	3.95	–	–	–	–
2a06	21.73	3.03	3.63	3.51	–	–	–	–	–	–
1sqx	22.75	3.47	3.92	4.01	–	–	–	–	–	–
3cx5	22.12	3.18	3.97	4.17	–	–	–	–	–	–
1 kb9	22.18	2.95	3.78	3.88	–	–	–	–	–	–
Position: PHDBT										
1p84	22.29	3.04	3.75	3.91	–	–	–	–	–	–
Position: ascochlorin										
3h1L	24.40	4.05	–	–	–	–	–	–	–	–
Famoxadone, fenamidone, JG144										
3 l74	21.05	2.91	3.00	3.30	3.13	3.34	–	–	–	–
3 l75	21.15	2.83	3.00	3.35	3.13	3.38	–	–	–	–
2fyu	21.02	2.73	3.26	3.67	3.85	4.48	–	–	–	–
Released position: MOA inhibitor in tetragonal crystals										
1sqb	16.91	*2.75*	3.18	3.64	3.29	4.18	4.08	3.13	3.74	3.58
1sqp	16.13	*2.85*	2.96	3.47	3.22	4.46	4.52	3.30	4.06	3.61
1sqq	17.97	*2.64*	2.87	3.78	3.55	4.38	4.02	3.00	4.34	2.95
c_1 position: empty site or MOA (or int position: 1bgy)										
1be3	13.72	3.26	2.68	3.63	3.18	3.54	3.89	3.65	3.37	2.50
1bgy	15.09	*2.96*	2.85	3.55	2.62	3.44	4.00	2.81	3.42	3.31
3h1h	16.79	*2.84*	2.98	3.19	2.96	3.60	3.91	2.98	3.30	2.90
3h1k	16.79	3.18	*2.94*	3.44	3.11	2.72	3.23	3.72	3.31	3.22
3 l70	16.84	*2.72*	2.96	3.39	2.87	3.36	3.66	2.87	3.32	2.82
3 l71	16.86	*2.72*	2.99	3.47	2.96	3.52	3.81	2.94	3.38	2.72
3 l72	17.01	2.98	*2.83*	3.13	2.99	3.12	3.51	3.59	3.55	2.95
3 l73	16.79	*2.65*	2.87	3.53	2.99	3.29	3.86	3.04	3.50	2.78
3tgu	17.12	3.06	*2.85*	3.29	2.95	3.25	3.67	3.19	3.65	2.77

The first column lists the structures used, arranged by the position of the ED. The second column gives the overall neck length, measured from a point in the transmembrane helix to a point in the ED. Then for each residue from 65 to 69, the length of two putative hydrogen bonds, one (3_{10}, marked*) from the amide N of the residue 3 ahead to the carbonyl of that residue, and one (α-helix) from the N of the residue 4 ahead is given. A typical α — helical H-bond is ~2.7 Å. Values over 3.5 Å are not compatible with a strong H-bond, and values over 4.5 Å are not shown. For the yeast structures, the residues aligning with these vertebrate residues were used

The shortest are the beef orthorhombic crystals. In these crystals there are contacts (described in Bowman et al. 2004) which are likely to be pushing the back end of the ISPED down, shortening the neck. Notably the cluster positions, and the residues around the clusters, superimpose very closely in all these *b*-position structures (Fig. 1 of Berry et al. 2013). It seems that in the *b*-position the ISPED is anchored by interactions of

the cluster binding domain with the binding crater and pivots about that contact, while the other end is not much constrained by the stretched-out linker, accounting for the variability in its length in this position.

Table 11.2 also lists the lengths of potential helical H-bonding pairs in the variable helix. As mentioned above only the single 3_{10} bond between 65 and 68 remains in the b position, and that is really stretched out of existence in the longest necks (3.82 in 3h1i, 4.05 in 3h1L). As the ED moves to the released or c_1 position, H-bonds to the carbonyl O's of residues 66–69 begin to form. Distances are listed for each of these from the residue three ahead in the sequence (3_{10} helical bond) as well as the residue four ahead (α-helical). In many cases the two are nearly equal, indicating the helix is still more stretched than a canonical α-helix.

The neck region has been subjected to extensive site-directed mutagenesis to probe its role in the function and assembly of the complex. While these mutations were originally conceived and discussed mainly with respect to their effect on flexibility of the linker, it has been argued (Berry et al. 2013) that they can better be rationalized in terms of the tension exerted by the tether on the extrinsic domain, pulling it away from its binding crater on the surface of cytochrome b. As discussed above, docking of the ISP in the b position results in dissolution of all but one turn of the α-helix that is present in the linker in the c_1 position or "released" state. This partial melting of the helix requires energy, which must detract from the energy of binding the ISP at the b position.

The three movable residues in the helix, 69–71, are usually Leu, Ala, or Lys69; Ala70; and Met or Leu71. These four amino acids are among the five with the highest helical propensity (O'Neil and DeGrado 1990), providing a relatively strong force for helix formation and the resulting dissociation of the ISP from its b position. Substitution of these helical residues with low-helical-propensity Gly or Pro (Darrouzet et al. 2000a) or Ile (Obungu et al. 2000), or insertion of additional Ala residues (Darrouzet et al. 2000b;

Obungu et al. 2000), results in a partially or completely inactive complex.

Replacement of these residues individually with Ala (which has a high helical propensity) had no effect (Darrouzet et al. 2000a), ruling out the need for a specific side chain. Insertion of extra residues in the neck led to loss of function. The 6-Pro or 6-Gly substitutions regained function by spontaneous loss of three of the residues to give a shorter than normal chain. This would not be expected if the defect is due to loss of flexibility in the 6-Pro mutant, but is consistent with the deletion overcoming excessive length due to failure of the Pro or Gly residues to form a helix.

Investigation of the 6-Pro substitution by EPR spectroscopy and flash kinetics indicated that the [Fe_2S_2] cluster is present in the Q_P site and unable to transfer electrons to cyt c_1 (Darrouzet et al. 2000a). EPR spectra supported that position for the 6-gly (Darrouzet et al. 2000a) and alanine insertion (Darrouzet et al. 2000b) mutants as well. This is consistent with stabilization of the ISP at its docking site on cyt b as a result of the reduced tension due to low helix-forming propensity or the increased length of the neck.

On the other hand the "box 1" mutations (in the cluster-bearing loop 1 of the ISP) around the first His cluster ligand (Liebl et al. 1997) (140 and 142 in the vertebrate sequence, 134 and 136 in Rhodobacter) or mutations in the ef loop of cyt b (Darrouzet and Daldal 2002, 2003), produce results consistent with destabilizing the b position relative to the c_1 position, such as weak or no interaction with quinone at the Q_P site and rapid re-reduction of cyt c_1 in the presence of myxothiazol. While either class of mutation is deleterious, they apparently counterbalance each other, so that 2nd site revertant to the box 1 mutations L136G or H arise in the neck region, while the +1Ala neck mutation reverted via a mutation in the ef loop (Darrouzet and Daldal 2002). Such complementary balancing interactions, together with complementary effects on the apparent midpoint potential of the ISP cluster- from

about 320 mv in wild type to 370, 410, and 460 mv in the $+$ Ala, $+$2Ala, and 6Pro neck mutants (Darrouzet et al. 2002), or 255–270 mv in T288 ef-loop mutants (Darrouzet and Daldal 2002, 2003) – can be rationalized in terms of relative stabilization of the b vs c_1 positions (Berry et al. 2013). While the ef loop and box 1 residues affect the equilibrium because they are involved in different interfaces in the two states, the neck residues are proposed to affect the equilibrium by adjusting the tension of the tether.

Deletion of up to five residues in the neck was tolerated but with considerably decreased activity and with increased tendency to lose the ISP during purification (Darrouzet et al. 2000a). Deletion of the conserved Asp making the H-bond with Ser had a similar effect (Obungu et al. 2000). Even with no helix, it seems unlikely that a neck shortened by five residues would be compatible with the b position, unless the neck binds higher up in the cyt b clamp by a few residues. Such non-specific binding might result in easy dissociation, as might loss of the turn enforced by the Asp-Ser H-bond.

One series of neck mutations which originally seemed to demonstrate the need for movement may need to be re-evaluated in light of current knowledge of the structures. Tian et al. (1999) substituted pairs of cysteine residues in the *Rb.s.* ISP in the region 40–46 (corresponding to 64–70 in the bovine sequence, see Table 11.1). The mutant with cysteines at 40 and 42 was inactive under oxidizing condition, and that with 42 and 44 had reduced activity. Those with 44 and 46, or 42 and 46, were unaffected. Now it is clear that 40 and 42 are in the immobile region within the cytochrome b clamp, so the effect was more likely due to interference with binding than to decreased flexibility. The two mutants involving 46 would cross-link the variable helix and prevent it from extending, but given the results with deletion mutants this would not be expected to abolish activity. Notably all three showed loss of ISP from the bc_1 complex upon purification (Tian et al. 1999).

Given the requirement of the neck residues in the bc_1 complex for high helical propensity and defined length, it is surprising that the linker in the b_6f ISP consists mainly of glycines and is insensitive to substitution with proline or changes in length. The mutations which change the length of the linker do affect sensitivity to inhibitors in a way consistent with the idea that a long linker favors binding to inhibitors in the Q_P site, but clearly there is no need for tension from the tether to facilitate dissociation of the product complex during turnover. It could be that compensating mutations in the interface between cyt b_6/SU$_{IV}$ and the ISP destabilize the b position. In this regard the "box 1" region of the ISP is exactly the same in the b_6f and bc_1 complexes, but in fact the ef loop shows the same mutation as the L286F mutation found in a revertant of the $+$1Ala mutation in *Rb.c.*: In the b_6f complex the corresponding residue is F69 in subunit IV. This could be a contributing factor.

V. Does the ISP-ED Undergo Internal Conformation Changes?

In describing the original high-resolution structure of bovine Rieske fragment, Iwata et al. (1996) emphasized the "high stability ... maintained both through a strong salt bridge/hydrogen-bonding network as well as through the presence of a disulfide bridge". In most mitochondrial bc_1 structures in which the ISP is sufficiently ordered to discriminate, it appears that the structure is the same as in the bovine soluble fragment, regardless of the position of the ISP-ED or the presence of inhibitors. This suggests that the ED moves as a rigid body during electron transfer.

There were some early indications to the contrary, however (Iwata et al. 1998, 1999). In structure 1BGY the cluster binding fold was found rotated by 6° relative to the base fold, resulting in 2 Å displacement of the cluster. In this structure the peptide bond linking conserved proline P175 with the previous residue was modeled in a *cis* config-

uration, where the soluble fragment and the other bc_1 structures have *trans*.

No other structures of mitochondrial ISP show significant deviation from the structure of the high-resolution ISP fragment (1rie), and the diffraction data were never released for structure 1BGY so it is impossible to evaluate the evidence. It is tempting to discount it as an artifact of refining a moving target: the cluster-bearing tip is most ordered when contacting cyt b in the fixed position, while the end to which the linker is attached is most ordered in the released and c_1 positions, when the linker is short and helical. Thus given a mixture of positions, the observed density might shift between them as you go from one end of the ED to the other. The ISP of 1BGY was not in the fixed or released position but in a unique "int" position, but the same principle could apply if there is some heterogeneity in its position.

Interestingly, the high resolution structure (1RFS, 1.83 Å) of the b_6f Rieske protein (Carrell et al. 1997) has the residue corresponding to P175 terminating a cis peptide as in the 1BGY Rieske. The soluble Rieske fragment from a cyanobacterium, *Thermosynechococcus elongatus* (3AZC, 2.0 Å), also has a cis peptide here (Veit et al. 2012), while that from *T. thermophilus* (1NYK, 1.3 Å) has a trans peptide in this place (Hunsicker-Wang et al. 2003) Structures of the entire b_6f complex are evenly divided as to whether this should be a cis peptide in the b_6f protein. However, given the lower resolution of these structures and the relatively high B factors of the ISP-ED in the complex due to its mobility, this should probably not be taken as evidence of cis – trans conformational switching in the protein.

There is some evidence for proline isomerization, if not large scale conformational change, in the *T. thermophilus* ISP. Markley and coworkers (Hsueh et al. 2013) used NMR to look at possible changes in conformation with oxidation and reduction of the soluble *T. thermophilus* Rieske fragment, and found evidence suggesting that the residues P152 and P170, corresponding to vertebrate P159 and P175, might go from *trans* in the oxidized state to *cis* in the reduced state. However no evidence was seen by NMR for a large-scale conformational change of the protein.

Acknowledgements

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Structural Perspective of Ferredoxin NAD(P)H Reductase Reactions with Cytochrome b_6f Complexes

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Summary

The reaction of FNR with the Cytochrome b_6f complex (Cyt *bf*) implies a role in cyclic electron transport. FNR is not a membrane protein but is also involved as a component of a cyclic electron transport 'super-complex.' It has been reported that FNR in higher plant chloroplasts is localized peripherally on the stromal side of thylakoid membranes through association with an intrinsic protein. Although several high-resolution X-ray structures of plant-type FNR are available, the exact binding mode of FNR to Cyt *bf* is still elusive. However, as high-resolution structures of Cyt *bf* and membrane bound FNR (L-FNR1) have become available, and possible patterns of the interaction can be structurally discussed through them.

I. The Perspective

In green plants, algae, cyanobacteria, and photosynthetic bacteria, light energy is trapped by the antenna proteins and converted to a stable chemical form. The principal function of the light-dependent reactions of photosynthesis is to generate the NADPH and ATP required for carbon reduction. This is accomplished through a series of reactions that constitute the electron transport chain which can be classified in two groups: (i) oxygenic photosynthesis in green plants, algae and cyanobacteria, and (ii) anoxygenic photosynthesis in purple and green sulfur bacteria.

Abbreviations: CEF – Cyclic electron transport; Cyt – Cytochrome; FAD – Flavin adenine dinucleotide; Fd – Ferredoxin; FNR – Ferredoxin NAD(P)$^+$ reductase; LET – Linear electron transport; LHC – Light harvesting chlorophyll protein; NADH – Nicotinamide adenine dinucleotide; NADPH – Nicotinamide adenine dinucleotide phosphate; PAGE – Polyacryl amide gel electrophoresis; PBS – Phycobilisome; PC – Plastocyanin; PDB – Protein Data Bank; PS – Photosystem; Tic – Translocon at the inner envelope of chloroplast; Trx – Thioredoxin

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In oxygenic photosynthesis, there are two photosystems (photosystem II and I in series) that are linked by the cytochrome b_6f complex (Cyt bf). A sequential arrangement of three macromolecular membrane protein complexes extracts low-energy electrons from water and reduces Ferredoxin (Fd) that is finally used to produce a strong reductant, NADPH, via Fd NAD(P)H reductase ([EC 18.12.1.2], FNR). This flow of electrons is known as linear electron transport (LET). In the thylakoid membrane, however, photosystem II (PSII) and photosystem I (PSI) are not always physically linked, but are unevenly distributed between grana and stroma thylakoids. To produce extra ATP per NADPH molecule, PSI and Cyt bf can transport electrons independently of PSII, a pathway known as a cyclic electron transport (CET). The FNR reaction with Cyt bf implies a role in CET, and it has been suggested that FNR could be involved in modulating the two photosynthetic energy flows. FNR is not a membrane protein but is also involved as a component of CET super complex, which generates a proton gradient that results in ATP synthesis, with minimal water splitting and reduction of $NADP^+$. The functional implication of FNR in photosynthetic electron partitioning has

led to interest in the mechanism and location of FNR binding to the thylakoid membrane (Fig. 12.1).

FNR is an ubiquitous Fd-dependent enzyme containing a flavin adenine dinucleotide (FAD) as a prosthetic group, which generally catalyzes the reversible redox reaction between the electron carrier protein, Fd, and $NAD(P)^+/NAD(P)H$. Based on phylogenetic and structural information, FNRs are classified into two families; glutathione reductase (GR)-type and plant-type (Aliverti et al. 2008). The former is further divided into three groups; Adrenodoxin reductase (AdR)-like, Oxygenase-coupled NADH-ferredoxin reductase (ONFRs)-like and Thioredoxin reductase (TrxR)-like FNR. The latter is also divided into two groups; bacterial FNR that is involved in nitrogen fixation and response to oxidative stress, and plastidic FNR (Fig. 12.2). The group of plant-type plastidic FNR contains FNRs from green plants, algae and cyanobacteria. High-resolution crystal structures of several plant-type FNRs have shown that the FAD binding and NADPH binding domains are arranged sequentially in the primary structure (Karplus et al. 1991; Bruns and Karplus 1995; Deng et al. 1999; Dorowski et al. 2001; Kurisu et al. 2001) (Fig. 12.3).
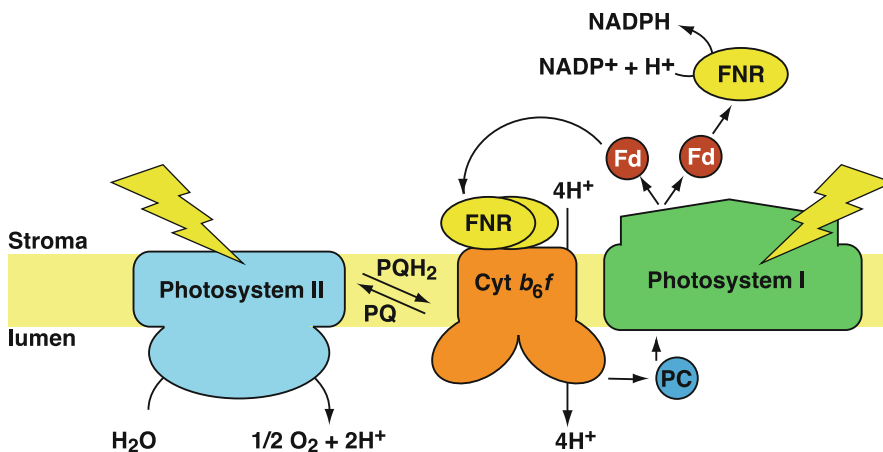
Fig. 12.1. Photosystem II, Photosystem I, and the cytochrome b_6f complex (Cyt b_6f) in the thylakoid membrane are responsible for oxygenic photosynthetic electron transport. The small electron transfer proteins, Ferredoxin (Fd) and Plastocyanin (PC), mediate electron transfer on the membrane. FNR functions in the two electron transfer pathways; linear and cyclic electron transport.

Plant-type FNR
— Plastidic FNR
— Bacterial FNR

GR-type FNR
— AdR-like FNR
— ONFRs-like FNR
— TrxR-like FNR
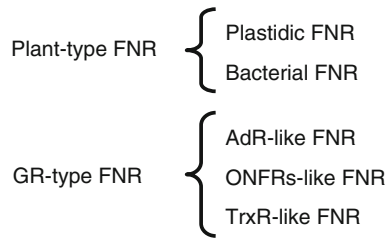
Fig. 12.2. Classification of FNRs. FNRs from higher plants and cyanobacteria are classified as Plastidic FNR. Those from purple and green sulfur bacteria are classified as Bacterial FNR and TrxR-like FNR, respectively.
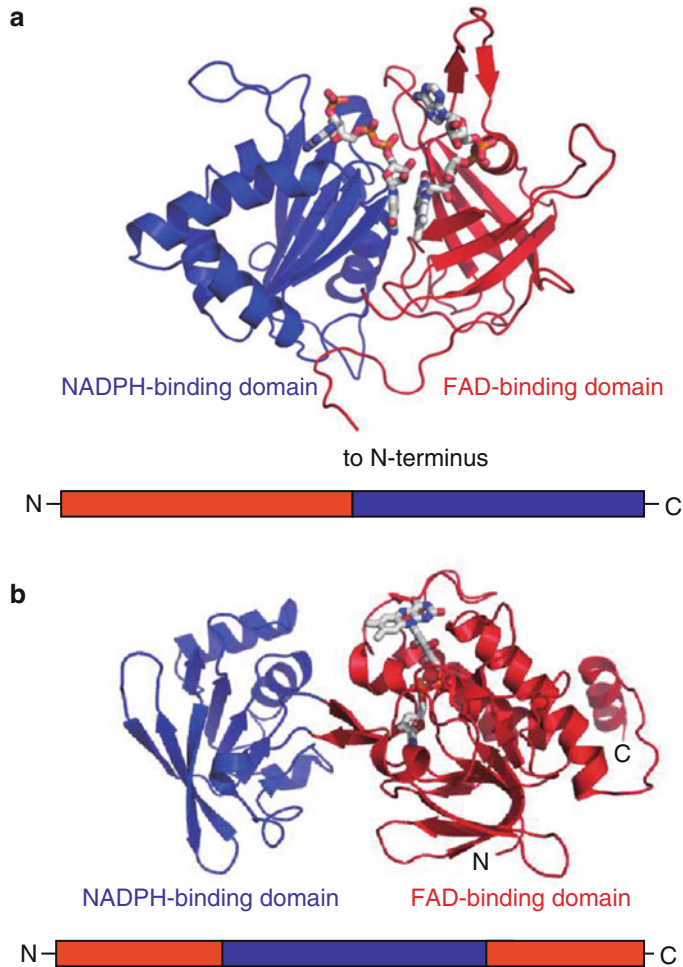
Fig. 12.3. Three-dimensional structure and domain organization of plant-type and GR-type FNRs. FAD and NADPH molecules are shown in ball-and-stick models. (**a**) Y308S mutant of pea FNR complexed with NADPH (PDB ID 1QFZ, chain A). (**b**) FNR from the green sulfur bacterium *Chlorobaculum tepidum* (PDB ID 3AB1, chain A.).

FNRs from purple bacteria belong to plant-type bacterial FNR (Nogués et al. 2005; Bortolotti et al. 2009, 2014), while that from green sulfur bacterium is classified as GR-type TrxR-like FNR (Seo and Sakurai 2002; Muraki et al. 2010) (Fig. 12.3).

FNR from green plants is localized mainly in chloroplasts. FNR in the leaf (L-FNR), which is bound to the thylakoid and also present as a soluble protein in the chloroplast stroma, catalyzes the photosynthetic reduction of $NADP^+$ to NADPH via reduced Fd (Hanke et al. 2005; Lintala et al. 2007). It has been reported that FNR in higher plant chloroplasts is localized peripherally on the stromal side of thylakoid membranes through association with the intrinsic proteins; PSI (Andersen et al. 1992), Cyt *bf* (Zhang et al. 2001; Okutani et al. 2005), NAD(P)H dehydrogenase complex (Quiles et al. 2000), TROL (Juric et al. 2009) and Tic62 (Benz et al. 2009). Among them, only Tic62 has been structurally characterized as an FNR binding-protein (Alte et al. 2010), and this protein was recently identified in the model plant organism, Arabidopsis (*Arabidopsis thaliana*). The others protein directly participate in photosynthesis, but Tic62 has no direct photosynthetic role. It seems rather to stabilise FNR. An FNR binding region containing a Ser/Pro-rich motif has been identified in both TROL and Tic62. In the X-ray structure of the complex between FNR and Tic62 polypeptide, FNR dimerizes around this Ser/Pro-rich motif of the peptide (Fig. 12.4). This interaction increases the surface area available for FNR:FNR binding from 380 $Å^2$ to 2070 $Å^2$. The strength of this binding interaction is reported to be pH-dependent (Alte et al. 2010).

In the detergent-soluble Cyt *bf* from spinach (*Spinacia oleracea*) leaves, L-FNR was tightly bound after exposure to hydrophobic extraction at high ionic strength, hydrophobic chromatography, and sucrose gradient separation. L-FNR bound to Cyt *bf* was enzymatically active (Zhang et al. 2001), implying a functional role of the Cyt *bf* complex in the CET pathway.

FNRs from green plants are encoded by a small multiple gene family as shown by the recent whole-genome analysis of model plants (Duvick et al. 2008). The presence of multiple isoenzymes at the protein level has been reported in several plant species. Maize (*Zea mays*) leaves contain three separate FNR isoproteins (L-FNR1, L-FNR2, L-FNR3). Although their capacity for electron transfer is similar, these three FNRs have distinct membrane association patterns. L-FNR1 is restricted to the thylakoid, L-FNR3 is only recovered from chloroplasts as a soluble protein, while L-FNR2 is found in both membrane-bound and unbound states. In order to determine whether the interaction between L-FNR and Cyt *bf* is isoenzyme specific, a Cyt *bf* partially purified from maize leaves was analyzed by SDS-PAGE (Okutani et al. 2005). Proteins of L-FNR1 and L-FNR2 in roughly equal quantities were co-purified with Cyt *bf*, and their identity was confirmed by western blotting.

Unlike C3 plants such as spinach or Arabidopsis in which FNR has been studied extensively, maize is a C4 plant and therefore contains two differentiated photosynthetic cell types. Mesophyll cells perform predominantly LET, while bundle sheath cells have very low PS II activity, and so utilize mainly CET. Using maize as a model therefore provides an opportunity to discover what determines the different membrane association behavior of FNR proteins, and how Fds contribute to two different photosynthetic electron transport pathways, LET and CET. In maize (*Zea mays*), two ferredoxin isoproteins, Fd I and Fd II, are expressed specifically in mesophyll and bundle-sheath cells, respectively (Kimata and Hase 1989). The two cell type-specific Fds differentially modulate electron flow around photosystem I (Fig. 12.5). Two cDNAs encoding Fd I and Fd II were introduced separately into the cyanobacterium *Plectonema boryanum* with a disrupted endogenous Fd. The resulting transformants expressing maize Fd I and Fd II in place of the endogenous cyanobacterial Fd showed significant differences in the two
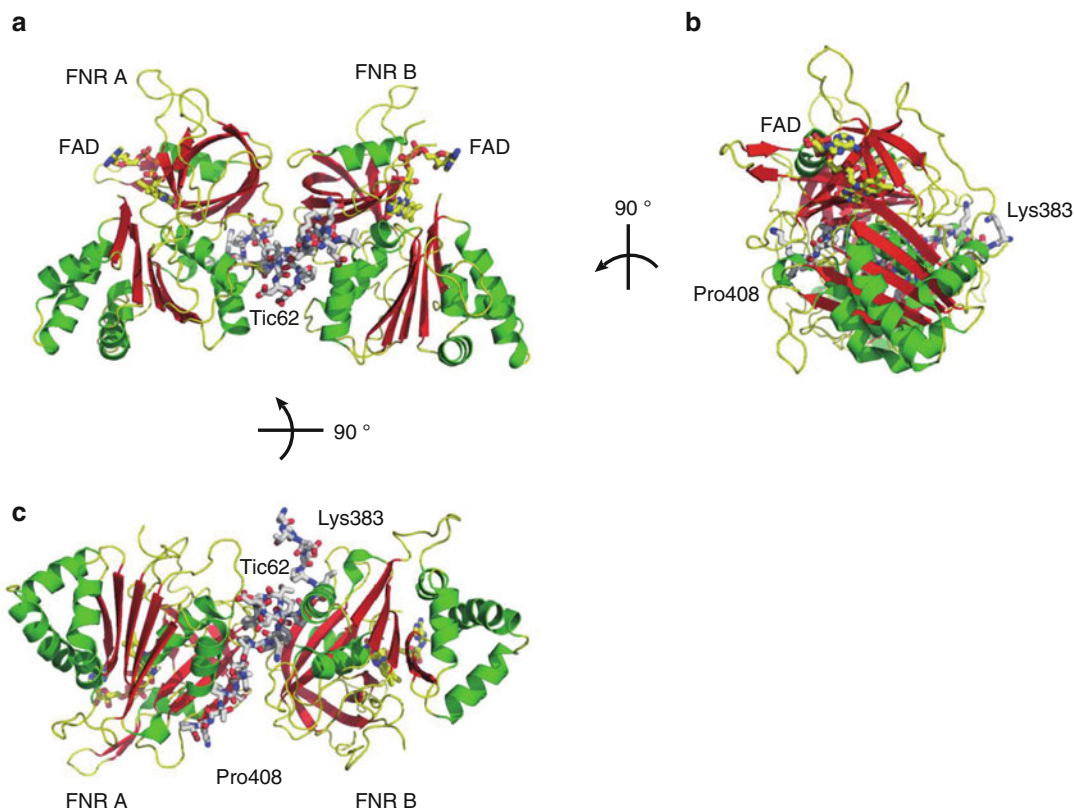
Fig. 12.4. Complex structure of the pea FNR and the C-terminal peptide of Tic62. Ribbon drawings of the complex viewed from (**a**) the front, (**b**) the side, and (**c**) the bottom (PDB ID 3MHP) of the complex. Peptide of Tic62 peptide drawn in ball-and-stick models with *white carbon atoms* is sandwiched by two FNR molecules. FAD molecules are also drawn in ball-and-stick with *yellow carbon atoms*.

pathways, thus causing marked changes in the balance of cellular levels of ATP and NADPH (Kimata-Ariga et al. 2000).

As mentioned above, maize contains three chloroplast L-FNR proteins with totally different membrane association. It has been confirmed that these three proteins have a variable distribution between cells conducting predominantly CET (bundle sheath) and LET (mesophyll), which is similar but not so simple as the case of Fd I and Fd II. Membrane-bound forms were detected in both the bundle sheath and mesophyll cells, with the ratio of L-FNR1 to L-FNR2 higher in the bundle sheath than the mesophyll cells. By comparison, soluble proteins were only detected in the mesophyll cells, where L-FNR2 is abundant and L-FNR3 is poor (Twachtmann et al. 2012).

The higher portion of membrane bound L-FNRs distributed together with Cyt *bf* in the bundle sheath therefore correlates with a higher demand for CET, while the greater abundance of soluble FNR occurs in cell types with a greater demand for LET in mesophyll cell.

Although there are several high-resolution X-ray structures of plant-type FNR are available, the exact binding mode of L-FNR to Cyt *bf* is still elusive (Karplus et al. 1991; Bruns and Karplus 1995; Deng et al. 1999; Dorowski et al. 2001; Kurisu et al. 2001). A large hydrophobic cavity in the $NADP^+$ binding domain of the crystal structure of spinach L-FNR was suggested as a possible binding region to the membrane, when the first structure of plastidic FNR was solved (Karplus et al. 1991; Bruns and Karplus

```
Fd2  1  ATYNVKLITP EGEVELQVPD DVYILDF AEE EGIDLPFSCR AGSCSSCAGK 50

Fd1  1  ATYNVKLITP EGEVELQVPD DVYILDQ AEE DGIDLPYSCR AGSCSSCAGK 50

         ********** ********** ******  *** :*****:*** **********


Fd2 51  VVSGSVDQSD QSFLNDNQVA DGWVLTC AAY PTSDVVIETH KEDDLL   96

Fd1 51  VVSGSVDQSD QSYLDDGQIA DGWVLTC HAY PTSDVVIETH KEEELTGA 98

         ********** **:*:*.*:* *******  ** ********** **::*
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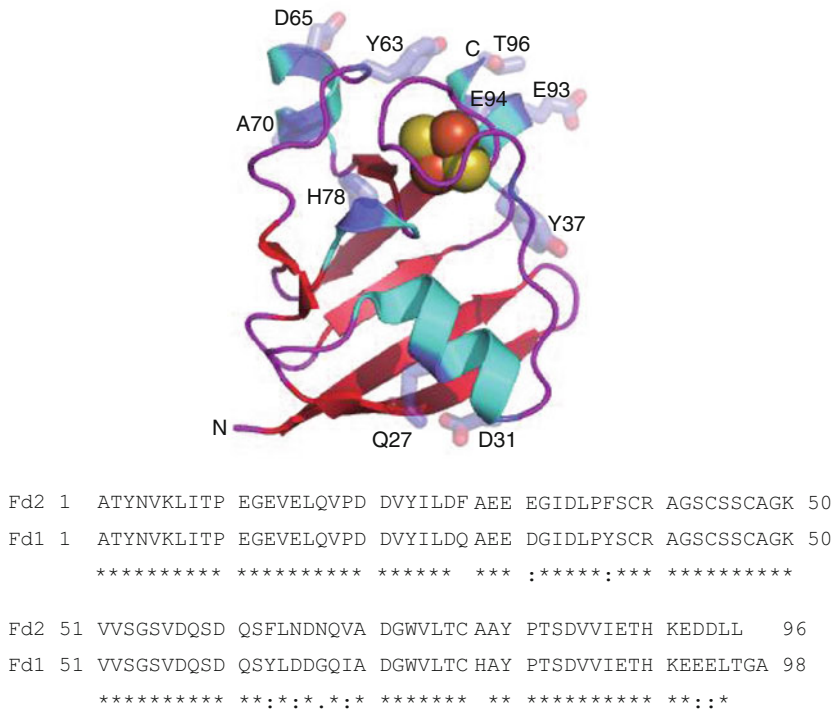
Fig. 12.5. Crystal structure of maize Fd I (PDB ID 3B2F) and amino acid sequence alignment of Fd I and Fd II from maize. Side chains of non-conserved residues are shown in transparent stick models that are mainly distributed on the surface surrounding the [2Fe-2S] cluster on the top of the molecule.

1995). Then, the crystal structure analysis of L-FNR from pea, paprika and maize was obtained (Deng et al. 1999; Dorowski et al. 2001; Kurisu et al. 2001). Later, the crystal structures of maize L-FNR isozymes, L-FNR1-3, became available (Twachtmann et al. 2012). The three structures of L-FNR1-3 resemble each other with the major differences between them being the orientation of the N-terminal 20 residues and the interface of back-to-back dimer formation (Fig. 12.6). All three maize FNRs were crystallized as a dimer in the crystallographic asymmetric unit. The dimeric arrangement of L-FNR1 and L-FNR2 is very similar to that of pea L-FNR complexed with the C-terminal peptide of Tic62, but that of soluble L-FNR3 is obviously different from the others. It should be noted that two molecules of maize L-FNR1 proteins interacted with two N-terminal regions extended from crystallographic neighboring molecules (2:2 stoichiometry). This

type of interaction at the crystallographic dimer interface of L-FNR can be found also in the structure of pea L-FNR mutants (PDB ID:1QFY), and would be widely distributed in the case of membrane bound FNRs.

In the LET participated by soluble FNRs, the resulting NADPH fuels carbon fixation by the Calvin cycle and drives other reductive metabolism in the stroma or outside the chloroplast. In higher plant roots, genetically distinct root-type FNR (R-FNR) enzymes catalyze the NADPH-dependent reduction of Fd to provide reducing power to the many Fd-dependent enzymes involved in assimilation of nitrogen and sulfur, etc. Contrary to L-FNRs, R-FNR exists as a soluble form and it does not interact with the thylakoid membrane (Aliverti et al. 2001). Although the crystal structure of R-FNR from maize exhibited some unique structural features distinct from L-FNRs, the entire structure including the characteristic of molecular sur-
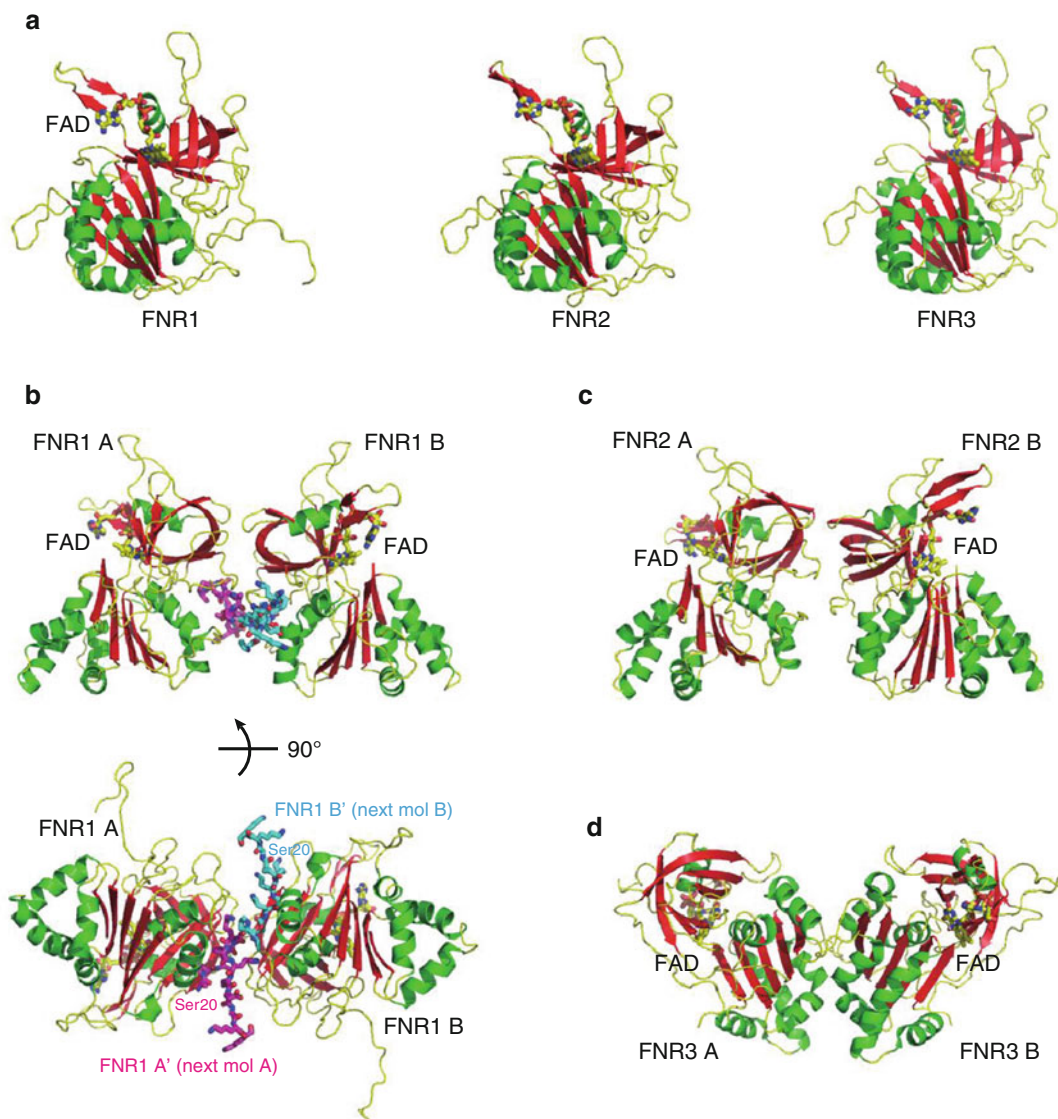
Fig. 12.6. Crystal structures of maize L-FNR1 (PDB ID 1GAW), L-FNR2 (PDB ID 3VO1) and L-FNR3 (PDB ID 3VO2). (**a**) monomeric forms, and (**b**) the crystallographic dimer of L-FNR1, (**c**) L-FNR2 and (**d**) L-FNR3. Interactions between the dimeric L-FNR1 and the N-terminal regions that extend from crystallographic neighboring molecules are highlighted in *cyan* and *magenta* stick models in (**b**).

face is quite similar to that of soluble L-FNR3 (Fig. 12.7). Soluble FNRs have shown an independent dimerization feature distinct from those of membrane-bound FNRs.

The Cyt *bf* is a dimeric trans-membrane complex consisting of eight polypeptide subunits per monomer; cytochrome *f*, cytochrome b_6, the Rieske iron-sulfur protein (ISP), and subunit IV as well as four small hydrophobic subunits, PetG, PetL, PetM, and PetN (latest structure, 2.5 Å, PDB ID 4OGQ). To date, X-ray crystal structures of the Cyt *bf* complex have been obtained from the cyanobacteria *Mastigocladus laminosus* (Hasan et al. 2013a; Yamashita et al. 2007; Kurisu et al. 2003), *Nostoc*
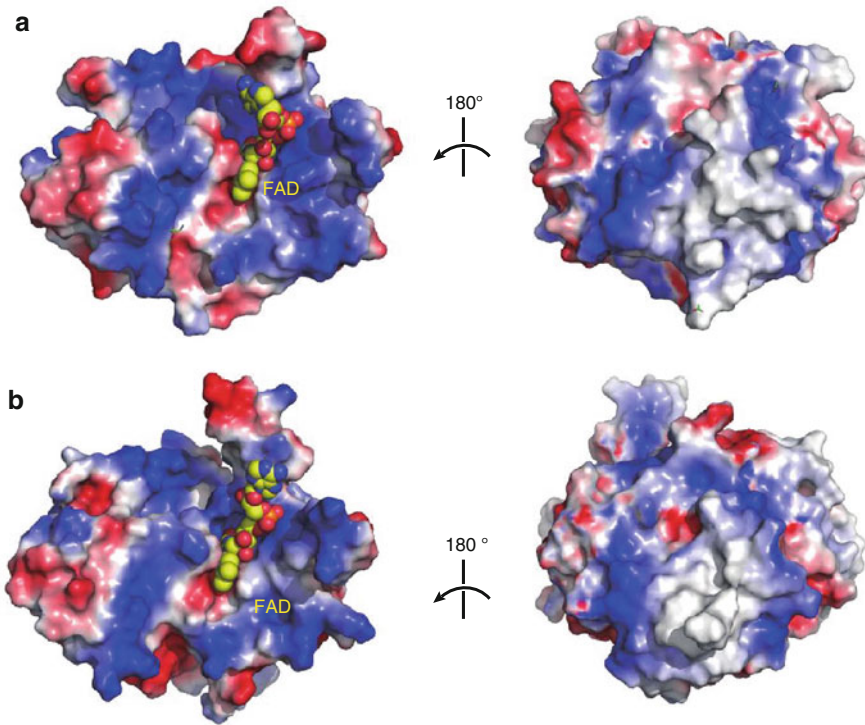
Fig. 12.7. Comparison of the molecular surfaces of soluble FNRs. Electrostatic potentials mapped on the molecular surface of (**a**) L-FNR3 (PDB ID 3VO2) and (**b**) R-FNR (PDB ID 3LVB), both of which exist in a soluble form.

PCC7120 (Hasan and Cramer 2014; Hasan et al. 2013b; Baniulis et al. 2009), and the green alga *Chlamydomonas reinhardtii* (Stroebel et al. 2003). Although there are several differences between these X-ray structures, the arrangement of eight subunits and composition of prosthetic groups are fundamentally the same. In the recently solved X-ray structure of Cyt *bf* from *Nostoc* PCC7120 at the highest resolution of 2.5 Å, it is proposed that the niche formed between the F and G trans-membrane helices (subunit IV) may represent the binding site for the LHCII kinase for state transition, based on the structural comparison between the mitochondrial Cytochrome bc_1 complex (Cyt bc_1) and Cyt *bf* and the non-physiological binding of augmented synthetic lipid (Hasan et al. 2013c). Compared to the case of LHCII kinase, biochemical evidence is not available to determine the binding site for FNR to Cyt *bf* complex. However, a possible pattern of

the interaction can be structurally predicted referring to the high-resolution structures of Cyt *bf* and membrane-bound FNR (L-FNR1).

Cyt *bf* has dimensions of 90×55 Å on the lumen side and of 120×75 Å on the stromal side. The lumen side of the membrane has two large extrinsic domains, cytochrome *f* and the ISP. The hydrophobic trans-membrane region has formed the functionally indispensable quinone/quinol-exchanging cavities between two monomers. In contrast, the stromal side has no globular domains but has only short loops and several chain termini, which have not been functionally annotated yet. If the back-to-back arrangement of FNR tethering discussed above is common to the case of interaction with Cyt *bf* complex, the structure of Cyt *bf* must have interacting terminal polypeptides on the stromal side of the membrane. There are four candidates
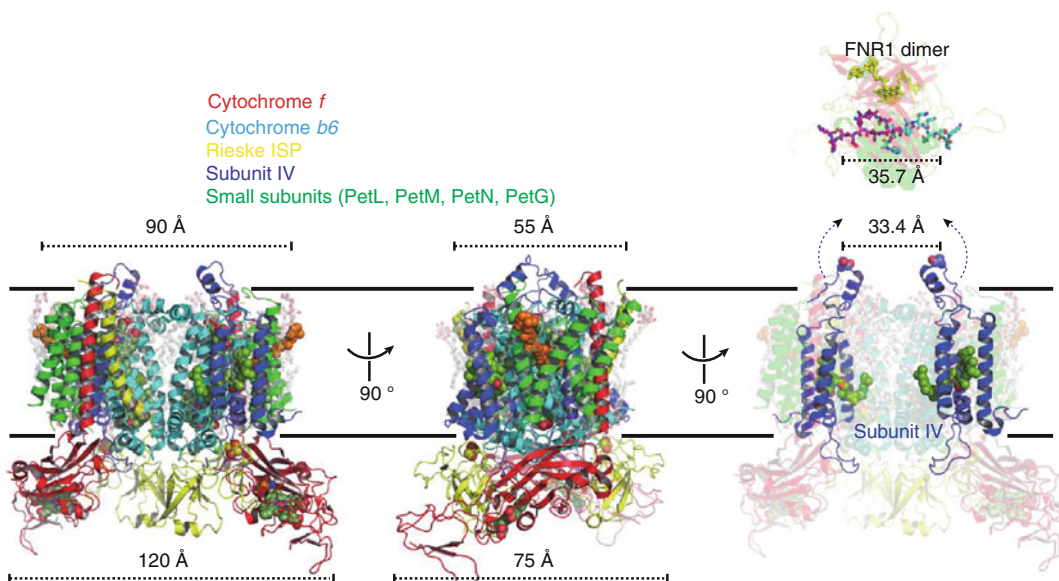
Fig. 12.8. Crystal structure of cyanobacterial Cyt *bf* (PDB ID 4OGQ) and the membrane-bound FNR (L-FNR1, PDB ID 1GAW) viewed normal to the plane of the membrane. Cyt *bf* is colored in each subunit: Cytochrome *f* in *red*, Cytochrome b_6 in *cyan*, the Rieske-ISP protein in *yellow*, subunit IV in *blue*, and four small subunits in *green*. Molecules of detergents and lipids are shown in transparent stick models. Hemes, iron-sulfur clusters, chlorophylls and carotenoids are drawn in CPK models. In the *right panels*, two pairs of subunit IV molecules in the Cyt *bf* and the neighboring N-terminal peptide proposed to interact with FNRs are highlighted. Dimensions of Cyt *bf* and the particular distances written in the text are also shown.

from major functional subunits of Cyt *bf*; the N-terminus of cyt *f*, the C-terminus of the ISP, the N-terminus of cyt b_6 and the N-terminus of subunit IV (Fig. 12.8). The C-terminal end of cyt b_6 can be excluded because it is structurally occluded by subunit IV and PetG.

Based on the L-FNR1 crystal structure, the length of the N-terminal polypeptides interacting with crystallographic adjacent FNR molecule is 35.7 Å (Fig. 12.8, Cα carbon of Ser 20, Maize numbering). Possible counterparts of Cyt *bf* must have the flexible terminal regions with corresponding distances. The two ends of the secondary structure (Fig. 12.8, Cα carbons of Ser 10, *Nostoc* PCC7120 numbering) of subunit IV are 33.4 Å apart, while those of cyt *f*, the ISP and cyt b_6, are separated by 77, 66 and 58 Å, respectively. Furthermore, the N-terminal region of subunit IV protrudes from the trans-membrane region. It looks favorable for the interaction of globular L-FNR1.

According to this structural information, it is proposed that L-FNR/Cyt *bf* interacts though the N-terminal regions of subunit IV. Supporting structure evidence can be obtained from the comparison between two structures of Cyt *bf* from *Nostoc* and *Chmalydomonas*. Structural plasticity indicated between two structures around Pro12 of subunit IV has suggested the possible rearrangement of polypeptide when interacting with membrane-bound L-FNRs. Based on these structural observation, the N-terminal region of subunit IV may have the highest probability for a binding site of L-FNR.

Involvement of subunit IV in the L-FNR/Cyt *bf* interaction implies that Cyt *bf* activity might be tuned by complex formation. The rate-limiting step of the oxygenic photosynthetic electron transport chain is located in the proton-coupled electron transfer through the Cyt *bf* complex. There is a consensus that the coupling of the redox-

coupled trans-membrane proton transfer to the p-side aqueous phase limits the rate of electron transfer. In the presence of low intensity illumination and/or small $\Delta \mu_H^+$, the 26 Å distance measured between the [2Fe-2S] cluster and the heme of cytochrome f in the Cyt bf crystal structure would seem to impose a major barrier to electron transfer (Heimann et al. 2000; Yan and Cramer 2003). A second possibility for a kinetic limitation is oxidation of the bulky lipophilic quinol. Intra-molecular quinone/quinol exchange cavity has a portal to the Q_p site of Cyt bf. The bent trans-membrane helix (F-helix) of Subunit IV forms a wall of the exchange portal. Based on the mutagenesis of this F-helix, it is proposed that a rate limitation of electron transport in Cyt bf may arise from steric constraints in the portal to Q_p site (Hasan and Cramer 2012). Since the N-terminal region of subunit IV is structurally close to the stromal end of F-helix (for example, an Cα distance between Lys20 and Lys119 is 10.95 Å), it may also be relevant for possible tuning of the activity in the membrane through the L-FNR/Cyt bf interaction.

In the case of cyanobacterial FNR, early reports suggested the unique membrane localization pattern of FNR is totally different from that of plastidic FNR. The molecular mass of a typical cyanobacterial FNR is similar to that of higher plants (ca. 35 kDa) (Serre et al. 1996; Hermoso et al. 2002). However, the FNR encoded by a cyanobacterial gene (*petH*) was reported to have an estimated molecular mass of 45 kDa. The larger than anticipated molecular mass of this protein arises from the presence of a small domain attached to the N-terminus of NAD(P)H binding domain, which displays significant sequence similarity with the phycobilisome (PBS) binding protein CpcD. It has been proposed that the full-length cyanobacterial FNR is located in close proximity to the thylakoid membrane through the binding to PBS (van Thor et al. 1999, 2000; Arteni et al. 2009). Three forms of FNR can be purified from the thermophilic cyanobacterium

Thermosynechococcus elongatus BP-1: (i) a 34 kDa form lacking the CpcD-like domain (probably due to proteolytic cleavage at the hinge region); (ii) a 45 kDa form with an intact CpcD-like domain; and (iii) a 78 kDa form in complex with phycocyanin, which is a major pigment-protein complex of PBS (Nakajima et al. 2002). Although the three-dimensional arrangement of the CpcD-like domain relative to the other domains is still unclear, it is noted that cyanobacteria have a unique system utilizing PBS to tether the FNR close proximity to the membrane probably suitable for CET.

Although the apparatus of anoxygenic photosynthesis is very diverse, coupling of electron and proton transfer mediated by the Cyt bf or bc_1 complex is indispensable for the photosynthetic electron transfer pathways except for the green thermophilic photosynthetic bacterium *Chloroflexus aurantiacus* that has an alternative complex III (Gao et al. 2010; Tang et al. 2011). In contrast to the case of Cyt bf or bc_1, purple and green sulfur bacteria may or may not have FNRs for the light-dependent reactions. The green sulfur bacterium *Chlorobaculum tepidum* (formerly *Chlorobium tepidum*) possesses an iron-sulfur type reaction center which is evolutionarily related to photosystem I in chloroplasts and is able to reduce Fd photosynthetically. It was anticipated that *C. tepidum* might possess a homologue of the plant-type FNR because the electron transfer reactions involving Fd are quite similar to those that occur in the chloroplast. Instead, *C. tepidum* has a GR-type FNR distinct from previously characterized plant-type FNRs distinct in its oligomerization state and amino acid sequence (Seo and Sakurai 2002; Muraki et al. 2010). Since anoxygenic phototynethetic bacteria like *C. tepidum* are living under much simpler photosynthetic conditions, i. e., shallow lakes and marshes near volcano producing H_2S gas, it seems that there is no need and no chance for *C. tepdium* FNR to interact with the Cyt bc complex by environmental adaptation.

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Chapter 13

Alternative Rieske Iron-Sulfur Subunits and Small Polypeptides of Cyanobacterial Cytochrome b_6f Complexes

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Summary

The cytochrome (cyt) b_6f complex is a central component of both, photosynthetic and respiratory electron- and proton transport processes in cyanobacteria. Among its eight *bona fide* subunits, the Rieske [2Fe-2S] protein is encoded by multiple genes in most cyanobacterial genomes. However, the significance of the resulting protein heterogeneity is essentially not yet understood. The following chapter provides an overview on the Rieske [2Fe-2S] protein diversity in cyanobacteria, and related aspects. In addition, potential roles of small cyt b_6f complex subunits are discussed.

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I. Introduction

About 50 years ago, John S. Rieske and his co-workers discovered a new type of iron-sulfur protein, which later became known as 'Rieske' proteins (Rieske et al. 1964). The catalytic center of these proteins is formed by a unique [2Fe-2S] cluster. In contrast to the bis-cysteine ligation found in ferredoxin-type [2Fe-2S] clusters, one of the two Fe ions is coordinated by two histidine residues in Rieske-type [2Fe-2S] clusters (Fig. 13.1) (Liu et al. 2014). Two inorganic sulfide ions bridge the two Fe ions, forming together a flat, rhombic cluster. Rieske proteins are essential subunits of different types of bacterial non-heme oxygenases (Ferraro et al. 2005), and of all cyt *bc* complexes, *i.e.,* cyt bc_1 (complex III), cyt b_6f and related complexes. Hence, Rieske proteins have a central role in biological energy transduction in most living organisms (Dibrova et al. 2013).

3D structures of various dimeric cyt *bc*-type complexes are available at high resolution, including structures of the cyt b_6f complexes of the green alga *Chlamydomonas reinhardtii* and three cyanobacteria *Mastigocladus laminosus*, *Nostoc* sp. PCC
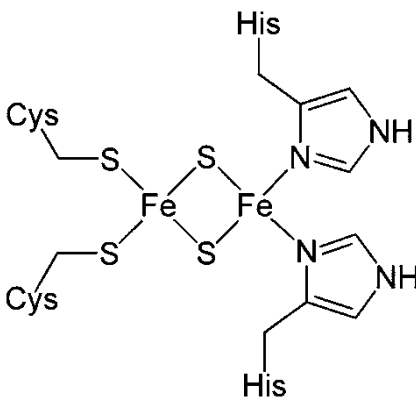
Fig. 13.1. Catalytic center of Rieske-type [2Fe–2S] proteins.

7120, and *Fremyella diplosiphon* SF33 (Fig. 13.2) (Stroebel et al. 2003; Kurisu et al. 2003; Yamashita et al. 2007; Baniulis et al. 2009; Hasan and Cramer 2014; Agarwal et al. 2015). A cyt b_6f monomer consists of the four large (17.5–32 kDa) subunits: cyt f (PetA), cyt b_6 (PetB), the Rieske [2Fe-2S] protein (PetC) and subunit IV (SU IV, PetD), as well as of four small (3.3–4.1 kDa) subunits (PetG, −L, −M and −N) at unit stoichiometry (for a more detailed description see the Chap. 9 by Cramer and Hasan in this volume). The typical Rieske [2Fe-2S] protein consists of 180–183 amino acids and has a molecular mass of ∼19 kDa (Schneider and Schmidt 2005). The protein consists of a single N-terminal transmembrane helix and a large soluble domain of ∼140 residues, located in the thylakoid lumen (Fig. 13.2). The two domains are connected by a flexible hinge region (Fig. 13.3). Within a cyt b_6f homodimer, the membrane-spanning α-helical anchor is swapped and the soluble domain of one Rieske monomer is anchored to the membrane by a transmembrane helix, which is part of the adjacent monomer. According to recent data, the domain-swapped structure is necessary for function (see below) but not essential for maintaining the dimeric structure of the complex (Agarwal et al. 2015). The soluble Rieske protein domain mainly consists of β-sheets and can be further divided into a large and a small subdomain (Carrell et al. 1997) with the Rieske [2Fe-2S] center being localized in the small subdomain (Fig. 13.3). Its ordinary form (see below) has a midpoint potential of 300–320 mV (Nitschke et al. 1992; Zhang et al. 1996; Schneider et al. 2002).

As discussed in greater detail in the Chap. 9 by Cramer and Hasan, a central feature of the cyt b_6f complex is the bifurcation of electron transport at the Q_o (or Q_p) plastoquinol (PQH$_2$) binding site, which is located close to the lumenal surface of the thylakoid membrane. One of the two electrons carried by PQH$_2$ is transferred to the energetically favored, so-called high-potential chain, which gives rise to a downhill electron transport
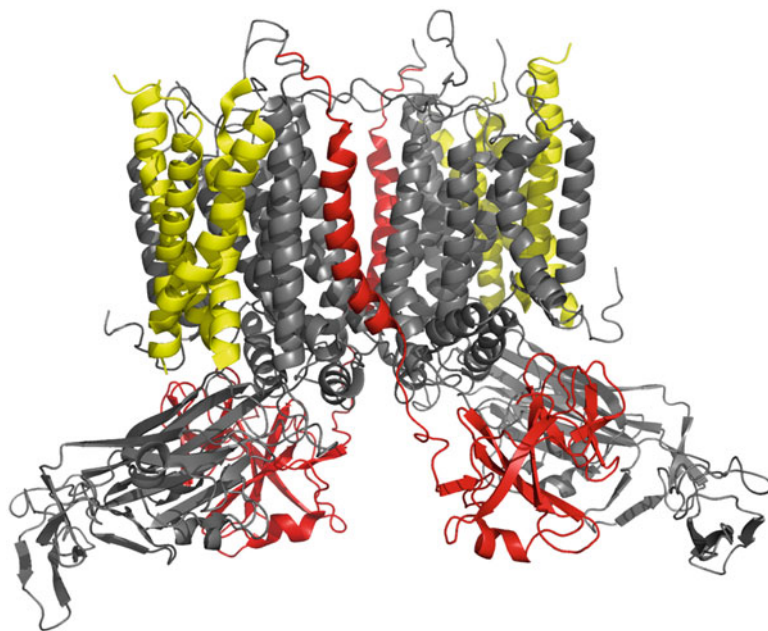
Fig. 13.2.   Side view of a dimeric cyt b_6f complex (PDB, 1vf5; Kurisu et al. 2003). Most α-helices of the complex are membrane integrated, whereas the β-sheet-dominated parts are the water-soluble domains, which extend into the thylakoid lumen. The two Rieske subunits of the complex are colored in *red*, the small subunits are marked in *yellow*, and all other subunits are shown in *gray*. While each small subunit has only a single membrane spanning transmembrane helix, the Rieske [2Fe—2S] polypeptide consists of two domains: a membrane-anchored transmembrane helix and a large water-soluble domain, which are connected by a flexible hinge region. These two domains are swapped between the two monomers in the cyt b_6f homodimer. For further details see Fig. 13.3 and the text.

towards plastocyanin/cyt c_6 and photosystem I (PS I) via the Rieske [2Fe-2S] cluster and cyt *f*. The Rieske [2Fe-2S] center is 8–10 Å (edge-to-edge) away from the bound quinol with a leucine and a histidine residue in between (Fig. 13.3). After reduction of the Rieske [2Fe-2S] cluster, the electron is subsequently transferred to cyt *f* via movement ("tethered diffusion") of the soluble domain of the Rieske [2Fe-2S] protein towards cyt *f* (Heimann et al. 2000; see also the Chap. 11 by Huang and Berry in this volume). Beyond participating in electron transport, the Rieske [2Fe-2S] center is also involved in PQH_2 deprotonation. As proposed by Crofts (Crofts et al. 2000; Crofts 2004), one of the two electrons and protons of the quinol, respectively, is transferred in a concerted action to the [2Fe-2S] center, which results in protonation of one of its histidine ligands. This proton can thereafter be released into

the thylakoid lumen via an internal water bridge located within cyt *f* (Martinez et al. 1996; Ponamarev and Cramer 1998; Cramer et al. 2006). The second electron is "recycled" through the so-called low-potential chain, which consists of a *b*-type heme (cyt b_L) and a *b*-type/*c*-type heme couple (cyt b_H/cyt c_i) transferring electrons across the membrane to the Q_i (or Q_n) quinone-binding site, where PQ molecules are reduced back to PQH_2 in the so-called Q-cycle (Mitchell 1975; see also the Chaps. 9, 10 and 21 by Cramer and Hasan, Esser et al. and Fisher et al.). The second proton may predominantly be released via Glu78, which is part of a conserved Pro-Glu-Trp-Tyr (PEWY) sequence in the *ef* loop of SU IV (Zito et al. 1998; Finazzi 2002; Cramer et al. 2006).

The four small cyt b_6f subunits, PetG, −L, −M and −N, are located at the periphery of the complex and form a so-called "picket
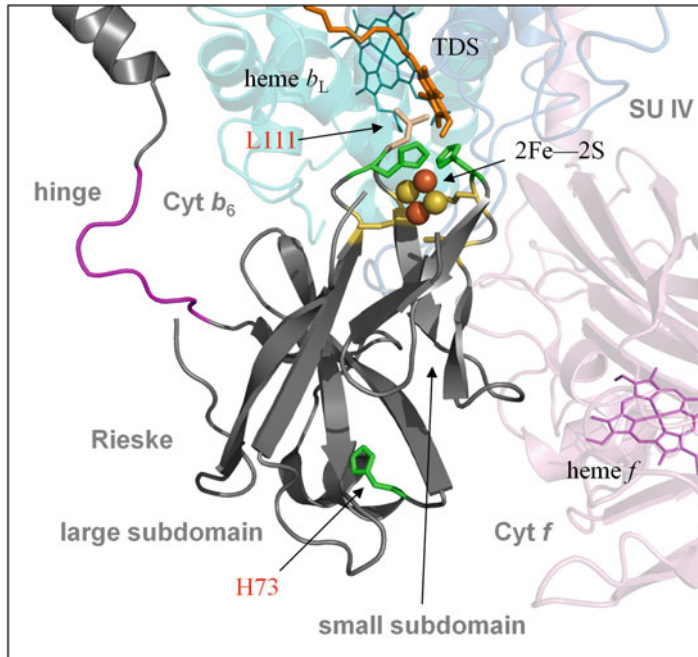
Fig. 13.3. The soluble domain of the Rieske [2Fe—2S] protein and its molecular environment (PDB, 1vf5 and 2e76; Kurisu et al. 2003 and Yamashita et al. 2007, respectively). The Q$_o$ plastoquinol binding site is shown with the bound quinone analog tridecyl-stigmatellin (TDS, *orange*). While subunits cyt b_6, SU IV, and cyt f are marked by transparent *cyan*, *sky blue*, and *pink*, respectively, the Rieske [2Fe—2S] subunit is colored in *grey*. For clarity, residues 142–144 and the last 16 residues of the Rieske C-terminus are omitted. The following color codes have been used: Rieske [2Fe—2S] center (*yellow/orange spheres*), hemes b_L and f (*cyan and pink lines*, respectively), four cysteine and two histidine residues around the [2Fe—2S] cluster (*yellow* and *green sticks/cartoons*, respectively), His73, part of a potential metal binding site (*green sticks/cartoon, red identifier*), the leucine residue (Leu111) located between the [2Fe—2S] center and the bound quinol (*wheat sticks/cartoon, red identifier*), hinge region between water soluble domain and transmembrane helix of the Rieske [2Fe—2S] protein (*magenta*). Transmembrane helix (*upper left*) and the water soluble domain of the Rieske protein (*center*) belong to opposing monomers. For details see text.

fence" structure (Fig. 13.2) (Stroebel et al. 2003; Kurisu et al. 2003; Yamashita et al. 2007; Baniulis et al. 2009; Hasan and Cramer 2014). Besides their important role in assembly and/or stability of the cyt b_6f complex, there are also experimental findings suggesting regulatory functions of these polypeptides. Beyond the eight *bona fide* cyt b_6f subunits, there is evidence for further protein components interacting transiently and/or loosely with the cyt b_6f complex. Though data strongly indicate involvement in electron transport and/or signal transduction processes of some of these partners, the nature of most of these interactions are still poorly understood.

II. Multiple Rieske-Proteins and Cytochrome b_6f Heterogeneity

In higher plants and green algae, as well as in some cyanobacteria, such as *Thermosynechococcus elongatus* and *Gloeobacter violaceus*, the Rieske protein of the cyt b_6f complex is encoded by a single *petC* gene. In chloroplasts as well as in cyanobacteria, this canonical *petC* gene is organized in an operon together with the *petA* gene coding for cyt f (Kallas et al. 1988). However, the vast majority of cyanobacterial genomes display a family of different *petC* genes. In most of these cyanobacteria, including the well-

```
PetC1    1   MTQISGSPDVPDLGRRQFMNLLTFGTITGVAAGALYPAVK   40
PetC2    1   -MDNTQAIAPPSYSRRQLLNFLAGTTVAVTASAGAYAMGK   39
PetC3    1   ----------MVKRRKLISYTAFSTAIAVITGCF-----   24

PetC1   41   YLIPP-SSGGSGGGVTAKDALGNDVKVTEFLASHNAGDRV   79
PetC2   40   FFVPPAEKGGAGGGIIAKDVLGNPIPASQILA-EAPGTRA   78
PetC3   25   ---GGNSSNGQGQ----------TVNVGTMADLKAKG--E   49

                                              Box I
PetC1   80   LAQGLKGDPTYIVVQGDDTIANYGINAVCTHLGCVVPWNA  119
PetC2   79   LVAGLAGDPTYLIVKEDGSLDSIGIVDSCTHLGCTFPWNG  118
PetC3   50   LKGNTPKGP-VTVVPNGNSGQISAVNPTCTHNGCQVNWKK   88

         Box II
PetC1  120   SENKFMCPCHGSQYNAEGKVVRGPAPLSLALAHATVTDDD  159
PetC2  119   NDQEFQCPCHGSRYHPDGSVARGPAPLPLKIVQVAVVD-D  157
PetC3   89   ANGKFVCPCHGAEFAATGKVLKGPAIRDLPTYATQVSGNN  128

PetC1  160   KLVLSTWTETDFRTDEDPWWA                     181
PetC2  158   QIFISPWTDLDPRTGEKPWWV                     179
PetC3  129   ILVKA----------------                     133
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Fig. 13.4. Sequence alignment of the three PetC proteins of *Synechocystis* sp. PCC 6803. Identical residues, as well as conservative substitutions, are marked by the colored background. Iron-sulfur binding sites (Box I and II) are highlighted in *yellow*, while amino acids forming putative transmembrane helices are shown in *bold*. Potential metal binding PetC1-His73 and lipid binding PetC3-Cys23 residues are highlighted by *red* color. The position of the hinge region is *underlined*.

characterized cyanobacteria *Synechocystis* sp. PCC 6803, *Nostoc punctiforme* and *Synechococcus* sp. PCC 7002, three Rieske isoforms are encoded (PetC1–3), from which PetC1 is organized with *petA* in an operon and shows the highest similarity to the chloroplast PetC (Schneider et al. 2002). In *Synechocystis* sp. PCC 6803 PetC1, PetC2 and PetC3 are encoded by the open reading frames *sll1316*, *slr1185* and *sll1182* localized in the chromosome at positions 1166186–1166728 (543 bp), 3527045–3527581 (537 bp) and 1848301–1848702 (402 bp), respectively. Nucleotide sequences show 53 %, 49 % and 42 % identities between *petC1* vs. *petC2*, *petC1* vs. *petC3* and *petC2* vs. *petC3*, corresponding to 44 %, 33 % and 24 % identity on the amino acid sequence level. This indicates a much closer relationship between PetC1

and PetC2 than between any of them and PetC3. In some other cyanobacteria, such as *Nostoc* PCC 7120, even an additional fourth Rieske protein (PetC4) is encoded (for a review see (Schneider and Schmidt 2005); for a sequence alignment of PetC1-PetC3 see Fig. 13.4).

Homologous genes encoding different protein isoforms and the resulting heterogeneity in protein complex compositions are well described. One of the most prominent examples in biological energy transduction is the *psbA* gene family, which encodes different (up to five) copies of the D1 protein of PS II in cyanobacteria, expressed differently according to the light conditions (see e.g., Mulo et al. 2009; Nowaczyk et al. 2010). In general, heterogeneity within protein complexes allows organisms to acclimate to changing environmental

conditions. Under normal conditions, typically one gene product is predominantly active, whereas the other (typically silent) gene(s) are expressed only under certain physiological (often stress) conditions. In case of the cyanobacterium *Synechococcus* sp. PCC 7002, the deletion of the canonical *petC* gene (i.e., *petC1*) (Yan and Cramer 2003) was not tolerated, suggesting that only *petC1* encodes a functional Rieske protein. In contrast, *petC1* could be deleted successfully in *Synechocystis* sp. PCC 6803. In this organism, all three *petC* genes (*petC1–3*) were deleted individually; also, two *petC* double deletion strains could be generated, resulting in the generation of five Δ*petC* mutant strains in total. Importantly, only the Δ*petC1*/Δ*petC2* double deletion strain turned out to be lethal (Schneider et al. 2004a), suggesting that either PetC1 or PetC2 have to be present and they can, at least partially, substitute each other. This is in good agreement with their high sequence similarity (see above and Fig. 13.4) and their predicted similar midpoint potentials (Schneider et al. 2002, 2004b).

The above mentioned *Synechococcus* data, as well as an in depth *in vivo* analysis of the various *Synechocystis* PCC 6803 Δ*petC* mutant strains further supported the assumption that PetC1, which is phylogenetically closest to the group of chloroplast Rieske proteins (Schneider et al. 2002), is the major Rieske iron-sulfur protein in cyanobacteria.

Very recently, the structure of the water-soluble extrinsic domain of the canonical Rieske [2Fe–2S] protein of *T. elongatus* was determined at high (2 Å) resolution (Veit et al. 2012, PDB: 3azc). Three internal water molecules (wat202, wat204, and wat208) have been identified in the structure as well as an unidentified metal ion in close proximity to wat202, which is hydrogen bonded to Tyr69, Asp77, and Val91. Interestingly, His73 (Figs. 13.3 and 13.4), which is located in a loop together with Tyr69 and Asp77, has already been proposed to be part of a potential inhibitory Cu^{2+} binding site (Rao et al. 2000). Thus, the metal binding site identified in the crystal structure might have a regula-

tory function in the Rieske protein. Notably, a membrane facing hydrophobic pocket has also been identified, suggesting either the binding of a lipophilic ligand (e.g., plastoquinone, lipid, chlorophyll or carotenoid molecule), or of hydrophobic residues from another protein to this hydrophobic cavity. Especially plastocyanin, which binds to cyt b_6f (as it does to PSI) via both hydrophilic and hydrophobic domains (Crowley et al. 2001; Haddadian and Gross 2006), would be a good candidate as binding partner (see also the Chaps. 30 and 31 by Bendall and Howe, and Diaz-Moreno et al. in this volume). This is also in agreement with experimental data, which suggest an electron transfer through the cyt b_6f complex without involvement of cyt f (Fernández-Velasco et al. 2001). Remarkably, both the proposed metal-binding motif and the hydrophobic pocket are absent in the PetC2 Rieske variant, indicating differences in the function and/or regulation of PetC1 and PetC2, respectively.

Although cyt f re-reduction kinetics indicated very similar catalytic activities of PetC1- and PetC2-type [2Fe–2S] centers under near-single turnover conditions (Ranade and Kallas, unpublished), the δ*petC1* mutant shows a remarkably low electron transport rate through the cyt b_6f complex. However, it could be shown that this is caused by the low cyt f content in the δ*petC1* strain, due to co-expression with *petC1* in the *petCA* operon (Schneider et al. 2002). Eventually, this results in low amounts of functional PetC2– cyt b_6f complexes (Schultze et al. 2009; Tsunoyama et al. 2009), causing concomitant accumulation of PQH_2 and a severely reduced capability ($\leq$30 %) to perform state transitions (Tsunoyama et al. 2009; Krause, Bernát and Rögner, unpublished; Ranade and Kallas, unpublished). These are accompanied by a significantly increased sensitivity towards high light stress, a decreased PS II/PS I ratio, and an enhanced cyt *bd* oxidase activity (Schneider et al. 2004a; Tsunoyama et al. 2009).

Besides this, there is also a low amount of PetC2 even in wild type *Synechocystis* PCC 6803 cells under stress conditions

with significantly increased transcript levels, which suggests a regulatory role of this polypeptide. This is indirectly confirmed by an atypically high growth rate of the $\Delta petC2$ strains under high light (HL) conditions (Tsunoyama et al. 2009). Generally, stress conditions, including HL treatment (Tsunoyama et al. 2009), low oxygen tension (Summerfield et al. 2008), H_2O_2- (more than 200-fold increase!) or Zn^{2+}-treatment (Houot et al. 2007), and cold- or heat stress in the presence of novobiocin (Prakash et al. 2009), induce high PetC2 transcript levels in wild type *Synechocystis*. While novobiocin may induce changes in DNA supercoiling (Prakash et al. 2009), all other observations link the *petC2* expression to the PQ redox level. Notably, Zn^{2+} (like Cu^{2+}, see above) has been shown to inhibit electron transport via the high potential chain (Rao et al. 2000; Roberts et al. 2002). Possibly, binding of these metal ions affects the interaction of the Rieske [2Fe-2S] protein with the Q_o site of the complex, preventing PQH_2 binding and oxidation (Rao et al. 2000; Roberts et al. 2002). Replacing PetC1 by PetC2, which lacks the inhibitory metal binding site (see above), might therefore maintain photosynthetic electron transport through the cyt $b_6 f$ complex even under metal stress conditions. However, the benefit of a metal-sensitive variant among two otherwise similar proteins is quite unclear and has to be explored in more detail. Nevertheless, the ability of the $\Delta petC1$ mutant to perform state transitions – albeit to a lower extent (see above) – indicates that both PetC1- and PetC2-containing cyt $b_6 f$ complexes play a role in redox-regulated light energy distribution between the two photosystems in cyanobacteria (Mao et al. 2002).

For PetC3, an involvement in electron transport processes was indicated by combined ferredoxin (Fd)-chromatography/mass spectrometry analysis showing Fd-PetC3 interaction (Hanke et al. 2011) and by significant (fourfold) upregulation of *petC3* expression in a $\Delta lepB1$ *Synechocystis* strain with a low functional cyt $b_6 f$ population

(Zhang et al. 2013). Besides, PetC3 was reported to induce an upregulation of the cyclic electron flow and to be involved in long-term light adaptation processes (Tsunoyama et al. 2009). However, the distinct features of PetC3 prevent a direct substitution of PetC1 or PetC2 in a cyt $b_6 f$-complex. Notably, the N-terminus of PetC3 lacks more than ten residues in comparison to PetC1 and PetC2, the sequence of its putative transmembrane helix is poorly conserved and the connected hinge region is absent (Fig. 13.4; Schneider et al. 2002). Furthermore, the midpoint redox potential of 135 mV (vs. 300–320 mV for PetC1/PetC2) excludes the oxidation of PQH_2, i.e., a role in the linear electron transport chain (Schneider et al. 2002, 2004b). In contrast to PetC1 and PetC2, which apparently are exclusively located in the thylakoid membrane, PetC3 is exclusively located in the cytoplasmic membrane, with all other major cyt $b_6 f$ subunits being absent (Aldridge et al. 2008; Schultze et al. 2009). Consequently, the cyanobacterial cytoplasmic membrane most probably does not contain a functional cyt $b_6 f$ complex, which, in turn, excludes a linear or cyclic photosynthetic electron transport and allows only a truncated electron transport chain via the PQ-pool (Schultze et al. 2009; for a review see Bernát and Rögner 2011). In conclusion, PetC3 is probably not a component of a classical cyt $b_6 f$ complex. Rather, as predicted by its N-terminal sequence, it might be a lipoprotein with a potential lipid binding site at Cys23 (Fig. 13.4). Based on sequence alignments [e.g., with KILIM_005_00710 of *Kineosphaera limosa* or SMUL_3119 of *Sulfospirillum multivorans* (accession numbers: K6X6L5 and W6EI55, respectively, at UniProt (http://www.uniprot.org))], PetC3 is predicted to be a subunit of an arsenite oxidase. Typically, arsenite oxidases are membrane-bound periplasmic enzymes, where the Rieske-type [2Fe–2S] center has a midpoint potential of +60 to +100 mV (Lebrun et al. 2003). The midpoint redox potential of PetC3 (135 mV, see above)

is close to this range, which supports the prediction that the PetC3 Rieske-type protein is a component of an arsenite oxidase. As arsenite was proposed as a potential electron donor to the PQ-pool (Nagy et al. 2014), PetC3 might be involved in this alternative electron transport pathway.

The PetC4 protein in *Nostoc* PCC 7120 has a high sequence similarity to PetC1 and PetC2 and apparently plays a role in nitrogen fixation and/or heterocyst formation and is proposed to be a redox sensor (Arnold et al. 2005).

III. Small *bona fide* Cytochrome b_6f Subunits

The four small *bona fide* cyt b_6f subunits PetG, −L, −M and −N are located at the periphery of the dimeric cyt b_6f complex and form a so-called "picket fence" structure (Fig. 13.2). While PetM and PetL are positioned peripherally, PetG and PetN are located more deeply inside the complex (Stroebel et al. 2003; Yamashita et al. 2007; Baniulis et al. 2009). This localization is also reflected in the phenotypes of the deletion mutants: While deletion of *petG* or *petN* genes, encoding the partly buried subunits, was impossible and/or showed dramatic effects (*i.e.,* lethal/bleached phenotypes, which are unable to grow photosynthetically) in *Synechocystis* sp. PCC 6803 (Schneider et al. 2007), *C. reinhardtii* (δ*petG,* Berthold et al. 1995), and tobacco (Hager et al. 1999; Schwenkert et al. 2007) deletions of *petL* resulted only in moderate phenotypes. Irrespective of pro- or eukaryotic sources, PetL-depleted cyt b_6f complexes were functional, although complex accumulation and stability as well as electron transport rates were affected (Takahashi et al. 1996; Zito et al. 2002; Schneider et al. 2007; Schöttler et al. 2007; Schwenkert et al. 2007). In contrast, deletion of *petM* resulted in different phenotypes in cyanobacteria and higher plants. While PetM is non-essential in the cyanobacterium *Synechocystis* sp. PCC 6803 and the Δ*petM* deletion strain

accumulated wild type levels of cyt b_6f complex (Schneider et al. 2001), *petM* RNAi tobacco plants were unable to assemble functional cyt b_6f complexes. This may result from functional differences in the corresponding PetM subunits (Hojka et al. 2014) and/or the different chemical nature of the essential carotenoid prosthetic group (9-*cis* β-carotene in *C. reinhardtii* and higher plants and echinenone in cyanobacteria (Wenk et al. 2005)), which is bound between the PetG and PetM helices.

As with other small proteins (see e.g., Hobbs et al. 2011), the physiological role of the four low molecular weight cyt b_6f complex subunits is only little understood. Besides their important role in assembly and/or stability of the cyt b_6f complex (as discussed above), other – still to be elucidated – functions of these small polypeptides may be predictable or can be suggested based on available experimental data. One example is the proposed regulatory role of PetM in *Synechocystis* sp. PCC 6803, based on a significantly altered stoichiometry of photosynthetic protein complexes in the *petM* knockout strain, while the activity of the cyt b_6f complex was unchanged (Schneider et al. 2001). Furthermore, large-scale protein interaction analyses revealed at least six *Synechocystis* proteins interacting with PetG (Sato et al. 2005). Three of them (gene products of the open reading frames *sll0040*, *slr0594*, and *slr1866*) are classified as unknown/hypothetical proteins, while three others are members of known protein families, among them a RND (Resistance-Nodulation-Division) multidrug efflux transporter (Slr0454, AcrB) and a penicillin-binding protein (Sll0002, PonA). As the functions of these polypeptides are not directly related to known cyt b_6f functions, further data are required for the elucidation of these interactions. Also the function of the sixth interacting partner (Sll0798, RppB, NrsS, Hik30) is still under debate. It is suggested to be a two-component sensor histidine kinase, functioning possibly as a nickel sensor (López-Maury et al. 2002) or as a redox-responsive regulator

of photosynthesis and photopigment related gene expression (Li and Sherman 2000). Based on the finding that low CO_2 reduces expression of *sll0798* – similar to other two-component regulators – a multiple regulatory role was proposed (Wang et al. 2003), potentially involving an up-regulation of the cyclic electron flow, as observed under these conditions (Eisenhut et al. 2007). As the cyt b_6f complex is considered the branching point for cyclic *vs.* linear electron flow (see e.g., Cramer and Zhang 2006; Cramer et al. 2006, 2010), a control directly at the complex would be straight forward. Present results indicate that PetG and Sll0798 might be involved in such a regulatory mechanism in cyanobacteria. Notably, interaction of Sll0798 with Fd was also reported (Hanke et al. 2011), which again suggests an involvement of this protein in the regulation of electron transport processes.

IV. Loosely Bound Subunits

Besides the eight *bona fide* cyt b_6f complex subunits identified in the X-ray structures, there is evidence for further protein components, interacting transiently and/or loosely with the cyt b_6f complex. As discussed above, protein interaction analysis revealed proteins interacting with the small subunit PetG in *Synechocystis* PCC 6803 (Sato et al. 2005). While the physiological role of these interactions is still enigmatic, only one of the three categorized proteins is directly related to electron transport and/or signal transduction processes (see above), *i.e.,* the basic functions of the cyt b_6f complex.

Although it cannot be considered as a small subunit, it should also be noted that the cyt b_6f complex and the ferredoxin: $NADP^+$ oxidoreductase (FNR; MW = 32–36 kDa) can be co-isolated from higher plants (Clark et al. 1984; Zhang et al. 2001; Iwai et al. 2010; Szymańska et al. 2011; see also the Chap. 23 by Minagawa on cyt b_6f supercomplexes). A central role of

this interaction in cyclic electron transport processes was suggested, which is discussed in detail elsewhere (Cramer et al. 2006, 2010, 2011; Cramer and Zhang 2006; see also the Chaps. 9 and 12 by Cramer and Hasan, and Kurisu).

Besides mediating electron flow, the cyt b_6f complex also has a central role in regulation of state transitions, the short-term regulation of light energy distribution between the two photosystems (Vener et al. 1995; for reviews see Wollman 2001; Allen 2003; Finazzi and Forti 2004; Lemeille and Rochaix 2010; Rochaix 2011; Minagawa 2011; and Chaps. 22 and 24 by Finazzi et al. and Rochaix in this volume). In eukaryotic photoautotrophs, occupation of the cyt b_6f Q_o site by PQH_2 leads to activation of the Stt7/STN7 kinase, which is a prerequisite for phosphorylation of the light harvesting system of PSII (LHCII) and the subsequent LHCII displacement from PSII to PSI (i.e., state 1 to state 2 transition) (Depège et al. 2003; Bellafiore et al. 2005; Lemeille et al. 2009). Activation of Stt7/STN7 includes transient association with the cyt b_6f complex. Notably, both cyt b_6f and Stt7 have dimeric structures (see Lemeille and Rochaix 2010); however, due to steric reasons it is very unlikely that the dimeric Stt7 can match the C2 symmetry of the cyt b_6f complex. This cyt b_6f complex-associated Stt7/STN7 kinase is inactivated under high light by the ferredoxin-thioredoxin system, most probably in order to prevent potential damage under these conditions (Martinsuo et al. 2003; Puthiyaveetil 2011). Further details are discussed in the Chap. 24 by Rochaix. In *C. reinhardtii*, state 1 to state 2 transitions are also combined with phosphorylation of a 15.2 kDa transmembrane protein (PetO), which is peripherally associated with the cyt b_6f complex (Hamel et al. 2000). PetO was shown to be a component of the cyt b_6f–PSI–FNR–LHCII supercomplex, which is engaged in cyclic electron flow (Iwai et al. 2010). This protein was suggested as *Chlamydomonas*-analog of the PGR5 regulator, which was found attached to the analogous supercomplex in higher plants

(DalCorso et al. 2008; Iwai et al. 2010). Thus, phosphorylation of PetO might be part of the signaling pathway in *Chlamydomonas* (Hamel et al. 2000). Interestingly, while neither PetO nor PGR5 is encoded in cyanobacterial genomes, the hypothetical cyanobacterial protein Ssr2016, which may have a role in cyclic electron flow (Yeremenko et al. 2005), shows a remarkably high similarity to the amino acid sequence of the PGR5 N-terminus. In contrast to the dogma that state 1 to state 2 transitions are generally combined with an increased cyclic vs. linear electron flow (Finazzi et al. 1999, 2002; Wollman 2001), recent results suggest an independent regulation of cyclic electron flow and state transitions (Takahashi et al. 2013; see also Rochaix 2011). Notably, while the molecular regulatory mechanism of state transitions in cyanobacteria is essentially unknown, the triggering mechanism may be similar to that in chloroplasts (Mullineaux and Emlyn-Jones 2005).

Highly purified cyt b_6f complexes from *Synechocystis* sp. PCC 6803 and *T. elongatus* contain an additional associated polypeptide with a molecular mass of ∼7 kDa. This potential 9th subunit of the (cyanobacterial) cyt b_6f complex was named PetP (Volkmer et al. 2007; Gendrullis et al. 2008). It is encoded by the open reading frame *ssr2998* in *Synechocystis* PCC 6803 and *tsr0524* in *T. elongatus*, respectively, and is highly conserved in all cyanobacterial genomes (Volkmer et al. 2007). In accordance with its amino acid sequence, predicting a cytoplasmically localized soluble polypeptide, proteomic analyses suggest that PetP is a membrane associated (*i.e.,* not membrane integral) protein (Wang et al. 2009). Combined cross-linking and mass spectrometry analyses, showing direct interaction between the N-termini of SU IV and PetP in *T. elongatus*, further support this view (Rexroth et al. 2014). Although this subunit has not been identified in any of the solved structures of cyanobacterial cyt b_6f complexes (Kurisu et al. 2003; Yamashita et al. 2007; Baniulis et al. 2009; Hasan and Cramer 2014), selected reaction monitoring (SRM) shows a 1:1:1 stoichiometry between

Rieske protein, SU IV and PetP (Rexroth et al. 2014), which therefore appears to be only loosely/temporarily associated with the complex. Functional analysis of *petP* deletion strains showed twofold slower growth rates than wild type cells, especially under HL conditions (Volkmer et al. 2007; Gendrullis et al. 2008; Rexroth et al. 2014). Also, flash-induced absorption change measurements indicated slower re-reduction kinetics relative to wild type of both cyt f^+ and P_{700}^+ by a factor of 1.7–3.1 in the mutants (Volkmer et al. 2007; Gendrullis et al. 2008; Rexroth et al. 2014), whereas the PQ-pool was highly reduced. Interestingly, the electron transport rates through the high-potential/linear electron transport chain was much more affected than the cyclic electron transport around the cyt b_6f complex (Rexroth et al. 2014) and through the low-potential chain within the complex which was basically unaffected (Nowaczyk et al. 2010). These kinetic alterations are accompanied by a strongly decreased stability of the complex isolated from PetP-depleted *T. elongatus* (Rexroth et al. 2014). Thus, although PetP is not an essential cyt b_6f subunit, these observations suggest a combined structural and regulatory role for PetP, which seems to be indispensible for maintaining the maximal photosynthetic activity under changing environmental conditions. In good accordance with this, time-lagged profiles of the transcriptional response of *ssr2998* indicate a close correlation with some genes encoding different PSII and phycobilisome subunits under fluctuating light (Schmitt et al. 2004). Also, screening for interaction partners of heterologously overexpressed PetP revealed, besides essential phycobilisome and cyt b_6f subunits, three PSI subunits, NdhF3, and Fd as potential interaction partners of PetP (Rexroth et al. 2014). Putting these together with the kinetic data above suggests a potential role of PetP in electron transport processes and, like PetO in *C. reinhardtii* (see above), formation of a cyt b_6f–PSI supercomplex in cyanobacteria.

It should also be mentioned that depletion of the integral membrane protein Ycf34, a

prototype of a new class of [4Fe-4S] proteins, shows – depending on light conditions – a similar phenotype as several cyt b_6f mutants (see above), *i.e.,* impaired electron transport through the complex (Wallner et al. 2012). This suggests a – direct or indirect – interaction between Ycf34 and the cyt b_6f complex.

Acknowledgements

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Inter-Monomer Electron Transfer in Cytochrome *bc* Complexes

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Summary

Crystal structures of cytochrome bc_1/b_6f revealed that these complexes assemble as homodimers. An intriguing feature of redox cofactor spatial arrangement in the dimers is the relatively short distance between two low potential hemes b_L located in separated monomers. Theoretical calculations suggested that with this distance a two-heme bridge can support inter-monomer electron transfer on the catalytic time scale. While this electron exchange was incorporated in several models describing the molecular mechanism of catalysis, its experimental verification has been hampered by a high degree of structural symmetry and the fact that such a reaction can be considered as virtual self-exchange. Recently, the construction of cytochrome bc_1 derivatives with broken symmetry between the monomers provided experimental evidence that the heme b_L-b_L electron transfer does take place on the catalytically-relevant time scale. In this chapter we summarize these recent developments and discuss the mechanistic consequences and possible physiological meaning of this inter-monomer connection.

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I. Introduction

All known cytochrome bc_1/b_6f complexes are homodimers characterized by high structural symmetry between the monomers. The symmetry concerns both the architecture of cofactor chains and the amino-acid composition/structure of the proteins (Berry et al. 2004; Xia et al. 2013). Intriguingly, the architecture of cofactor chains, as described by crystal structures of bc_1/b_6f complexes (Xia et al. 1997; Zhang et al. 1998; Hunte et al. 2000; Kurisu et al. 2003; Stroebel et al. 2003; Hasan et al. 2013), indicated that the two monomers are separated in such a way that they do not exchange electrons, except for one point where closely-positioned hemes b_L (each coming from different monomer) make catalytically-relevant electron transfer between monomers possible. Accordingly, several groups have suggested that this electronic connection may be an inherent part of enzymatic turnover, either as a straightforward electron transfer route that competes with intra-monomer electron transfer (Osyczka et al. 2004, 2005; Shinkarev and Wraight 2007), or as an element of more elaborate pathways involving conformational changes or substrate exchanges that gate various processes (Gopta et al. 1998; Covian et al. 2004; Covian and Trumpower 2006, 2008; Cooley et al. 2009; Castellani et al. 2010).

However, from the structural point of view, as each monomer is equipped with all elements necessary to perform the catalytic

Q-cycle described in detail in other chapters in this volume (see also Sarewicz and Osyczka 2015 for a recent review), both the existence of the inter-monomer electron transfer and its possible role became a matter of intense debate (Shinkarev and Wraight 2007; Crofts et al. 2008, 2013; Mulkidjanian 2010; Cramer et al. 2011; Khalfaoui-Hassani et al. 2012; Bazil et al. 2013; Hasan et al. 2014). In searching for the ways of verifying whether the inter-monomer electron transfer exists, the experiments based on studying the effects of mutated residues lying between the two hemes b_L at the monomer:monomer interface (Gong et al. 2005), or the effects of sub-stoichiometric concentrations of inhibitors were initially described (Covian and Trumpower 2005). However, the results of those experiments are opened to interpretation (Shinkarev and Wraight 2007; Crofts et al. 2008).

Recently, a direct evidence of inter-monomer electron transfer was provided by the experiments in which the structural symmetry of the dimer was broken to expose the inter-monomer connection for kinetic testing *in vitro* and functional testing *in vivo* (Świerczek et al. 2010; Lanciano et al. 2011, 2013a; Czapla et al. 2012a, 2013; Ekiert et al. 2014). In this chapter, we focus on those recent advances and present them along with background of structural and theoretical rationales to describe the current state of knowledge on inter-monomer electron transfer in cytochrome bc complexes and its possible physiological meaning.

II. Implications from Structures for Inter-Monomer Electron Transfer

As the structural details of cytochrome bc complexes are described in other chapters of this book, we highlight just those elements that are essential to the topic of this section.

Cytochrome bc_1 complex assembles as a homodimer, where each monomer consists of three catalytically active subunits: cytochrome b, cytochrome c_1 and the Fe-S protein. The accessory subunits that are

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Abbreviations: A – Electron acceptor; B-B – Cytochrome bc_1-like complex containing fusion protein of two *R. capsulatus* cytochromes b (cytochrome bb) in place of two separate cytochrome b subunits; B_S-B – Cytochrome bc_1-like complex containing a hybrid-fusion protein of *R. sphaeroides* and *R. capsulatus* cytochromes b (cytochrome b_Sb) in place of two separate cytochrome b subunits; D – Electron donor; Fe-S – Rieske protein, subunit of cytochrome bc_1 complex containing [2Fe-2S] cluster; Q_i – Quinone reduction site of cytochrome bc_1 also known as Q_n; Q_o – Quinol oxidation site of cytochrome bc_1 also known as Q_p; *R.* – *Rhodobacter*; ROS – Reactive Oxygen Species; UQ – Ubiquinone; UQH_2 – Ubihydroquinone

present in some structures, especially in mitochondrial complex III, do not play a role in redox reactions catalyzed by the enzyme. The core cytochrome b subunit in each monomer contains two catalytic quinone-binding sites, Q_i and Q_o, localized near the two opposite sides of the membrane: inside (matrix) and outside (inter-membrane). These two sites are electronically connected by heme b_L and heme b_H that form the low potential b-chain. The cofactors of other core subunits, heme c_1 of cytochrome c_1 and Rieske [2Fe-2S] cluster of the Fe-S protein, form the high potential c-chain connecting the Q_o site with soluble electron carriers (such as cytochrome c) (Berry et al. 2000; Xia et al. 2013).

Despite the differences in the genetic and protein amino-acid sequences, the general spatial arrangement of cofactors and catalytic sites in mitochondrial complex III, cytochrome $b_6 f$ and bacterial cytochrome bc_1 is highly conserved. When crystal structures of bc_1 homologues from various organisms are superimposed, the catalytic core of the enzymes reveals no significant differences, especially within the central hydrophobic protein core of cytochrome b and within the arrangement of all four hemes b in the dimer (Fig. 14.1a). The arrangement of cofactors in cytochrome $b_6 f$ exhibits some differences in the peripheral parts of the chains: the location of heme f is shifted in comparison to heme c_1, and additionally, an atypical heme c_i is present near heme b_H and the Q_i binding site of cytochrome $b_6 f$ (Kurisu et al. 2003; Stroebel et al. 2003) [also see Hasan et al., this volume]. However, the other cofactors of cytochrome $b_6 f$ overlay with those from cytochrome bc_1 counterparts. Interestingly, the edge-to-edge distance between the two hemes b_L in the dimer varies between 13.2 and 15.2 Å, depending on the structure. Such distances fall within the limits of the electron transfer rate required for the fast catalytic turnover of enzymes (Page et al. 1999). Conversely, other cofactors of the opposite monomers are positioned farther apart, preventing any catalytically-relevant electron exchange.

The cofactor arrangement suggests the existence of a highly symmetric H-shaped electron transfer system built of two upper and two lower branches connected by a bridge formed by the two hemes b_L (Fig. 14.1b). In this system, the heme b_L-b_L bridge constitutes a structural element supporting the inter-monomer electron transfer.

III. Theoretical Prediction for the Time Scale of Heme b_L-b_L Electron Transfer

Direct measurements of the rate of possible electron transfer between hemes b_L in cytochrome bc_1 are generally difficult because of several reasons, including the high symmetry of the dimer and the fact that such reaction can be considered as virtual self-exchange. The magnitude of the rate of electron transfer between two hemes b_L can, however, be estimated by theoretical calculations.

The general view of electron transfer theory is discussed in detail in Sect. II of this book. Here, we introduce those aspects that directly relate to calculations of heme b_L-b_L electron transfer. The nature of electron transfer process is quantum mechanical and relies on quantum mechanical tunneling phenomenon, which means that electron can cross an insulating barrier between donor (D) and acceptor (A) even when the thermal energy is much lower than the energy gap between those of the electron donor and acceptor (Marcus and Sutin 1985; Moser et al. 1992; Barbara et al. 1996). The characteristic rate constant of such electron tunneling between D and A molecules follows Fermi's golden rule and depends on a product of two terms: the electronic (V^2) and the nuclear (FC):

$$k_{ET} = \frac{2\pi}{\hbar} V^2 FC \qquad (14.1)$$

where, k_{ET} is the rate of electron tunneling, V^2 is the electronic coupling between D and
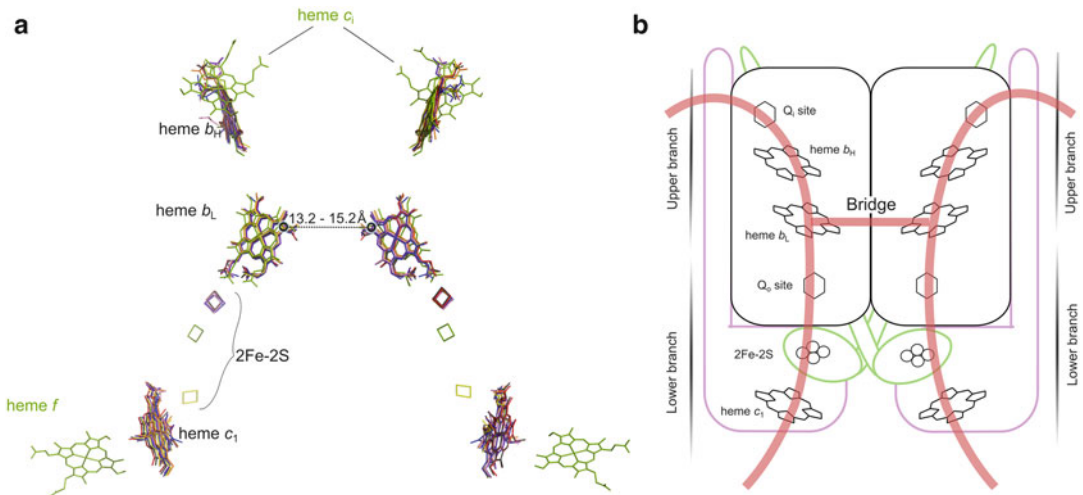
Fig. 14.1. Arrangement of cofactors in dimeric cytochrome bc_1/b_6f complexes. (**a**) Cofactors from the super-imposed crystal structures of cytochrome bc_1 of bacteria (*Rhodobacter capsulatus*, 1ZRT (Berry et al. 2004), *Rhodobacter sphaeroides*, 2QJP (Esser et al. 2008), *Paracoccus denitrificans*, 2YIU (Kleinschroth et al. 2011)), yeast (*Saccharomyces cerevisiae*, 1KYO (Lange and Hunte 2002), 3CXH (Solmaz and Hunte 2008)), chicken (*Gallus gallus*, 3H1H (Zhang et al. 1998)), bovine (*Bos taurus*, 2A06 (Huang et al. 2005)) and cytochrome b_6f of the cyanobacterium (*Mastigocladus laminosus*, 1VF5 (Kurisu et al. 2003)). Numbers above the dashed line show the range of the edge-to-edge distances between hemes b_L in those structures. (**b**) Cofactors embedded in cytochrome bc_1 subunits of a dimer form an H-shaped electron transfer system (*thick lines*) consisting of two upper branches, two lower branches and the heme b_L-b_L bridge.

A electronic states, FC is density of states weighted Frank – Condon factor and $\hbar$ is the reduced Planck constant.

The first electronic term V^2 is temperature independent and falls exponentially with increasing the distance between D and A:

$$V^2 = V_0^2 \exp(-\beta R) \qquad (14.2)$$

where: V_0^2 is the maximum coupling in the case when D and A are in van der Waals contact, R is the edge-to-edge distance between D and A and β is the scaling factor, which determines how fast the coupling decays with the distance R. The scaling factor β depends on the nature of the medium between A and D and is in the range between $\sim$2.8 $Å^{-1}$ for vacuum to $\sim$0.7 $Å^{-1}$ for covalently bonded system. Extensive studies of electron transfer in proteins showed that $\beta = 1.4$ $Å^{-1}$ is a good approximation and may vary slightly with packing density (Page et al. 1999). This equation is similar to those describing the decrease of the frequency of spin-spin

exchange interaction (detected by electron paramagnetic resonance) with increased distance between two paramagnetic species (Likhtenshtein 1996). If so, then β should also be somewhat sensitive to the possible hydrogen bonds formed between D and A molecules, as they facilitate propagation of electronic density over larger distances along the bonds (Fournel et al. 1998; Calvo 2007).

The second term, FC, is associated with changes in the nuclear coordinates of D and A and surrounding solvent molecules caused by electron transfer. It depends on the reorganization energy, λ, and on the free energy change of the reaction, ΔG, which is the difference between energy of products and reactants. The rate of electron transfer is expected to be the largest when ΔG is equal to the reorganization energy, i.e. $\Delta G = -\lambda$. In case of electron transfer between hemes b_L, the reaction can be considered as self-exchange, assuming that the properties of hemes b_L do not depend significantly on conformational changes in the other parts of

the protein; therefore, in this case, $\Delta G = 0$ (Marcus and Sutin 1985).

Calculation of reorganization energy is generally difficult due to the enormous complexity of the system. However, in proteins, studies on electron transfer between cofactors separated by different distances and of different redox potentials allowed estimation of the general value of λ of about 0.7 eV. These studies also provided the general phenomenological equation useful for calculating the electron transfer rate between most of the protein cofactors (Page et al. 1999; Moser et al. 2006, 2010):

$$\log(k_{ET}) = 13 - (1.2 - 0.8\rho)(R - 3.6)$$
$$- 3.1 \frac{(\Delta G + \lambda)^2}{\lambda} \qquad (14.3)$$

In this formula parameter ρ defines the packing density factor of protein between cofactors (Page et al. 1999). The value $\rho = 1$ corresponds to the fully packed matrix ($\beta = 0.9\ \text{Å}^{-1}$), while $\rho = 0$ corresponds to vacuum ($\beta = 2.8\ \text{Å}^{-1}$). For most proteins the packing density factor is about 0.76 (Page et al. 1999).

Taking the typical values of ρ and λ determined for proteins, the equation (14.3) can be simplified and k_{ET} is given by the following formula that has only a single parameter (distance R) that influences the electron transfer rate between cofactors (Moser et al. 2006):

$$\log(k_{ET}) = 12.8 - 0.6R \qquad (14.4)$$

Despite the fact that this equation is only a crude approximation of k_{ET} for electron transfer between cofactors, it can be used to obtain an order of magnitude for the rate of electron transfer between hemes b_L in cytochrome bc complexes. The rate of this process calculated for edge-to-edge distances between hemes from various cytochrome bc_1 structures (Fig. 14.2, dotted lines) exceeds the range of catalytic turnover rate. This means that the heme b_L-b_L electron transfer, when connecting the catalytic Q_o and Q_i sites, should not limit the overall turnover rate of the enzyme.

IV. Experimental Evidence for Inter-Monomer Electron Transfer

Mechanistic studies on cytochrome bc_1 catalysis are often performed with photosynthetic bacteria *Rhodobacter capsulatus* or *Rhodobacter sphaeroides*. These simple organisms allow straightforward genetic manipulations while the light-induced catalytic cycle makes detailed analyses of the electron transfer reactions possible (Gray and Daldal 1995; Berry et al. 2009). Experimentally, the most unambiguous way to expose the inter-monomer electron transfer for functional testing would seem through the cross-inactivation of complementary parts of electron transfer chains of the dimer to enforce the inter-monomer connection as the only possible link between the Q_o and Q_i catalytic sites. This, however, required specific modifications to the genetic system used for mutagenesis which, due to the fact that the genes of cytochrome bc_1 naturally do not differentiate between the monomers, only enabled introducing mutations symmetrically in both monomers. Breaking the symmetry of the dimeric bc_1 complex has been accomplished recently in experiments that used two approaches. One approach was based on an idea of fusing two cytochrome b subunits of the dimer (Świerczek et al. 2010; Czapla et al. 2012b, 2013), the other – on using two different plasmids (Castellani et al. 2010; Lanciano et al. 2011, 2013a; Khalfaoui-Hassani et al. 2013).

Construction of the fusion protein involved replacing the cytochrome b gene in *R. capsulatus* with its duplicated version, in which two parts of the same gene were connected by a linker (Fig. 14.3a). Such protein folded into an enzymatically-active complex (named B-B) consisting of the cytochrome bb core containing all four quinone catalytic sites, to which cytochrome c_1 and Fe-S subunits assembled in a way that overall topography resembled the dimeric bc_1 complex (Świerczek et al. 2010; Czapla et al. 2012b). A variant of the fusion protein system was also developed using cytochrome b genes from different, but closely related
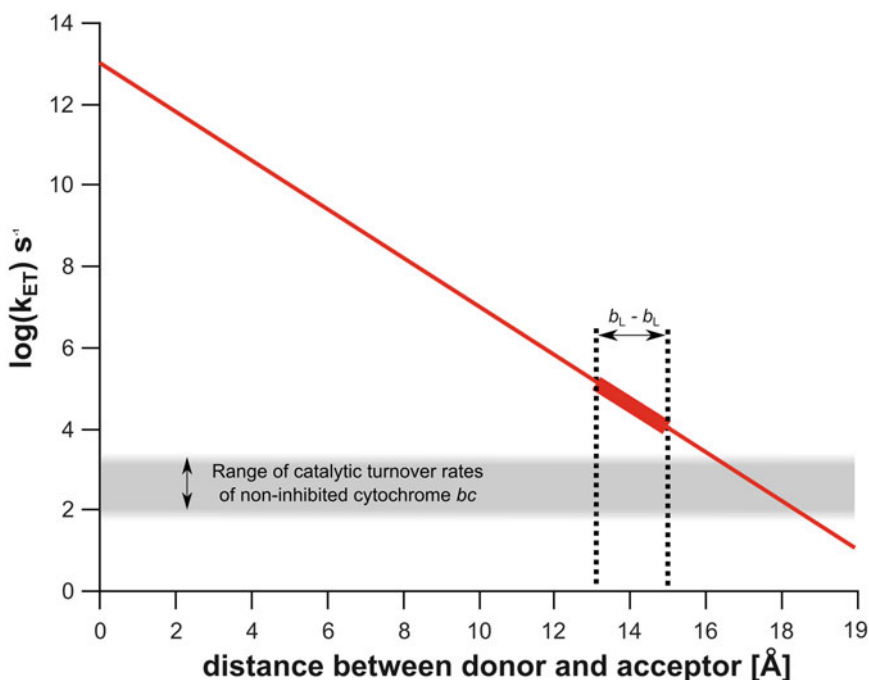
Fig. 14.2. The distance dependence of the rate for heme b_L-b_L electron transfer. The distance dependence of the rate (*red line*) was calculated using simplified equation (14.4). The range of edge-to-edge distances between hemes b_L (*dotted vertical lines*) defines the range of expected rates for heme b_L-b_L electron transfer (*thick red line*). The range of distances corresponds to that shown in Fig. 14.1a. *Gray* area represents the span of cytochrome bc_1 catalytic turnover rates implicated from flash-induced electron transfer measurements and steady-state enzymatic turnover assays.

species: *R. sphaeroides* and *R. capsulatus* (Fig. 14.3b). Such hybrid fusion protein (cytochrome b_Sb) was also assembled into functional bc_1-like complex (named B_S-B) (Czapla et al. 2013). With these systems, it was possible to create cross-inactivated forms in which mutations knocking out the individual cofactors were placed asymmetrically in different halves of the fusion protein. These mutations inactivated the upper branch of one monomer (H212N mutation, preventing heme b_H assembly, thus disrupting the electronic connection with the Q_i site (Osyczka et al. 2004)) and the lower branch of the other monomer (G158W, rendering the Q_o site inactive (Ding et al. 1995)) and leaving the path involving the inter-monomer electron transfer as the only connection between the active Q_i and Q_o catalytic sites.

Flash-induced kinetic measurements in membranes with cross-inactivated B-B or

B_S-B revealed that the electron transfer between the monomers takes place in the millisecond time scale, matching the enzymatic turnover time and thus suggesting its catalytic relevance (Świerczek et al. 2010; Czapla et al. 2013). The measurements of the enzymatic activities of the purified cross-inactivated B-B mutant confirmed that inter-monomer electron transfer can support multiple turnovers (Czapla et al. 2012a). In addition, using cells expressing cross-inactivated B_S-B complexes, it was possible to demonstrate that the inter-monomer electronic connection can support the bc_1-dependent photoheterotrophic growth. Such growth was not supported when the same branches were knocked out in B_S-B (both upper or both lower branches) (Ekiert et al. 2014).

The "two-plasmid" system involved the use of two plasmids coding for the
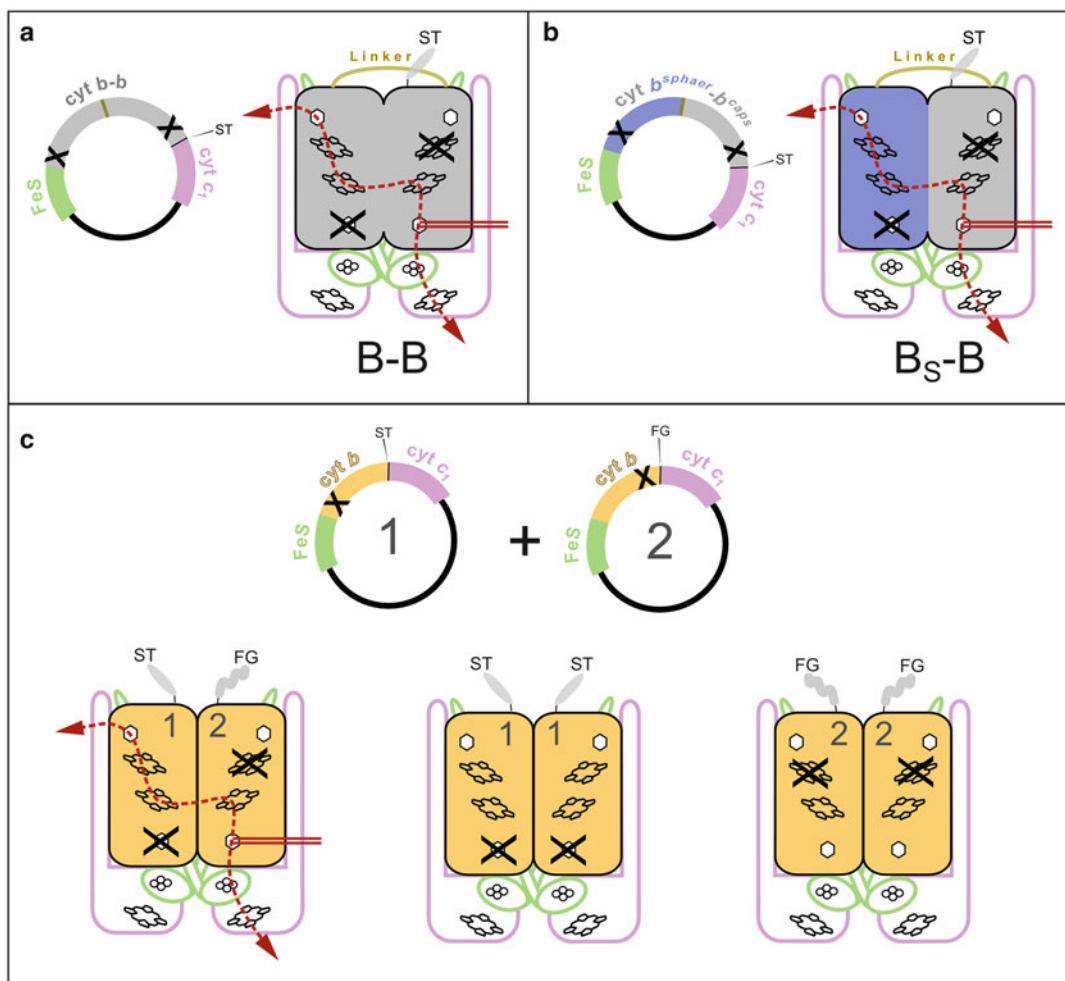
Fig. 14.3. Experimental systems used to expose the inter-monomer electron transfer in the *Rhodobacter capsulatus* cytochrome bc_1. For each system, mutations disabling individual branches of the H-shaped electron transfer system (*black crosses*) are shown at the levels of plasmid and protein complex. Cross-inactivation disrupts the functional link between Q_o and Q_i sites within each monomer, leaving the path involving inter-monomer electron transfer via the heme b_L-b_L bridge as the only pathway to connect the catalytic sites in a dimer (*dotted arrow lines*). *ST* Strep-tag, *FG* FLAG-tag. (**a**) Fusion protein system – two cytochrome b subunits in the dimer are replaced by a fusion consisting of two cytochromes b identical in primary amino-acid sequence. (**b**) Hybrid fusion protein system – two cytochrome b subunits in the dimer are replaced by a hybrid fusion of cytochromes b of *R. sphaeroides* and *R. capsulatus*. (**c**) Two-plasmid system – mixture of homo- and heterodimers identifiable by different composition of polypeptide tags.

cytochrome b protein tagged with different polypeptide labels (Fig. 14.3c). Mutations inactivating opposite branches of the electron flow used in this system affected the Q_o site (Y147A; (Saribas et al. 1995)) or inactivated the Q_i site or heme b_H (H217L or H212N, respectively (Gray et al. 1994; Osyczka et al. 2004)). Mutated cytochromes

b were randomly distributed into dimeric bc_1 complexes assembled in *R. capsulatus* cells. Flash-induced kinetic measurements in membranes showed the millisecond electronic connection between the monomers of bc_1 complex (Lanciano et al. 2011, 2013a). Two rounds of protein purification, using the tags distinctive for each of the

mutated plasmids, resulted in obtaining the cross-inactivated complexes, which were enzymatically active. Moreover, it was observed that these complexes were able to support the photoheterotrophic growth of the cells (Lanciano et al. 2013a).

The results of these independent sets of experiments using both approaches not only provided consistent evidence for the existence of inter-monomer electron transfer in the catalytic time scale, but also indicated that inter-monomer electron transfer can support function of cytochrome bc_1 *in vivo*.

V. Mechanistic Consequences of Inter-Monomer Electron Transfer

Electron transfer between the two hemes b_L has several important mechanistic consequences. It converts the independent cofactor chains in the dimer into an H-shaped electron transfer as indeed implicated from the structure (Fig. 14.1). This system connects electronically all four quinone catalytic sites. It consists of two uprights connected by a bridge. Each upright is formed by one upper and one lower branch that together encompass the c- and b-chains of one monomer. The bridge allows for connection of the lower branch of one monomer with the upper branch of the other monomer. It also links two upper branches together and two lower branches together. All these additional links increase, in principle, a number of possible electron transfer paths connecting the catalytic sites of the dimer, if compared to a simple connection of upper and lower branch within the upright of one monomer.

The catalytic Q-cycle requires functional connection of the Q_o site with the Q_i site (Mitchell 1975; Crofts et al. 1983; Brandt and Trumpower 1994), which, given all possible links of the H-shaped electron transfer system, can involve intra- and/or inter-monomer electron transfers. Intra-monomer electron transfer (within one

upright) connects the Q_o and Q_i sites of the same monomer, while the path involving inter-monomer electron transfer connects the Q_o and Q_i sites across the dimer. From the experimental point of view it appears that both ways of connecting the sites support enzymatic function. This means that the H-shaped electron transfer system, in its basic engineering principle, allows any connection between the catalytic sites of the opposite sides of the membrane to be enzymatically competent (Świerczek et al. 2010). On thermodynamic grounds, the net electron flow in this system can be understood as a result of consecutive changes in transition probabilities between the fully reversible partial reactions in the chain (Osyczka et al. 2004; Shinkarev and Wraight 2007; Cieluch et al. 2010), with the main rationale that electron "blindly" uses any path that is available at a given point in time.

However, an obligatory coupling of two turnovers of the Q_o site with one turnover of the Q_i site, inherent to the Q-cycle, opens the possibility for other, more elaborated, mechanistic schemes for the operation of the H-shaped electron transfer system. Indeed, specific sequences of electron transfer within the dimer, involving the inter-monomer electron transfer, are proposed in various models that assume allosteric interactions within/between monomers. In these schemes, conformational constraints at certain steps act to impose the specific direction of electron flow. One mechanistic element often evoked is an allosteric control over the position of the Fe-S head domain by the occupants of the Q_i site, which in various models is differently ascribed (Covian and Trumpower 2006, 2008; Cooley et al. 2009; Mulkidjanian 2010).

An intriguing consequence of inter-monomer electron connection is a possibility of electron transfer between the Q_i sites through reactions involving just the two upper branches and the bridge. Such tunneling equilibration between all four hemes b in a dimer, occurring on a time scale of Q_o site catalysis and in a manner

independent of the Q_o site, can in principle, lead to dismutation of two semiquinones that are present simultaneously at two Q_i sites of two monomers. In effect, this removes the strict coupling between two turnovers of one Q_o site and one Q_i site described in traditional scheme of the Q-cycle (Osyczka et al. 2004).

VI. Physiological Significance of Inter-Monomer Electron Transfer

The physiological meaning of the H-shaped electron transfer system remains an open issue. Considering currently available information on the thermodynamics of the reactions in cytochrome bc_1 it can be anticipated that electron transfer between hemes b_L and b_H of each monomer is favored over the heme b_L-b_L electron transfer (inter-monomer electron transfer) as long as there are no factors that influence the electron transfer route (Shinkarev and Wraight 2007). One such factor is membrane potential, inherently present and dynamically changing in bioenergetically active organelles in living cells. An increase in membrane potential impedes the electron transfer from heme b_L to heme b_H (Shinkarev et al. 2001; Rottenberg et al. 2009), but at the same time it may increase the fraction of electrons that travel through the heme b_L-b_L bridge. Therefore, the membrane potential can be considered as one of the prominent factors that effectively modulate the inter- *vs* intra-monomer electron transfer.

Inter-monomer electron transfer, creating the conditions that do not preclude the dismutation of two semiquinones that occupy two Q_i sites of the dimer, gives the enzyme a possibility to reduce one ubiquinone (UQ) molecule to ubihydroquinone (UQH_2) in one of the Q_i sites using two electrons, each delivered from a different Q_o site. This option of completing enzymatic turnovers may provide physiological advantage for the cells, as it enhances the efficiency of the Q-cycle under a shortage of UQH_2 in the quinone pool.

Another possible physiological advantage of the H-shaped electron transfer system is related with its flexibility to use more than one electronic path that functionally connects the Q_o site with the Q_i site. This built-in redundancy allows physiological function of the enzyme even after operational damage of one part (Fig. 14.4). Such damage can be associated with the occurrence of certain mutations, especially within the cytochrome b subunit. In eukaryotic cells, this is particularly relevant to the process of accumulation of mitochondrial damage inherent to aging and/or a progress of development of several mitochondria-related diseases (DiMauro 2004; Kujoth et al. 2007; Tuppen et al. 2010). Given that mtDNA-located gene coding for cytochrome b subunit does not differentiate between monomers, the presence of mutations in just a part of the copies of the gene (mtDNA is present in several copies in mitochondria) is likely to result in expression of both symmetrically and asymmetrically mutated dimers of cytochrome bc_1, as indeed observed in bacterial two-plasmid systems (Castellani et al. 2010; Lanciano et al. 2011, 2013a). It is thus possible that upon accumulation of mtDNA mutations, the amount of the asymmetrically damaged complexes increases. Such partially-damaged enzymes are likely to retain their physiological function as long as any of the connections between the operational Q_o and Q_i sites in the dimer is preserved (Sarewicz and Osyczka 2015).

Yet another possible physiological advantage of the H-shaped electron transfer system is related to the reactive oxygen species (ROS) generation by cytochrome bc_1. Current discussions on ROS generation by this enzyme consider the prolonged state of reduced heme b_L as condition increasing the probability of superoxide generation by the Q_o site (Kramer et al. 2004; Cape et al. 2006; Dröse and Brandt 2012; Bleier and Dröse 2013; Lanciano et al. 2013b). Such conditions arise when a semiquinone at the Q_o site (Cape et al. 2007; Zhang et al. 2007; Sarewicz et al. 2013) is given the opportunity to interact with oxygen. In one scenario, this occurs when reduced heme
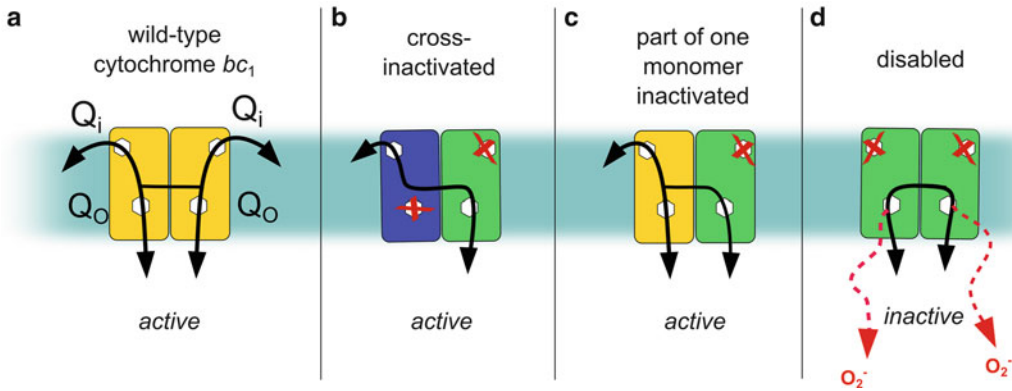
Fig. 14.4. Possible advantage of H-shaped electron transfer in cytochrome bc_1 in the context of mutational damage. (**a**) In wild-type cytochrome bc_1, four active branches and a bridge connect catalytic Q_o and Q_i sites. (**b**) Mutational inactivation of complementary branches on each of the monomers does not abolish enzymatic activity of a dimer, as long as the cross-communication between the opposite Q_o and Q_i sites is maintained through the bridge. (**c**) Inactivation of a part of one monomer does not inactivate a dimer. (**d**) Assembly of similarly damaged monomers (for example with both upper branches disabled) leads to the formation of an inactive enzyme, which additionally may become the source of superoxide radical.

b_L reduces ubiquinone to semiquinone as a part of reverse electron transfer (Dröse and Brandt 2008; Sarewicz et al. 2010). In this case, the highest probability of reaction of semiquinone with oxygen is when the [2Fe-2S] cluster is remote from the site and unable to interact with occupant of the Q_o site at the time of semiquinone formation (Borek et al. 2008; Sarewicz et al. 2013). The other scenario assumes that when semiquinone formed as a result of one-electron reduction of [2Fe-2S] cluster by UQH_2 cannot immediately reduce heme b_L (as this heme is already reduced and unable to take electrons from it), it reacts with oxygen instead (Muller et al. 2002; Forquer et al. 2006; Quinlan et al. 2011).

Typically, heme b_L becomes reduced for prolonged time when the electron outflow from the Q_i site is blocked or suppressed by inhibition or mutation and an active Q_o site is exposed to an excess of substrate UQH_2. If such damage or inhibition occurs asymmetrically in a dimer (is present only in one monomer), the connection between monomers may help removing electrons from hemes in the b-chain, decreasing the chance of superoxide formation by the enzyme. Considering that

cytochrome bc_1 evolved in anoxygenic environment, it is not clear whether possible protective role of geometric proximity of two hemes b_L against ROS was a coincidental effect or an evolutionarily-driven design. Nevertheless, one cannot exclude possibility that some tuning of energy levels might have occurred throughout evolution (Rutherford et al. 2012), for example to minimize the probability of direct reaction of reduced heme b_L with oxygen.

VII. Conclusions

The inter-monomer electron connection emerges as an integral part of the electron transfer system of cytochrome bc complexes. This intriguing feature of the dimer, implicated from the crystal structures and electron transfer calculations, is now supported by experimental evidence at both *in vitro* and *in vivo* levels. *In vitro*, the kinetic measurements established that inter-monomer electron transfer connects functionally the quinone binding sites on the opposite sides of the membrane and occurs on a time scale of milliseconds or faster and thus does not limit the enzymatic turnover.

In vivo, it was shown that specifically-mutated enzymes that rely on the inter-monomer electron transfer to connect the catalytic sites are competent in supporting the cytochrome bc_1-dependent growth of the cells. The existence of inter-monomer electron connection converts the independent redox chains of cytochrome bc_1 dimer into the H-shaped electron transfer system that enables any connection between the catalytic quinone binding sites on the opposite sides of the membrane to be enzymatically competent. Details of electronic distribution in this system in living cells and the physiological meaning of inter-monomer electron transfer, especially in the context of the radical-generating activity of cytochrome bc complexes, still remain to be elucidated.

Acknowledgments

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Heme c_i or c_n of the Cytochrome b_6f Complex, A Short Retrospective

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Summary

The unexpected existence of heme c_i (or c_n) of cytochrome b_6f complex was revealed to the scientific community in Spring 2003 at the Gordon Bioenergetics conference, taking place at Kimball Union Academy, NH, USA. The three-dimensional structures of the complex were published back-to-back by two independent groups in Fall 2003 (Kurisu et al. Science 302:1009–1014, 2003; Stroebel et al. Nature 426:413–418, 2003). Further information about the physico-chemical properties of this unusual heme was gathered in the following years. Here we shall re-examine early evidence for the presence of this unusual cytochrome, originally called G (Lavergne. Biochim Biophys Acta 725:25–33, 1983; Joliot and Joliot. Biochim Biophys Acta 933:319–333, 1988), which show the role that heme c_i plays in electron transfer in the cytochrome b_6f complex.

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I. Introduction

The cytochrome $b_6 f$ complex (plastoquinol-plastocyanin oxidoreductase) is the inter-system electron carrier *par excellence*. It provides photosystem II (PSII), oxidizing water, with plastoquinones as electron acceptors, and photosystem I (PSI), reducing ferredoxin, FNR and NADP$^+$, with reduced plastocyanins (or c_6 cytochromes) as electron donors. The molecular and functional features of the cytochrome $b_6 f$ complex are described in other chapters of this volume (see the Chap. 9 by Cramer and Hasan) and others in this series (Kallas 2012). Here the focus will be on a peculiar cofactor, present in the plastoquinone-binding pocket of the complex, the Q_i (or Q_n) site, on the stromal side of the thylakoid membrane, or the cytoplasmic side in cyanobacteria. Table 15.1 recapitulates the various terminologies used to discriminate between the quinol-oxidation "o" or "p" and quinone-reduction "i" or "n" sites of cytochrome *bc-bf* complexes. The "o" and "i" terminology originates from mitochondrial cytochrome bc_1 complexes whereas "n" and "p" refer to the electronegative and positive sides of the membrane.

Plastoquinols (the reduced and protonated form of plastoquinones) produced by PSII are oxidized and deprotonated at the cytochrome $b_6 f$ Q_o site. Whereas protons are released into the thylakoid lumen, therefore participating to the building of a pH gradient (ΔpH), only half of the electrons are directed towards the "high-potential chain" (consisting of the Rieske iron-sulfur protein and cytochrome f) and then further trans-

Table 15.1. The various names given to the quinone binding sites of cytochrome *bc-bf* complexes.

Quinol oxidation site	Quinone reduction site
"o" outside (used in this review)	"i" inside (used in this review)
Intermembrane space (top)	Mitochondrial matrix (bottom)
"p" positively charged	"n" negatively charged
Thylakoid lumen (bottom)	Chloroplast stroma (top)
Periplasm	Cytoplasm
Left (Mitchell 1976)	Right (Mitchell 1976)

ferred along the chain through plastocyanin (or cytochrome c_6) to PSI. The other half of the electrons are recycled to the "low-potential chain", namely through hemes b_L, b_H and c_i (Stroebel et al. 2003) or x (Kurisu et al. 2003), or c_n (Cramer and Zhang 2006), for the reduction and protonation of a quinone at the Q_i site. The plastoquinol formed at Q_i can be reoxidized at the Q_o site, therefore completing a cycle called the Q-cycle (Mitchell 1975). The bifurcation of electron flow at the Q_o site and the Q-cycle means that the cytochrome $b_6 f$ complex, like the cytochrome bc_1 complex, translocates two protons through the membrane per one electron passing through Cyt f or Cyt c_1, resulting in a major contribution to ΔpH.

The oxidoreductase activity and the overall structure of the cytochrome $b_6 f$ complex are not so different from that of the cytochrome bc_1 complex, although a c'-type heme is found in the Q_i site of the cytochrome $b_6 f$ complex. Similar to cytochrome $b_6 f$ complex, which transfers electrons with photosystems II and I, the cytochrome bc_1 complex is involved in photosynthetic electron flow in association with either type II or type I reaction centers in purple bacteria and in green-sulfur bacteria, respectively (see the Chaps. 3 and 10 by Majumder and Blankenship, and Esser et al., in this volume). Here we review some of the physico-chemical properties of heme c_i, discuss its role in redox chemistry of quinones, and its postulated specific roles

for cytochrome b_6f complex function in comparison with the cytochrome bc_1 complex.

II. Early Evidence: Heme c_i of the Cytochrome b_6f Complex, A Functional Retrospective

Early evidence for the presence of an unusual c'-type heme associated to the cytochrome b_6f complex came from a spectroscopic study by Lavergne (1983), and a subsequent one from Joliot and Joliot (1988), using intact cells of the green microalga Chlorella. These

thorough studies have provided most of the functional information about heme c_i. This discovery arose by the combined advances of two unique tools: firstly, the development of highly sensitive flash-spectrophotometers, and secondly the isolation of low-chlorophyll mutants of green microalgae. As shown in Figs. 15.1 and 15.2, heme c_i shows rather weak absorbance changes ($\Delta\varepsilon_{425\,nm} \approx 60$–$70$ mM^{-1} cm^{-1}), compared to hemes b ($\Delta\varepsilon_{433\,nm} \approx 150$ mM^{-1} cm^{-1}) or f ($\Delta\varepsilon_{422\,nm} \approx 150$ mM^{-1} cm^{-1}), and it is only observable in the blue region of the spectrum where chlorophyll absorbance is preponderant.
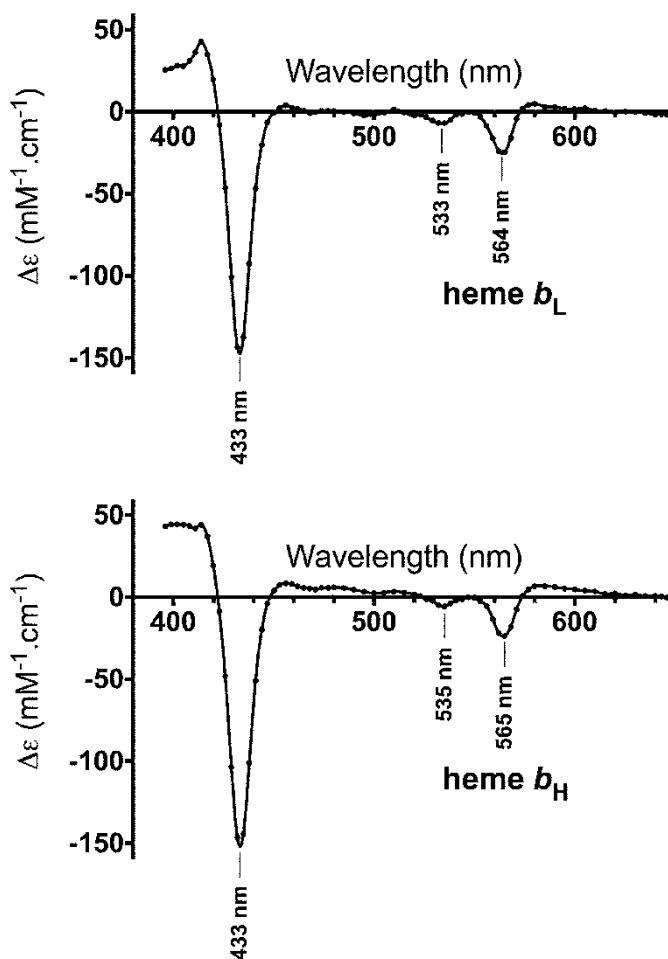
Fig. 15.1. Oxidized-minus-reduced difference spectra of the b hemes of the purified cytochrome b_6f complex from *Chlamydomonas reinhardtii* (Redrawn from Alric et al. (2005)).
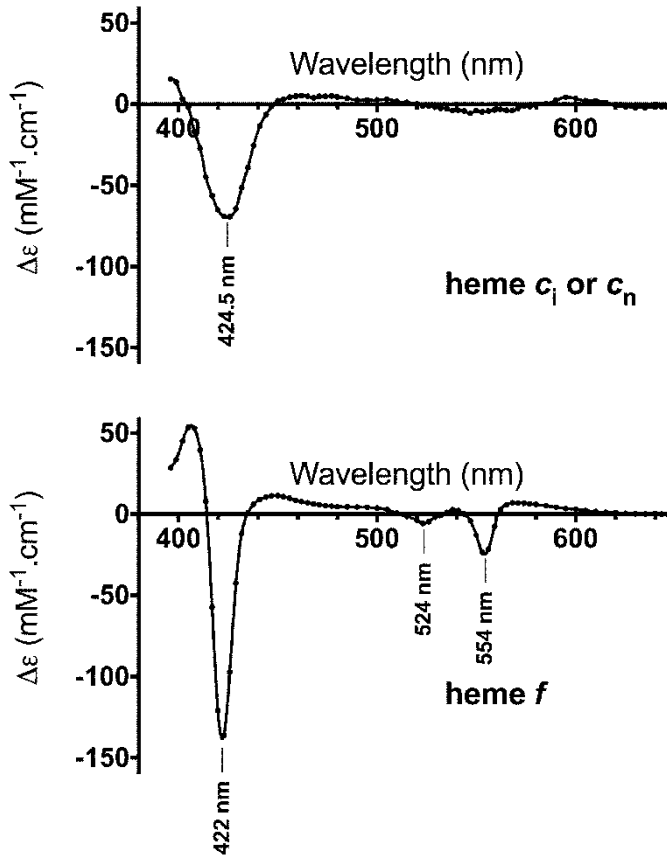
Fig. 15.2. Oxidized-minus-reduced difference spectra of heme c_i (or c_n or x) and Cyt f of the purified cytochrome b_6f complex from *Chlamydomonas reinhardtii* (Redrawn from Alric et al. (2005)).

Light-induced absorbance changes measured *in vivo* are dominated by the large contribution of the electrochromic shift (ECS) of pigments induced by the membrane potential ($\Delta\Psi$) formed during illumination. First reported by Duysens (1954), this signal was later (Witt 1971) ascribed to an absorbance shift (Stark effect) of chlorophylls a and b (bands around 630–700 nm), carotenoids (peak around 515–520 nm), chlorophyll b (trough around 480 nm), or a mixed contribution of chlorophylls and carotenoids (<450 nm) showing sharp bands. Those spectral contributions in the region below 450 nm hinder the absorbance changes in the γ-band of cytochromes specific to oxidation-reduction of hemes f (∼420 nm) or b_6 (∼430 nm) from the cytochrome b_6f complex. Furthermore, absorbance flattening

(Duysens 1956) in the highly absorbing blue region of the spectrum drastically reduces the contribution of the cytochrome's γ-bands (420–430 nm) relative to their α-bands (550–560 nm).

Chlorella sorokiniana has a small cell size compared to other eukaryotic algal species (Shihira and Krauss 1965). It was therefore quickly adopted by the *in vivo* spectroscopists, who appreciated the lower scattering and lower absorption flattening of these intact cells (Lavergne et al. 1984). At this time, mutants were produced and studied that were devoid of the major photosynthetic complexes PSI, PSII, cytochrome b_6f complex, LHC2 complexes (Lacambra et al. 1984; Lavergne et al. 1984), or chloroplast ATPase (Rappaport et al. 1999). In addition, almost all possible combinations of double

mutants were obtained, providing the experimentalist with a complete set of tools for the in vivo analysis of photosynthetic electron flow in the whole visible spectrum range.

Provided with a Chlorella strain lacking LHC2 and PSI, and with a Joliot-type spectrophotometer, a highly sensitive flash spectrophotometer designed and built by D. Béal (Joliot et al. 1980), J. Lavergne measured the absorption changes from ∼390 to ∼580 nm induced by 1 s of preillumination (Lavergne 1983). He observed that a spectral component, peaking around 425 nm and differing from the ECS contribution, appeared concomitantly with heme b oxidation and to the decay of the membrane potential. A pure spectral signature of this new component, that was named carrier G, of the photosynthetic chain was obtained 2 s after preillumination in the presence of the strong membrane uncoupler dicyclohexyl-18-crown-6 see Fig. 15.3 top panel (Lavergne 1983). The electron transfer from G to heme b_H was completed in less than 100 μs. This, with the requirement of a
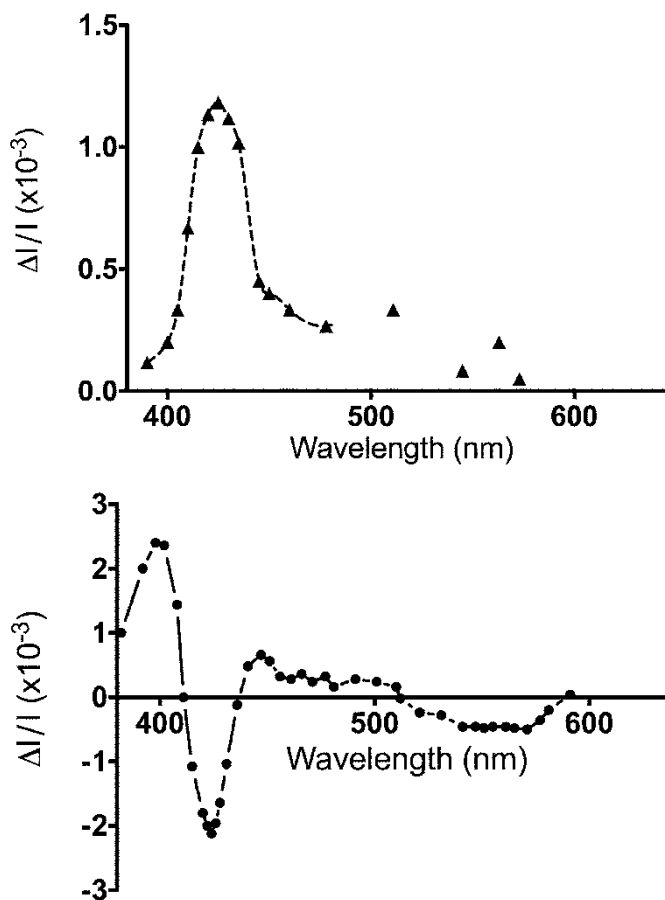
Fig. 15.3. Reduced-minus-oxidized (*top*) and oxidized-minus-reduced (*bottom*) difference spectra of heme c_i (or c_n or x) measured *in vivo* in intact cells of low-chlorophyll mutants of *Chlorella sorokiniana* (Redrawn from Lavergne (1983) and Joliot and Joliot (1988)). In the bottom panel, absorbance changes are distorted at shorter wavelengths (<410 nm) due to the difference between b_L and b_H contributions in this range, but the 500–600 nm range is better resolved than in the top panel, matching those of Fig. 15.2. *In vivo*, the amplitude of absorbance changes due to cytochromes is flattened in the γ-band compared to the α-band (observed here comparing Fig. 15.3, *bottom panel* to Fig. 15.2, *top panel*), for absorbance flattening, see (Duysens 1956).

transmembrane location of G, had suggested that it is embedded in (or tightly bound to) cytochrome $b_6 f$ (Lavergne 1983). A few years later, Joliot and Joliot (1988) observed G under similar uncoupled and preilluminated conditions: the same signal appeared in the difference spectrum between untreated and CO-treated samples (Joliot and Joliot 1988), see Fig. 15.3 bottom. The spectral features of G were similar to those of high spin c' cytochromes that were found in some photosynthetic bacteria.

Functional and biochemical evidence such as the apparent absence of G in spinach thylakoids, and no clear spectral contribution of G in the oxidized-minus-reduced spectra of purified cytochrome $b_6 f$, suggested that G was not ubiquitous in photosynthesis or that it could be a soluble protein loosely bound to cytochrome $b_6 f$ complex, lost during purification. Despite repeated attempts to further characterize this cytochrome, researchers mistook what was probably its most obvious manifestation: a blue-green smeary band in denaturing electrophoresis gels, appearing after TMBZ staining. Whereas the non-covalently bound b-type hemes of mitochondrial and bacterial cytochrome b are lost after denaturation of the complex (Berry et al. 1991), the cytochrome b_6-subunit of Cyt $b_6 f$ complexes can be stained by TMBZ after electrophoresis (Kuras et al. 1997). One of the b hemes seemed resilient to solubilization, but no matter how harsh the denaturing treatment was; the hazy blue-colored band appearing in TMBZ-stained gels would not fade away. At that time, the presence of a c-type heme covalently-bound to the b_6 subunit was so unthinkable that an unusual binding of one of the two b hemes to the cytochrome $b_6 f$ complex was postulated. For a recent update on the detection of heme c_i attached to cytochrome b_6 in denaturing gels, see (de Vitry et al. 2004). Other signs of the invisible heme c_i came from the difficulty of constructing Q_i-site mutants in *C. reinhardtii* on the basis of the homology with the Cyt bc_1 complex (de Lacroix de Lavalette et al. 2008), and from

mass spectrometry data (Whitelegge et al. 2002).

Despite these apparent inconsistencies, the pre-structure era did collect some solid information on this new cytochrome: (i) the new spectral component 'G' that was identified corresponded to a c'-type cytochrome (Lavergne 1983; Joliot and Joliot 1988), (ii) this cyt c' should be tightly bound to or embedded in the cytochrome $b_6 f$ complex, very close to heme b_H and located on the stromal side of the membrane (Lavergne 1983), (iii) the redox potential of this new cofactor was 30 mV higher than that of b_H, and the equilibrium constant between b_H and c_i was dependent upon the membrane potential (Lavergne 1983), and (iv) the redox potential of this cofactor could be modulated by the presence/absence of axial ligands of the heme iron, such as CO (Joliot and Joliot 1988).

III. Tridimensional Structure

The two structures of the cytochrome $b_6 f$ complex, published back to back in 2003 (Kurisu et al. 2003; Stroebel et al. 2003) confirmed the high structural homology between cytochrome bc_1 and $b_6 f$ complexes: the inter-cofactor distances are similar and the b hemes are oriented in the same way. Some differences in the $b_6 f$ complex relative to the cytochrome bc_1 complex were expected: a chlorophyll a molecule and a β-carotene, retained during purification of the $b_6 f$ complex (Huang et al. 1994; Pierre et al. 1995, 1997), were found in the structure. The big surprise from the crystal structure was a large flat electron density with two lateral extensions, in the Q_i site, at the stromal side of the complex. The general structure of the Q_i site is similar in $b_6 f$ and bc_1 complexes, with the exception of the active site showing a larger quinone-binding pocket formed by separate Cyt b_6 and subunit IV components rather than a single Cyt b protein, and an additional prosthetic group in $b_6 f$ complexes. The latter was likely to correspond to a heme according to the following observations: (i)

the shape and size of the flat density matched those of a porphyrin; (ii) a strong central electron density likely arose from an iron atom; (iii) well defined lateral extensions of the prophyrin ring were attributable to propionates and vinyl groups, one of which is located within bonding distance of the Cys35 from cytochrome b_6.

In contrast to other cytochromes, heme c_i iron does not have any proteic axial ligand, which makes it a curiosity of nature and suggests it could have unusual functions. Furthermore, a weak electron density appeared between heme c_i iron and the heme b_h propionate that could be attributed to a water molecule or a hydroxyl ion, making the c_i heme iron pentacoordinated (Fig. 15.4). On the other side of the heme iron, Phe 40 from subunit IV protrudes above the heme plane, likely gating the cavity and controlling quinone accessibility (de Lacroix de Lavalette et al. 2009).

The puzzling features of the heme c_i remain a challenge to be understood: the rather strict conservation of the architecture of the site between cyanobacteria (Kurisu et al. 2003) and green algae (Stroebel et al. 2003) strongly suggests that the Q_i-site of the cytochrome b_6f complex has distinctive functions which make the complex more fit to the energetic requirements of oxygenic photosynthesis.

IV. Heme c_i/Q_i Site Directed Mutants

The discovery of the additional heme c_i site in the Q_i site helped us understand the failure of site-directed mutagenesis approaches of the Q_i site during the pre-structure era (de Lacroix de Lavalette et al. 2008). The tridimensional structure of the quinone pocket now opens new opportunities for a ratio-

Fig. 15.4. Heme c_i environment of the *Chlamydomonas reinhardtii* cytochrome b_6f complex. The map shows potential ligands, water molecules around heme c_i and an electron density corresponding to a plastoquinone head and possibly interacting with F40 (Reprinted from Stroebel et al. 2003, with permission from the publisher).

Fig. 15.5. Difference Fourier map between F40Y mutant and wild type of heme c_i environment. Difference density is contoured at 4 Å positive signal in *green*, negative signal in *red*. The positive electron density around Tyr40, shows a higher occupancy of Tyr for this site than the native Phe, the negative signal suggest the absence of the quinone.

nal redesign of the cavity, to understand the redox chemistry of quinone reduction. The Phe40 from subunit IV was the obvious first mutagenesis target to modulate the interaction between c_i and the quinone or quinol molecules. When Phe40 was replaced by a Tyr (F40Y mutant) (de Lacroix de Lavalette et al. 2009), Tyr40 served as an axial ligand to the heme, which induced a downshift of the c_i heme midpoint-potential.

Figure 15.5 shows a stick model of the Q_i site of the F40Y mutant (Daniel Picot personal communication), where the stick model of the polar head of the quinone is added from the native structure. Here the quinone tail, too disordered in the crystal, could not be modelled (Stroebel et al. 2003). The 3D-contours show the positive (green) and negative (red) differences in electron density maps obtained from mutant and wild type crystals (F_{40Y}-F_{wt}). The negative signal shows that the mutation to Tyr has released the quinone. An unexpectedly strong positive electron density also appeared in the difference map, around Tyr40, showing a higher occupancy of Tyr for this site than the native Phe, which had a weaker electron density.

This can be explained because Tyr is tightly coordinated with the c_i heme group whereas the native Phe at position 40 is somehow more flexible and more readily allows the binding of a quinone to the Q_i site.

Following a single flash reduction of the b hemes in the F40Y mutant, their reoxidation was 50 times slower than in the wild type (de Lacroix de Lavalette et al. 2009), showing that Tyr 'locks' the cavity and impedes access by quinone. However, under continuous illumination, when the reducing pressure increases on the Cyt $b_6 f$ complex low-potential chain, the turnover of the mutant b_6 f complex was largely sufficient to sustain photosynthesis (de Lacroix de Lavalette et al. 2009), suggesting that the Tyr 40 cavity lock can be forced open. Tyr 40 was apparently displaced by carbon monoxide, which ligated to the c_i heme iron. The kinetics of CO rebinding after photodissociation was only two times slower in the mutant than in the wild type (de Lacroix de Lavalette et al. 2009). All of these data support the idea that the steric hindrance related to the Tyr-c_i heme coordination is relieved under steady-state illumination conditions; in other

words, heme c_i reduction may control lig-
and binding and substrate accessibility. Data
from heme oxygenase and nitrite reductase
(Adachi et al. 1993; Liu et al. 1999; Das et al.
2001) show similar redox-induced changes in
heme ligation that control the accessibility of
the substrate to the catalytic site. In the wild
type cytochrome b_6 f complex, the Phe40
aromatic ring has to move away from the
heme iron to accommodate the quinone (D.
Picot, personal communication). This move-
ment requires energy that can be partly pro-
vided by the ΔG of heme c_i reduction.

V. NQNO, A Not-So-Good Inhibitor of the Q_i Site of the Cytochrome $b_6 f$ Complex

Perhaps a similar redox-control of the Q_i-
site occupancy accounts for the inhibitory
effect of NQNO (Jones and Whitmarsh
1988). Rich et al. (1991) noted that "*NQNO
concentrations which are sufficient to cause
a maximal effect on the quinone reduction
site completely inhibit steady state electronic
turnover of the bc complexes but have no
effect on steady state electronic turnover
(Jones and Whitmarsh 1988) or proton
translocating stoichiometry (Hope and Rich
1989) of the bf complex. Furthermore,
at these inhibitor concentrations oxidant-
induced reduction of both haems b is possible
in the bc complexes, but oxidant-induced
reduction of more than one haem b has never
been achieved with the bf complex (Rich
1988; Furbacher et al. 1989). These findings
have led to views that the cytochrome bf
complex may have a basic reaction cycle
which can be different to that of the bc
complexes*".

These early observations may be ratio-
nalized by the structural differences in the
quinone-binding pocket and from the redox
titrations of heme c_i. Redox titrations show
that the E_m of heme c_i is $\sim$240 mV more
electronegative in the presence of NQNO.
This shows that NQNO stabilizes the oxi-
dized form of heme c_i, or destabilizes the
reduced form of heme c_i. In other words,

NQNO binds to the oxidized form of c_i,
or reciprocally NQNO is released upon re-
duction of c_i. Because the shift in potential
is rather large ($\sim$240 mV), the changes in
terms of equilibrium constant of the reac-
tions (and therefore association/dissociation
constant of the substrate/inhibitor) are rather
important (*i.e.* $10^{240/60} = 10^4$-fold change).
For a structure of the complex with NQNO,
see (Yamashita et al. 2007).

In Fig. 15.6, we propose a series of
reactions that would depict the residual
turnover of the cytochrome $b_6 f$ complex in
an NQNO-poisoned sample. These involve
electron transfer reactions and binding or
release of quinone analogs. Let us first
describe the B, C, D, and E states as expected
from the redox titrations from (Alric et al.
2005). The -60 mV per pH unit dependence
of the redox potential of c_i, in the inhibited
or non-inhibited state show that heme c_i
is protonated while reduced (deprotonated
while oxidized). In the presence of NQNO,
at pH 7, c_i and b_L are nearly equipotential
(Alric et al. 2005) (see B and C states, K $\sim$ 1),
whereas in the absence of inhibitor the
reduction of c_i ($E_m \sim +100$ mV) is largely
favored at the expense of the reduction of
b_L ($E_m \sim -140$ mV) (see D and E states,
$K_{eq} \sim 10^4$). This translates into a dissociation
constant 10^4-fold stronger (greatly favoring
dissociation) for the reduced form of the
heme (see states C and E) than for its
oxidized form (see states B and D) and
accounts for the leakiness of NQNO. Under
steady-state illumination, when the reducing
pressure from PSII on the cytochrome
$b_6 f$ low-potential chain is strong (heme
c_i reduction), NQNO is released from its
site thereby exemplifying the structural
difference between cyt bc_1 and cyt $b_6 f$
foreseen by Rich et al. (1991).

VI. Concluding Remarks

Twenty years elapsed between the discov-
ery of an unusual c'-type cytochrome sig-
nal associated with cytochrome $b_6 f$ turnover
(Lavergne 1983) and the proper identification
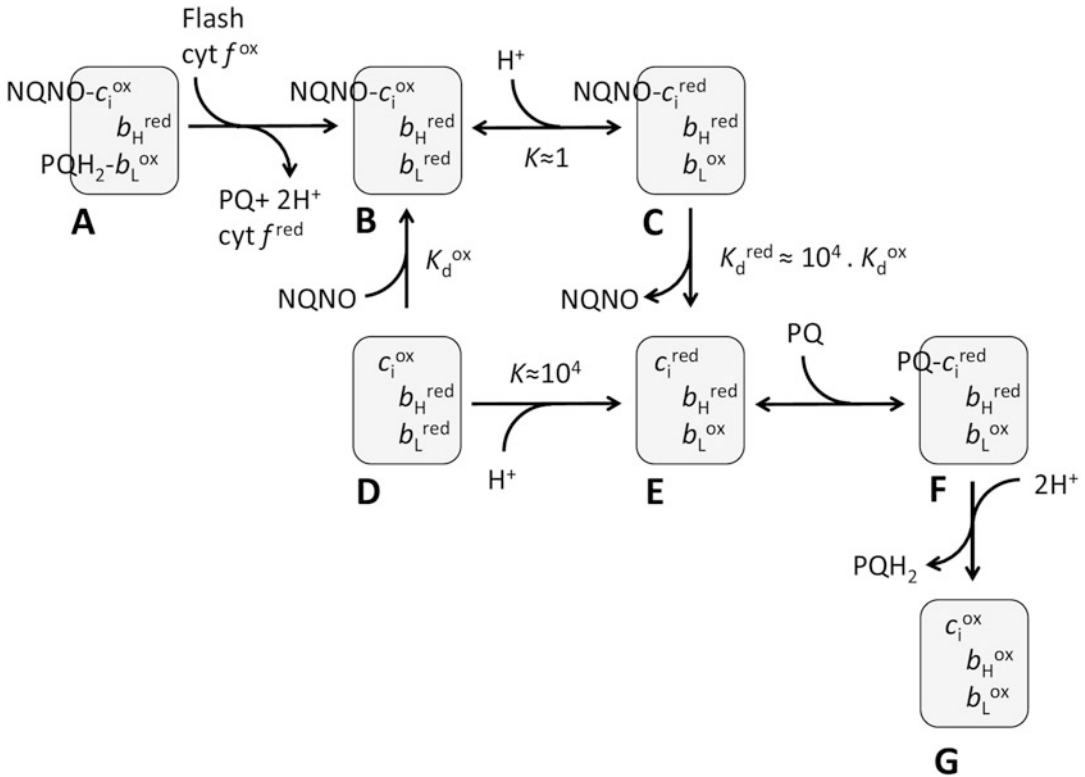
Fig. 15.6. Model for cytochrome $b_6 f$ turnover observed in the presence of NQNO. Upon oxidant-induced single-turnover of the enzyme (Cyt f oxidized by PSI following a single flash), state B is partly stable and the complex in its inhibited state. Upon continuous illumination, when the reducing pressure builds up on the cytochrome $b_6 f$ low-potential chain, steadily forming c_i^{red}, b_H^{red} and b_L^{red}, NQNO is released from the Q_i site, and oxidation of c_i-b_H (E-G reactions) is favored. The equilibrium constant, K, refers to the redox equilibrium between c_i and b_L and the dissociation constant, K_d, refers to the dissociation of NQNO from c_i.

of this cofactor as an intrinsic component of the complex (Kurisu et al. 2003; Stroebel et al. 2003). This rather long delay was partly due to the very nature of this peculiar heme c'. More probably, the delay was due to the unexpected existence of such a cofactor in such an improbable place. The specific role heme c_i plays in the cytochrome $b_6 f$ complex does not appear clearly from comparison with its counterpart, the ancestral cytochrome bc_1 complex. Perhaps the only satisfactory idea is that the closely coupled, two-electron reduction of the plastoquinone (PQ) to plastoquinol (PQH_2) in the Q_i site by the heme c_i-b_h "double barreled shotgun" would reduce the half-life of the semi-quinone and would make the cytochrome $b_6 f$ complex more fit for the oxic environment of oxygenic photosynthesis (Baymann et al. 2007).

Acknowledgments

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Structure and Function of Bacterial Cytochrome *c* Oxidases

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Summary

Cytochrome *c* oxidase or complex IV is the terminal enzyme of the aerobic respiratory chain performing the essential process of reducing molecular oxygen to water. The energy resulting from this reaction is exploited to drive proton pumping across the membrane, which in turn contributes to the generation of a proton motive force and the downstream synthesis

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of ATP. This chapter highlights current progress in the field of bacterial cytochrome c oxidase research from the perspective of the structural and functional characterisation of this family of essential enzymes.

I. Introduction

Energy is a fundamental requirement for all living organisms. Plants, algae and cyanobacteria derive their metabolic energy from photosynthesis while animals derive it from cellular respiration (Stryer et al. 2002). During photosynthesis, solar energy is harnessed by chlorophyll to produce carbohydrates from CO_2 and water, yielding oxygen as a by-product. In cellular respiration different food molecules such as glucose and fatty acids are metabolised into CO_2 and water, releasing energy. This energy is used to synthesise adenosine $5'$-triphosphate (ATP), the cell's chemical energy currency. ATP can later be used to drive a myriad of cellular functions ranging from biosynthesis to the active transport of ions and molecules across the membrane. A key protein in the bacterial respiratory chain, cytochrome c oxidase, forms the focus of this chapter and is described in detail from a structure and function perspective.

II. Respiratory Chain

The electron transport chain (Fig. 16.1), also referred to as the respiratory chain, catalyses the final steps of cellular respiration and is located in the plasma membrane of ar-

Abbreviations: ATP – Adenosine tri-phosphate; CcO – Cytochrome c oxidase; EPR – Electron paramagnetic resonance; FTIR – Fourier transform infra-red spectroscopy; HCO – Heme copper oxidase; HiPIP – High potential iron-sulfur protein; MD – Molecular dynamics; NAD^+ – Nicotinamide adenine dinucleotide (oxidised form); NADH – Nicotinamide adenine dinucleotide (reduced form); NMR – Nuclear magnetic resonance; N-side – Electrochemically negative side of the membrane; PDB – Protein Data Bank; P-side – Electrochemically positive side of the membrane; ROS – Reactive oxygen species; SU – Subunit

chaea and bacteria or the mitochondrial inner membrane in higher organisms. The respiratory chain comprises a series of integral membrane protein complexes and two mobile electron carriers required to couple electron transfer, from Complex I to Complex IV (see below), with transmembrane proton pumping and the reduction of oxygen (Fig. 16.1). Structures are available for both the soluble and membrane components of the respiratory chains to various resolutions (Fig. 16.1). The overall function of the respiratory chain is to generate the trans-membrane electrochemical gradient (proton motive force) that is used to drive thermodynamically unfavourable reactions such as the synthesis of ATP by the F_0F_1-ATP synthase, in a process known as oxidative phosphorylation. The membrane protein components of the bacterial electron transport chain are as follows (Fig. 16.1):

1. NADH:ubiquinone reductase (Complex I), the first and largest enzyme in the respiratory chain, functions by oxidizing NADH to NAD^+ and reducing ubiquinone to ubiquinol. The process is coupled to the pumping of four protons. In addition, two protons are used to reduce ubiquinone and one proton is released in the oxidation of NADH. Among the electron/proton transfer complexes, Complex I is capable of generating the largest amount of reactive oxygen species (ROS) (Hatefi et al. 1961; Hatefi 1985; Turrens 1997; Brandt 2006, 2011).

2. Succinate dehydrogenase (Complex II) is the second most reducing complex of the respiratory chain. It participates in both the citric acid cycle and the electron transport chain coupling the two processes (see Maklashina and Cecchini 2010). The enzyme reduces ubiquinone to ubiquinol using electrons derived from the oxidation of succinate to fumarate. Unlike Complex I, electron transfer in Complex II is not coupled to proton pumping.

Fig. 16.1. Overview of the electron transport chain. The chain comprises Complex I (*Thermus thermophilus,* PDB entry: 4HEA), Complex II (*Escherichia coli,* PDB entry: 1NEK), Complex III (*Saccharomyces cerevisiae*, PDB entry: 1EZV), cytochrome *c* (*T. thermophilus*, PDB entry: 1C52), Complex IV (*Paracoccus denitrificans*, PDB entry: 1QLE) and ATP synthase (*S. cerevisiae*, PDB entry: 2XOK).

3. The cytochrome bc_1 complex (Complex III) is the penultimate enzyme in the respiratory chain. It couples the one-electron reduction of cytochrome *c* to the step-wise oxidation of ubiquinol with the concomitant transfer of protons across the membrane via the Q cycle (Crofts 2004). Cytochrome *c* is a small heme containing protein that acts as a soluble electron carrier between Complex III and IV of the respiratory chain (Fig. 16.1). Like Complex I, Complex III also produce ROS (Hatefi et al. 1961; Hatefi 1985; Turrens 1997).

4. Cytochrome *c* oxidase (CcO, Complex IV) is a member of the Heme Copper Oxidase (HCO) superfamily. It is the terminal enzyme in the respiratory chain of mitochondria and aerobic prokaryotes (Ferguson-Miller and Babcock 1996). CcO uses electrons donated by cytochrome *c* to catalytically reduce molecular oxygen to water (Fig. 16.1). This electron transfer is coupled to the translocation of protons via conserved proton pathways across the membrane.

III. Heme Copper Oxidases

As the terminal enzyme of the electron transport chain, HCOs have a rich diversity in electron donors, subunit composition, and heme group types (Ferguson-Miller and Babcock 1996; Pereira et al. 2001, 2008; Sousa et al. 2012). Members of the HCO superfamily are defined by the presence in the most conserved subunit, subunit I (SU I), of a low-spin heme and a hetero-binuclear centre composed of a high spin heme and a copper ion, Cu_B. In bacteria, the heme group can be of type A, B or O (which differ in porphyrin ring substituents) or derivatives thereof. By contrast, the mitochondrial HCOs contain only A-type hemes (Calhoun et al. 1994; Lubben and Morand 1994).

HCO superfamily members are further classified as Type A, B and C based on amino acid sequence signatures and the degree of conservation of the D- and K-proton pathways (Sect. IV), (Pereira et al. 2001, 2008; Sousa et al. 2012) (Fig. 16.2 and Table 16.1). Type A HCOs include mitochondrial CcOs and their closely related counterparts from bacteria such as *P. denitrificans* and *Rhodobacter sphaeroides*, as well as the bo_3-type ubiquinol oxidase from *Escherichia coli*. This Type A group can be further divided into two subclasses, according to the amino acid composition at the hydrophobic end of the D-pathway. Type A1 contains a highly conserved glutamate in the sequence motif –XGHP**E**V– on helix

P-side

N-side
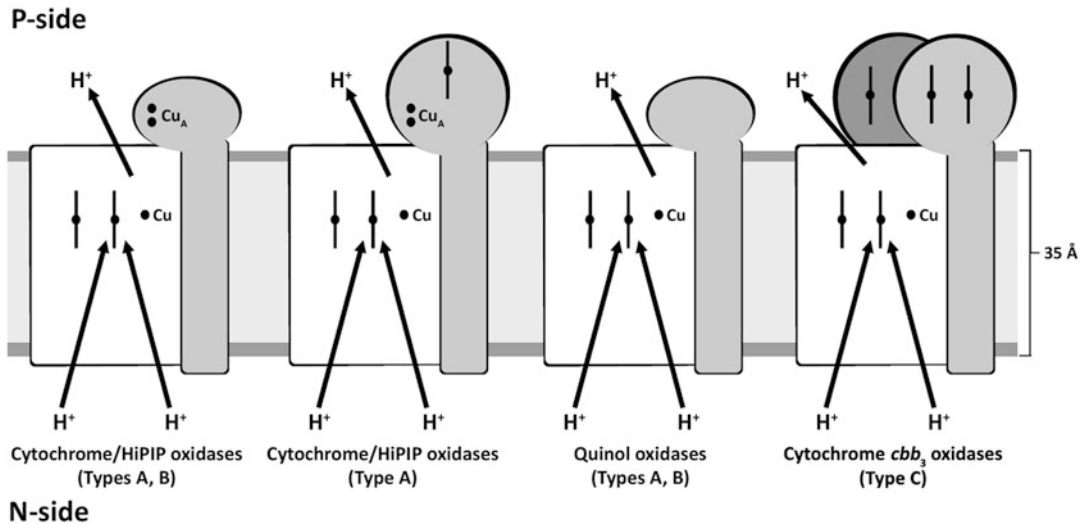
Fig. 16.2. Schematic representation of the architecture and overall mode of action of the various types of heme-copper oxidases. HCOs have been simplified to the catalytic subunits I (*white*) and II (*grey*). The structural and functional similarities of SU I are indicated and form the basis for HCO classification. Hemes are illustrated as a *black line* with a *black dot* in the centre for the metal. Proton movements are represented by *arrows*. For HCOs containing Cu$_A$ and/or heme *c* within their auxillary subunits, electron donation is via reduced cytochrome *c* or in some cases high potential iron-sulfur proteins (HiPIP). The *cbb*$_3$–type CcOs have an additional third subunit involved in electron transport (*dark grey*) (Redrawn from (Pereira et al. 2001)).

VI. In Type A2, the gating glutamate has been proposed to be replaced spatially by a consecutive tyrosine and serine in a – **YS**HPXV– motif (Pereira et al. 1999). Both A1 and A2 HCOs have a covalent link between one of the Cu$_B$ complexing histidines and a tyrosine on the same helix.

HCOs of the Type B variety are found in bacteria and archaea. *T. thermophilus ba*$_3$–oxidase is a Type B HCO. It has low sequence homology with the Type A mitochondrial CcO. The B-type HCOs do not contain the canonical K- or D-pathways. Instead they have a proton-pathway similar to the K-pathway of A-type HCOs. B-type HCOs have a His-Tyr cross-link, as observed with Type A HCOs.

Finally, the Type C HCOs are highly divergent from both the Type A and B oxidases with respect to amino acid and subunit composition. This group consists solely of *cbb*$_3$-type CcOs. They have an extra subunit that is involved in electron transport, while SU I contains the canonical His-Tyr cross-link and a single proton pathway analogous

to the Type A K-pathway. Unlike Type A and Type B HCOs, where the His-Tyr cross-link is between amino acids on the same helix, in Type C HCOs these residues are on different helices. This chapter will be limited to a discussion on the structure and function of the bacterial CcOs.

A. Overall Structure

Crystal structures for members of all three major HCO families are available (Type A (A1 and A2), Type B and Type C) (Fig. 16.3). The first bacterial cytochrome *c* oxidase structure was of the four subunit *aa*$_3$-type CcO from *P. denitrificans* (Iwata et al. 1995). The structures of the three canonical subunits (Subunits I-III) of the bacterial enzyme were remarkably similar to their mammalian counterpart (Tsukihara et al. 1996). Subsequent to the publication of the first structure of *aa*$_3$-type CcO, improvements were made to the quality of the crystallographic data and the models derived through the crystallisation of both

Table 16.1. Structural and biophysical characteristics of the HCO types and sub-types.

HCO family and subfamily	A		B	C
	A1	A2		
Examples	*P. denitrificans aa₃*	*T. thermophilus caa₃*	*T. thermophilus ba₃*	*P. stutzeri cbb₃*
	R. sphaeroides aa₃	*R. marinus caa₃*	*R. marinus ba₃*	
Minimal functional unit				
Subunit I	Low spin heme (*a*, *b*, and *a* derivatives)		Low spin heme (*b*)	Low spin heme (*b*)
	High spin heme (*a*, o and *a* derivatives)		High spin heme (*a*)	High spin heme (*b*)
	Cu$_B$		Cu$_B$	Cu$_B$
Subunit II	Cu$_A$ or Cu$_A$ and low spin heme *c*			FixO/CcoO[a]
	No prosthetic groups (quinol oxidase)			Low spin heme *c*
Subunit III	No prosthetic groups			FixP/CcoP[a]
				Two low spin hemes *c*
Proton channels:				
D-pathway	Yes	Yes	N/A	N/A
Residue(s) at end of D-pathway	E	YS	N/A	N/A
K-pathway	Yes	Yes	Alternative K-pathway	Alternative K-pathway
Pumping stoichiometry in reconstituted enzymes	0.75–1 H$^+$/e$^-$ Verkhovsky et al. (1999), Pawate et al. (2002), Richter et al. (2005), Capitanio et al. (2006)	0.8–1 H$^+$/e$^-$ Honnami and Oshima (1984), Pereira et al. (1999)	0.5–0.75 H$^+$/e$^-$ Kannt et al. (1998b)	0.2–0.4 H$^+$/e$^-$ Toledo-Cuevas et al. (1998)

Table 16.1. (continued)

HCO family and subfamily	A		B	C
	A1	A2		
Readily observable intermediate states	$P(Fe^{IV} = O)^b$ $F(Fe^{IV} = O)^b$		Not determined	Not determined
Tyr-His cross-link	Yes	Yes	Yes	Yes[c]

Modified from (Pereira et al. 2001)

[a]The FixO/CcoO and FixP/CcoP subunits found in cbb_3-type CcO refer to the mono- and di-heme c containing subunits and are not related to SU II and III of the Type A and B HCOs

[b]The P and F intermediates differ with regard to the protonation state of the ligand on Cu_B

[c]In Type C HCOs the Tyr-His cross-link is atypical; it bridges two helices
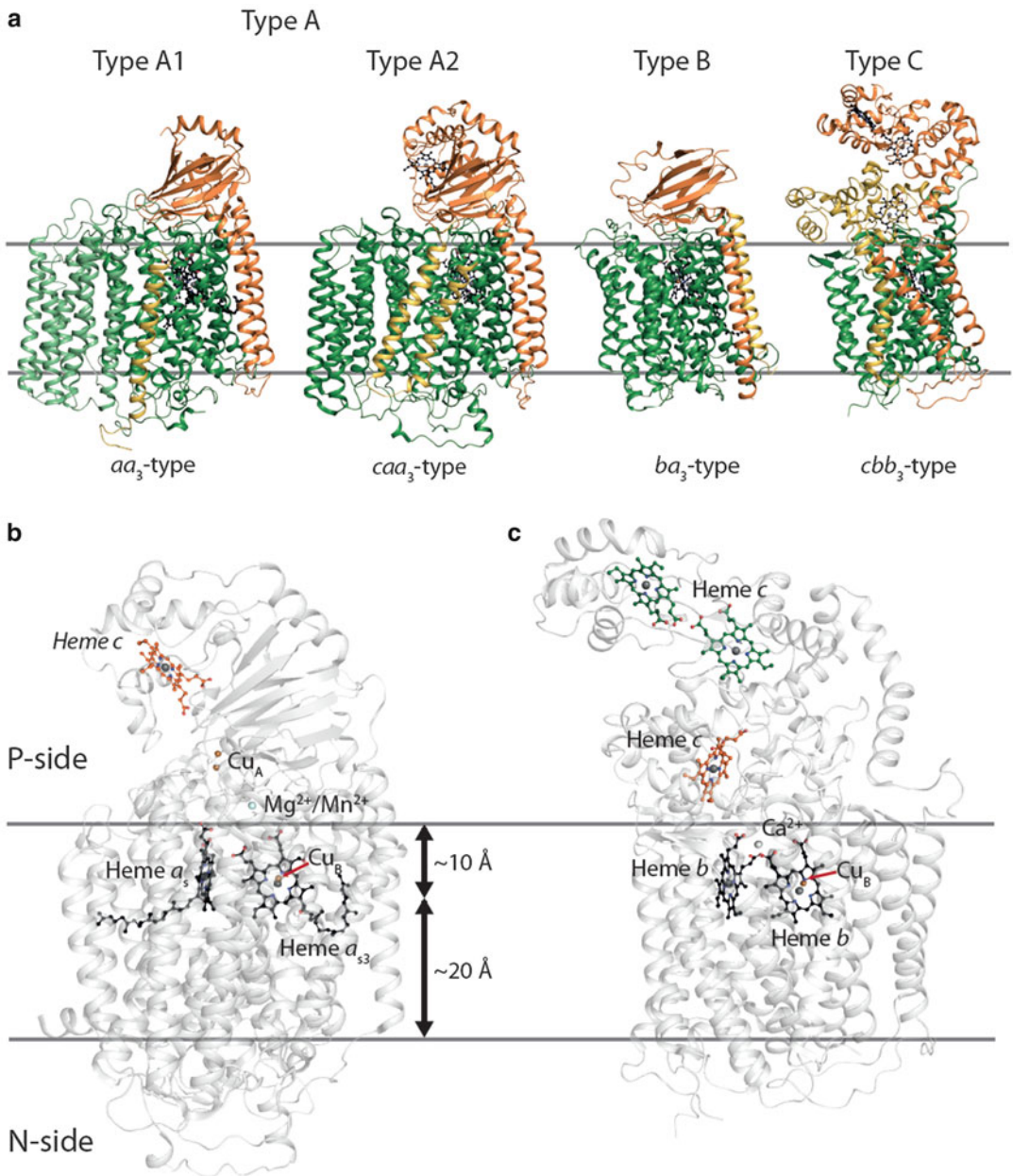
Fig. 16.3. Overview of CcO structures and prosthetic group arrangements. (**a**) Representative models of Type A1 (*aa₃*-type CcO; PDB entry 1QLE), Type A2 (*caa₃*-type CcO; PDB entry 2YEV), Type B (*ba₃*-type CcO; PDB entry 1EHK) and Type C (*cbb₃*-type CcO; PDB entry 3MK7). Protein is displayed as ribbons with SUs I–IV colored *green, orange, light green* and *yellow* for the Type A oxidases, SUs I–IIa colored *green, orange*, and *yellow* for Type B and CcoN-P colored *green, yellow*, and *orange* for Type C HCOs. (**b**) Architecture of Type A and B HCOs using *caa₃*-type CcO (*caa₃*-type CcO; PDB entry 2YEV) as an example. For *aa₃*-type and *ba₃*-type CcOs, electron transfer within the enzyme begins from Cu_A. (**c**) Architecture of Type C HCOs (*cbb₃*-type CcO; PDB entry 3MK7). In this figure, hemes are displayed as ball and stick with metal ions as spheres. Ligating residues are omitted for clarity. In **b** and **c**, the position of the metal centres relative to the sides of the membrane is indicated by *double headed arrows*.

the fully-assembled enzyme (SU I-IV) and a complex comprising the catalytically active subunits (SU I & II) of the *P. denitrificans* (Ostermeier et al. 1997; Koepke et al. 2009) and *R. sphaeroides* enyzmes (Svensson-Ek et al. 2002; Qin et al. 2006, 2008, 2009). Investigations into the overall structural similarities and subfamily-defining differences of HCOs were made possible upon the elucidation of the ba_3-type CcO (Type B) structure from *T. thermophilus* (Soulimane et al. 2000; Luna et al. 2008; Tiefenbrunn et al. 2011) and more recently the cbb_3-type CcO (Type C) from *P. stutzeri* (Buschmann et al. 2010) and the caa_3-type CcO from *T. thermophilus* (Lyons et al. 2012) (Fig. 16.3), both of which can function as nitric oxide (NO) reductases. Structural descriptions of bacterial CcO from the various HCO types and subtypes follow.

1. Subunit I

The HCO defining subunit, SU I, is similar for the three family types and consists of twelve transmembrane (TM) helices that house the aforementioned low spin (6-coordinate) and high spin (5-coordinate) hemes and the copper ion, Cu_B, as well as the proton pathways required for activity (Fig. 16.2). The heme types vary between Types A, B and C HCOs and are designated heme x (low-spin) and heme x_3 (high spin), respectively, where x denotes the heme type. All three redox centres are buried in the hydrophobic core of the protein and are located at a depth of approximately 1/3 of the low dielectric membrane thickness from the *P*-side of the membrane (Fig. 16.3b) (Iwata et al. 1995; Verkhovsky et al. 1999). The iron of the low spin heme is axially coordinated by two conserved histidines, one on each face of the heme. The high spin heme is axially coordinated by histidine (customarily called the proximal position), while the sixth coordination or distal site remains open for substrate binding. Cu_B is ligated by three histidine side-chains and is located about 5 Å from the distal side of the heme iron. Furthermore, one of the Cu_B

coordinating histidines is covalently linked to a neighbouring tyrosine side chain. Together, the high spin heme and Cu_B constitute the enzyme's active site. Both hemes are positioned with their propionates pointing towards the *P*-side of the membrane.

In addition to the redox centres described above, SU I houses a functionally relevant non-redox active metal centre in Type A and C HCOs. In Type A HCOs, this metal is typically a Mg^{2+} or Mn^{2+} ion and is found at the interface between SU I and SU II just above the high spin heme propionates. The metal is coordinated by three residues from SU I and SU II as well as three water molecules and resides within a larger water cluster. In Type C HCOs, however, the metal is a Ca^{2+} ion, which interacts directly with both the low and high spin hemes, and a glutamate and a serine side chain on SU I (also called CcoN in cbb_3-type CcO) and SU II, respectively. While the divalent cation is not essential for proton pumping in HCOs (Mills et al. 2000), it is most likely part of an exit pathway for water molecules produced during turnover (Florens et al. 2001). A calcium binding site is also present at the periplasmic side in transmembrane helix I of subunit I of the bacterial CcOs. This calcium ion has no known function.

2. Subunit II

In Type A HCOs, SU II comprises two N-terminal transmembrane helices and a globular cupredoxin domain that resides in the periplasm in bacteria. The cupredoxin domain, formed from a ten-stranded β-barrel, houses the mixed valence dinuclear copper site, Cu_A. The copper ions are ~2.5 Å apart and act as the primary electron acceptor of CcO from reduced cytochrome c. They are bridged by two cysteines and individually ligated by a histidine – methionine pair and a glutamate peptide carbonyl – histidine pair, respectively. In addition to the cupredoxin domain, Type A HCOs can also contain a C-terminally fused cytochrome c domain as in the structure of the caa_3-type CcO from *T. thermophilus* (Lyons et al. 2012).

SU II of Type B HCOs have a similar overall architecture to the Type A HCOs in that they contain a membrane anchored cupredoxin domain. However, the membrane spanning domain comprises a lone helix. Spacially, the missing helix is compensated by an additional subunit consisting of a single TM and is designated SU IIa (Soulimane et al. 2000).

Type C HCOs do not use a dinuclear copper site to receive electrons from reduced cytochrome *c*. Instead, redox centers housed in two auxillary subunits, designated subunit O and P (CcoO and CcoP), are used. CcoO comprises a single TM helix and a soluble cytochrome *c* domain carrying one heme *c*. In *cbb*$_3$-type CcO from *Pseudomonas stutzeri*, the linkage between the TM helix and the cytochrome *c* domain interacts with the high (active site) and low spin hemes in SU I (CcoN), as well as CcoP. CcoP consists of a pair of N-terminal TM helices connected by a 40 Å long helix to an extramembranal globular domain (Buschmann et al. 2010). The globular unit comprises two cytochrome *c* domains each housing a heme *c*. The third canonical helix in the two cytochromes is domain-swapped.

3. Subunit III

In Type A HCOs, SU III comprises seven TM helices that form a V-shaped cleft between two helical bundles and has been modelled with one or more integral phospholipids. The *P. denitrificans* enzyme contains a single phosphatidylcholine molecule while the *R. sphaeroides* enzyme contains two phosphatidylethanolamines (Iwata et al. 1995; Svensson-Ek et al. 2002). The *T. thermophilus caa*$_3$-type CcO has a three-chained thermophilic glycoglycerophospholipid in this region (Lyons et al. 2012). The rationale behind this V-shaped packing of helices in not obvious. However, it is possible that the bound lipid facilitates oxygen uptake into SU I to the binuclear centre. It has been shown recently that oxidase enzyme depleted of SU III undergoes suicide inactivation, where increasing amounts of inactive enzyme

appear during catalytic turnover (Haltia et al. 1989; Bratton et al. 1999; Hosler 2004), with a subsequent loss of Cu$_B$. Furthermore, the presence of SU III appears to influence the pH dependence of steady state oxygen reduction and is important for maintaining efficient proton flow to the active site via the D-pathway (Gilderson et al. 2003; Mills et al. 2003; Hosler 2004; Mills and Hosler 2005).

4. Subunit IV

SU IV, identified in the crystal structures of Type A1 HCOs as a single transmembrane helix, is loosely associated along one side of SU I and III (Fig. 16.3) and has no obvious effect on the enzymatic properties of the enzyme (Iwata et al. 1995). It bears no significant sequence homology with any other known peptide or protein. Hence, its function remains a mystery. Iwata et al. speculated on its role in stabilisation of the enzyme (Iwata et al. 1995). In the case of *caa*$_3$-type CcO from *T. thermophilus* SU IV consists of two TM helices located across the concave membrane exposed surface of SU I/III (Fig. 16.3) (Lyons et al. 2012). To date, no evidence for a SU IV has been found in Type B HCOs, while in the structure of *cbb*$_3$-type CcO electron density for an additional helix was detected. However, its sequence could not be assigned to CcoQ (Buschmann et al. 2010).

IV. Pathways Within HCO

Internal pathways through HCOs are paramount for protons and oxygen to reach the binuclear centre and for the release of product water and pumped protons across the membrane. These pathways will be described in the following sections for Type A, B and C HCOs.

Crystal structures of the various cytochrome *c* oxidases in conjunction with site-directed mutagenesis studies have identified conserved proton pathways within the assorted HCOs. Type A HCOs have two proton pathways designated as the D- and K-pathways while the Type B and C HCOs
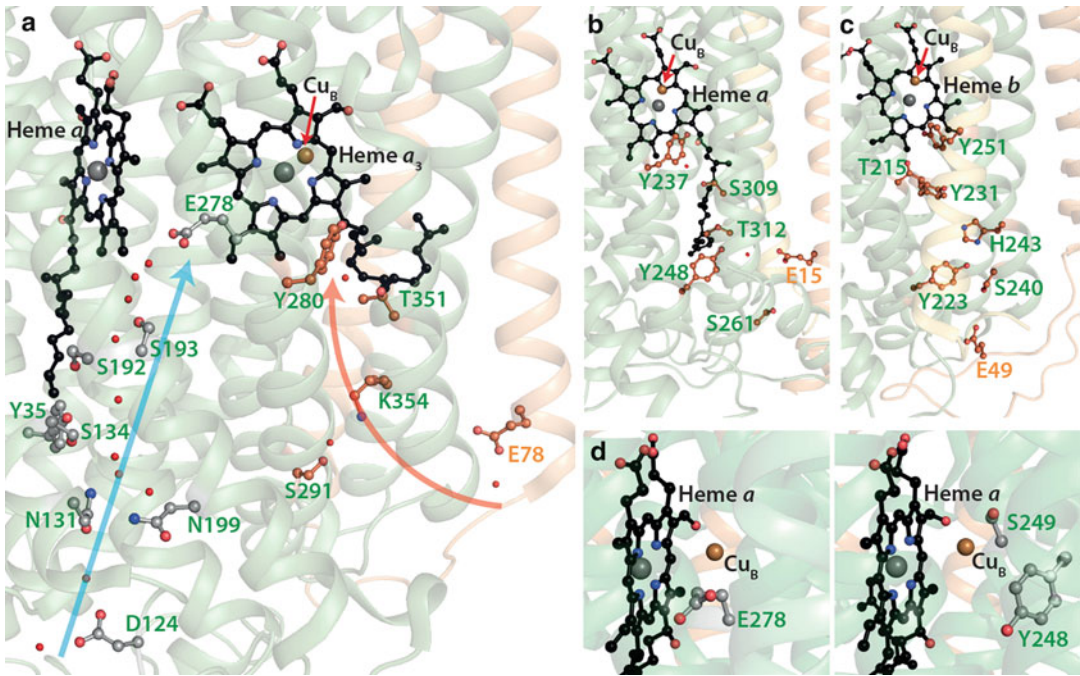
Fig. 16.4. HCO proton pathways. (**a**) Key residues of the D- and K-pathways for Type A HCO (*aa₃*-type CcO; PDB entry: 1QLE). The D- and K-pathways are identified by *blue* and *red arrows*, respectively. (**b–c**) The alternative K-pathway for Type B (*ba₃*-type CcO; PDB entry 1EHK) and Type C (*cbb₃*-type CcO; PDB entry 3MK7) HCOs. (**d**) Difference between Type A1 (*left*, *aa₃*-type CcO; PDB entry: 1QLE) and A2 (*right*, *caa₃*-type CcO; PDB entry 2YEV) HCOs in the composition of the hydrophobic end of the D-pathway. The linings for the K- and D-pathways are *gray* and *orange*, respectively. Hemes are displayed as ball and stick with metal ions as spheres. Waters are shown as *red* non-bonded spheres. Prosthetic group ligands have been excluded for clarity.

contain a single proton pathway analogous to the Type A K-pathway (Fig. 16.4).

A. Type A

1. D-Pathway

In Type A HCOs, the longer D-pathway is named for the conserved and functionally critical aspartate at the pathway entrance (Fig. 16.4a). The importance of the aspartate has been highlighted by an Asp132Asn mutation in *R. sphaeroides* that precludes proton translocation and significantly impairs enzymatic turnover (Thomas et al. 1993; Fetter et al. 1995; Garcia-Horsman et al. 1995). In the *P. denitrificans* enzyme, the pathway starts at Asp 124 on the *N*-side of the membrane and is connected through hydrogen bonding via a lone bridging water molecule to Asn 131. A continuous chain

of water molecules, hydrogen bonded to a number of flanking residues, links Asn 131 to the gating glutamate, Glu 278, (Fig. 16.4a and d). The gating glutamate is located approximately 10 Å from the binuclear centre and is essential for both the proton pumping and oxygen reduction events. The protons can traverse the pathway from the cytoplasm to the active site via the Grotthuss mechanism where protons transfer between hydrogen bonded molecules in the water chain (de Grotthuss 1806). While the water chain is well conserved within the known bacterial Type A1 HCOs structures, the number and arrangement of water molecules vary between the different structures. The most complete chain has been reported for the *aa₃*-type CcO from *P. denitrificans* where eight waters span the distance between Asp 124 to the gating glutamate Glu 278 (Koepke et al. 2009). Recently, it has been shown that

the structure of the water pathway may be modulated by changes in the redox state of the oxidase that has led to a proposed water gating mechanism (Kim et al. 2007, 2009) with elements of alternating access between the D- and K- pathways (Qin et al. 2009).

In the *T. thermophilus caa₃*-type CcO, a member of the Type A2 HCOs, the D-pathway, is similar to that of the Type A1 HCOs (Lyons et al. 2012). It is formed by a chain of ordered internal waters complete with orienting polar side chains that extend from Asp 103 at the cytoplasmic (N-) side of the membrane to the active site via the hallmark YS "gate" that differentiates the A1 and A2 HCO sub-types (Fig. 16.4d). This gate is functionally equivalent to the aforementioned gating glutamate (Glu 278 *P. denitrificans*) as determined by single-, double- and triple-mutant studies performed on the *P. denitrificans aa₃*-type CcO, which mimics the Type A2 pump configuration in both *T. thermophilus* and *Rhodothermus marinus* (Backgren et al. 2000).

The D-pathway is responsible for transmitting six protons, four of which are pumped to the *P*-side of the membrane; the remaining two are donated to the active site for use in oxygen reduction chemistry. In *P. denitrificans aa₃*-type CcO, Glu 278 has been identified as the gate or branch point responsible for routing protons to the active site or to the pump loading site which leads to the *P*-side of the membrane (Fig. 16.4). This glutamate points towards the *N*-side of the membrane ("down" conformation) from which it can accept protons as indicated by the crystal structures and molecular dynamics simulations (Iwata et al. 1995). For either proton pumping or proton loading of the binuclear centre to occur, the side chain of Glu 278 has been proposed to undergo a rotamer change during turnover to a position pointing towards the *P*-side ("up" conformation) (Riistama et al. 1996; Kaila et al. 2008). Here, it contacts both the active site and pump loading site through individual water networks. In contrast, the protonation state of the YS "gate" side chains in Type A2 HCOs are not modulated by the redox state of

the enzyme as shown by pK_a calculations on both homology and crystal derived models (Pereira et al. 1999; Lyons et al. 2012). This would indicate that its role is to stabilize particular water wires that are continuous with the active site or the proposed proton exit pathway, supporting a role for a water-gated mechanism.

2. K-Pathway

The shorter K-proton pathway (Fig. 16.4) is named after a conserved, centrally located lysine residue, Lys 354 (*P. denitrificans* numbering). It leads to the binuclear centre via the highly conserved residues Lys 354, Thr 351, and the His 276 cross-linked Tyr 280 in the *P. denitrificans* enzyme (Fig. 16.4a). In the known crystal structures, the pathway contains two structurally resolved water molecules. However, a continuous pathway is not evident without the transient movement of key side chains and/or rearrangement of structural waters. The K-pathway is responsible for supplying two protons to the catalytic site as part of the initial reduction of the binuclear centre. In fact, unless proton uptake occurs via the K-pathway, reduction of the binuclear centre is not possible (Adelroth et al. 1998). While the K-pathway is not involved in the oxidative phase of the catalytic cycle (Sect. VI) (Tuukkanen et al. 2006), movement of the protonated lysine side chain towards the binuclear centre has been reported to act as a "dielectric well" stabilizing/neutralizing an otherwise uncompensated electron transfer to the active site (Junemann et al. 1997; Brändén et al. 2001; Rich and Marechal 2013).

The entry point to the K-pathway has been determined by site-directed mutagenesis to be Glu 101 on SU II in *R. sphaeroides aa₃*-type CcO (Branden et al. 2002; Tomson et al. 2003). The entry point to the *P. denitrificans* enzyme is unclear as similar mutagenesis work on the homologous Glu 78 did not affect proton pumping or the rate of heme a_3 reduction even though a long range electrostatic interaction between the binuclear active site and Glu 78 has been identified based on

titration curves calculated by Monte Carlo sampling (Kannt et al. 1998a). A recent high resolution structure of the *P. denitrificans* enzyme has identified a chain of ordered water molecules close to Glu 78 that may form the entry for protons in the K-pathway without the need for direct involvement of the glutamate side chain (Koepke et al. 2009).

B. Type B and C

1. An Alternative K-Pathway

Interestingly, proton pathways within the Type B and C HCOs are markedly different from that of the Type A HCOs. Crystal structure analysis of the Type B HCO, (ba_3-type CcO from *T. thermophilus*) identified three putative pathways – a novel Q-pathway and homologs of the Type A D- and K-pathways (Soulimane et al. 2000). The lack of sequence conservation within the putative D- and Q-pathways suggested they were not functional. However, the alternative K-pathway, consisting of a number of polar residues that are conserved across the B-family, could form a proton pathway. These residues reside within SU I (Thr 312, Ser 309, Tyr 248, Ser 261 and Tyr 237) with the exception of the pathway entrance (Glu15) which is on SU II (Fig. 16.4b). Subsequent mutagenesis of these residues showed that this alternative K-pathway is critical for enzyme function (Chang et al. 2009).

For the Type C cbb_3-type CcO, the putative proton pathway is composed of residues in the CcoN subunit and Glu 49 of the CcoP subunit at the pathway entrance (*P. stutzeri* numbering). The pathway propagates through a number of polar residues (Tyr 223, Ser 240, His 243, Tyr 317, and Thr 215) to Tyr 251 (Fig. 16.4c), which is cross-linked to one of the histidine ligands of Cu_B and is located ∼4 Å from the active site (Rauhamaki et al. 2006; Buschmann et al. 2010). Recently, it has been shown that alanine and glutamine mutants of Glu 25 in the *R. sphaeroides* cbb_3-type CcO, homologous to Glu 49 of the *P. stutzeri* enzyme, have severely impaired proton uptake from

solution during oxidation of the fully reduced enzyme by O_2, consistent with this residue playing a critical role in the proton pathway (Lee et al. 2011). Interestingly, the enzyme's ability to reduce NO (a process requiring protons from the periplasmic side of the membrane) remains largely unaffected.

C. Proton and Water Exit Pathways

Given the sequence diversity across the various HCO types, it is logical to assume that if the pump loading site is common to all, the number of possible candidates for this site is limited. They consist of the six histidines that ligate the heme iron and Cu_B, the four propionate groups of the two hemes, as well as the internal water cluster over the hemes (Fig. 16.5a) (Chang et al. 2009). Various models have been proposed that have focused on one or more of the Cu_B ligating histidines, but no convincing evidence has emerged identifying these residues as the proton loading site (Wikstrom 2000; Makhov et al. 2006; Sharpe and Ferguson-Miller 2008; Popovic et al. 2010). The active site heme D-propionate has also been implicated as the pump loading site or as a transient site through which protons transfer to the proton loading site. In Type A and B HCOs, the area above the D-propionate contains two highly conserved arginines which form a salt bridge with the propionate (Ostermeier et al. 1997; Kannt et al. 1998a). However, these arginines are not conserved in Type C HCOs, with this site interacting with a Ca^{2+}. Furthermore, site directed mutagenesis of the arginines in the aa_3-type CcO from *R. sphaeroides* also indicate that the D-propionate is unlikely to be the proton loading site. However, its role as a transient site has not been excluded (Lee et al. 2009).

The active site A-propionate is a possible location for the pump loading site based on evidence from computational studies on the *P. denitrificans* enzyme and structural analysis of the HCO family (Wikstrom and Verkhovsky 2007; Chang et al. 2009; Kaila et al. 2011). The computational analysis identified a transient and reversible
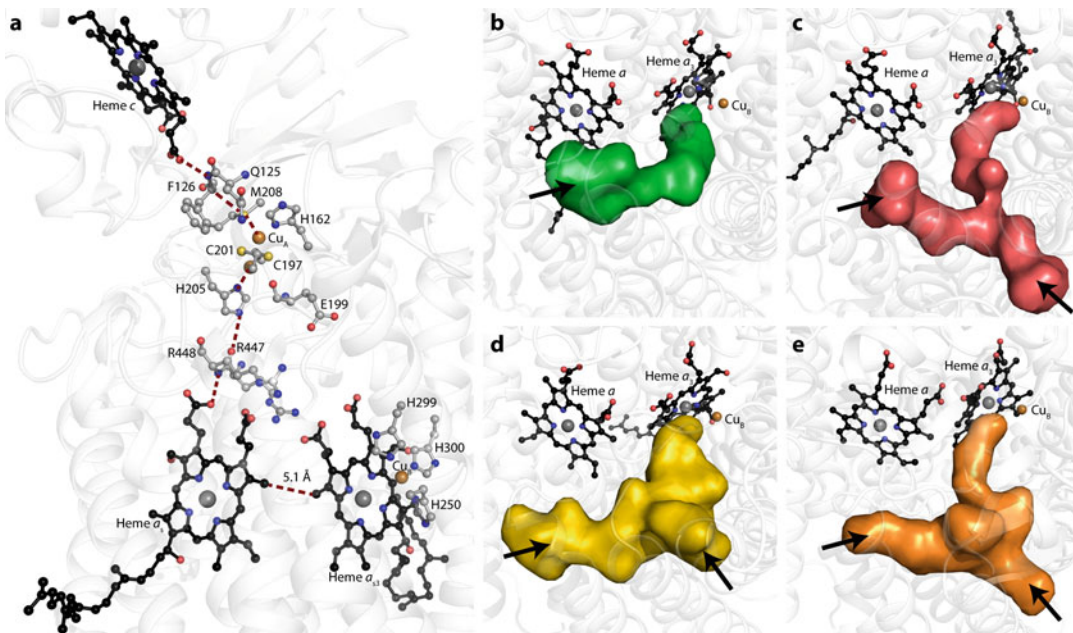
Fig. 16.5. Electron transfer pathway and oxygen channels. (**a**) Electron transfer pathway for *caa₃*-type CcO between heme *c*, Cu$_A$, heme *a* and the active site. Figure redrawn based on electron transfer pathways calculated for *P. denitrificans aa₃*-type CcO (Farver et al. 2006) and *caa₃*-type CcO (Lyons et al. 2012). The ion to ion distances are 17.2 Å, 19.5 Å and 13.6 Å for heme *c* iron to CuA, CuA to heme *a* iron, and heme *a* iron to heme *a₃* iron, respectively. (**b**–**e**) Calculated oxygen channels for *aa₃*-type CcO from *R. sphaeroides* (PDB entry 1 M56), *caa₃*-type CcO from *T. thermophilus* (PDB entry 2YEV), *ba₃*-type CcO from *T. thermophilus* (PDB entry 1EHK) and *cbb₃*-type CcO from *P. stutzeri* (PDB entry 3MK7). Entry points into the channels from the membrane hydrocarbon region are indicated by *black arrows*. Protein is represented as transparent *white* cartoon. Hemes and relevant residues are as ball and stick with ions as spheres. Channels were calculated using *Hollow* (Ho and Gruswitz 2008).

conformation change of the A-propionate of heme *a₃* that could facilitate proton pumping upon heme *a* reduction. This conformational change results in a breaking of the hydrogen bond shared between the propionate and a nearby aspartic acid (Asp 399 of SU I in *aa₃*-type CcO of *P. denitrificans*). This effectively increases the pK_a of the A-propionate, allowing it to be protonated, thus linking the pK_a of the A-propionate with the redox state of heme *a* (Kaila et al. 2011). The same study also showed that Arg 438 and the D-propionate dissociate. However, this did not correlate to a change in its pK_a implying that the A-propionate is likely the pump site (Kaila et al. 2011).

In the various HCOs, a hydrophilic cavity above the hemes, separating SU I and SU II, has been postulated as the beginning of the water exit pathway. This portal in Type A and C HCOs is centred on a conserved Mg^{2+}/Mn^{2+} and Ca^{2+} ion, respectively, while in Type B HCOs the cavity is filled with water molecules. These sites are continuous with the periplasmic space via discrete channels (Iwata et al. 1995; Ostermeier et al. 1997; Soulimane et al. 2000; Svensson-Ek et al. 2002; Koepke et al. 2009; Buschmann et al. 2010; Lyons et al. 2012).

V. Electron Transfer

The elucidation of crystal structures for cytochrome *c* oxidase has provided the distance separating the redox centres, the ligands and surrounding protein, and their relative orientation (Fig. 16.3b and c). This

has allowed for a more detailed exploration of hypotheses into the electron transfer pathways within the protein as outlined below.

Cytochrome c oxidase is reduced by four consecutive one-electron transfer events from cytochrome c, with the rate limiting step being the formation and disassociation of the cytochrome c/cytochrome c oxidase complex. In general, electron transfer through cytochrome c oxidase proceeds as follows: upon complex formation electrons are rapidly transferred from reduced cytochrome c to the Cu_A centre (in Type A and B HCOs). From the homobinuclear Cu_A centre, intra-molecular electron transport has been calculated for the *T. thermophilus* enzyme which proceeds via a hydrogen bond to the His 205 side chain of SU II to the amide carbonyl between Arg 447 and Arg 448 in SU I (Fig. 16.5a). Another hydrogen bond connects the peptide nitrogen of Arg 448 to the heme a A-propionate (Farver et al. 2006). It has been demonstrated that the inter-heme electron transfer is concomitant with vectorial proton transfer (Belevich et al. 2006). Analogous electron transfer calculations have been made for the bovine heart enzyme that reveal a similar electron transfer path. For an in-depth analysis of electron tunneling see (Moser et al. 2008; Gray and Winkler 2010).

While the cytochrome c/cytochrome c oxidase complex structure remains elusive to crystallographic methods, efforts have been made to explore the interactions between these two redox partners using mutagenesis, nuclear magnetic resonance (NMR) (Reincke et al. 2001) and molecular dynamics approaches (Roberts and Pique 1999; Flock and Helms 2002; Bertini et al. 2005; Lyubenova et al. 2007; van Dijk et al. 2007). From in vitro interaction studies of the cytochrome c oxidase Cu_A domain and its partner, the soluble cytochrome c_{552} from *P. denitrificans,* a multi-step mechanism was proposed (Maneg et al. 2003, 2004). First, oppositely charged surface potentials are responsible for the long-range orientation and initial interaction of the two redox partners. Secondly, hy-

drophobic patches on both surfaces govern a fine tuning of the docking. Finally, reduction of Cu_A proceeds through electron transfer from cytochrome c via the conserved Trp 121 side chain of the Cu_A domain in the *P. denitrificans* enzyme (Drosou et al. 2002). A different scenario has been proposed for the *T. thermophilus* cytochrome c_{552} and the ba_3-type CcO Cu_A electron transfer complex where docking is dictated primarily by hydrophobic interactions (Muresanu et al. 2006).

The fused cytochrome c/cupredoxin domain in caa_3-type CcO offers a unique view of this complex (Lyons et al. 2012). The cytochrome c domain is oriented such that its heme c propionates interact directly with the cupredoxin domain, with the most prominent interaction between the heme c D-propionate and the *cis*-amide of an aromatic residue conserved across SU II of Type A and B CcOs, i.e., Phe 126 and Trp 121 in *T. thermophilus* caa_3-type CcO and *P. denitrificans* aa_3-type CcO, respectively. This *cis*-amide was first identified in *R. sphaeroides* aa_3-type CcO (Qin et al. 2006). Based on this observation a pathway model was proposed with through-bond and through-space tunneling events.

In the case of cbb_3-type CcO, the primary electron acceptor from cytochrome c is the outer most heme c of CcoP, whose C and D pyrroles are exposed. This accepted electron is subsequently transferred via the inner heme c of CcoP to the heme c of CcoO. From CcoO, the electron is shuttled to the CcoN heme b. The edge-to-edge distance between heme c of CcoO and heme b of CcoN is shorter than that between Cu_A and heme a or b in aa_3-type CcO in cupredoxin domain containing HCOs. The electron is transferred between heme b and b_3, which are in Van der Waal's interaction with each other, and from heme b_3 it can equilibrate with Cu_B.

VI. Oxygen Channel

Non-polar molecules freely diffuse through the hydrocarbon rich environment of the biological membrane. Structural and computa-

tional analyses of Type A, B and C HCOs have identified possible channels that can be exploited by molecular oxygen to access the active site (Fig. 16.5b–e) (Iwata et al. 1995; Hofacker and Schulten 1998; Svensson-Ek et al. 2002; Luna et al. 2008; Lyons et al. 2012). Early suggestions and calculations for an oxygen pathway in cytochrome *c* oxidase identified a hydrophobic residue lined channel starting at the V-shaped cleft in SU III with the oxygen molecule entering SU I between helices IV and V (Hofacker and Schulten 1998).

For the aa_3-type CcO of *R. sphaeroides* (Svensson-Ek et al. 2002) and the ba_3-type CcO of *T. thermophilus* (Luna et al. 2008; Tiefenbrunn et al. 2011), hydrophobic cavities have been determined from xenon positions upon X-ray analysis of crystals pre-pressurised with the gas. Xenon is used as an oxygen analog. With a higher atomic number it is easier to identify in electron density maps than oxygen. In these studies, the end of the pathways, proximal to the active site, in both the *R. sphaeroides* and *T. thermophilus* enzymes are in close agreement with previous molecular dynamics calculations (Hofacker and Schulten 1998). A mutation of the highly conserved Val 287 to isoleucine in the *E. coli bo₃* oxidase resulted in an increase in the K_M for dioxygen with no effect on Vmax when compared to the wild type enzyme (Riistama et al. 1996). This valine, homologous to Val 279 in *P. denitrificans* aa_3-type CcO, is located one residue away from the gating glutamate position on helix VI in Type A1 HCOs. Given its proximity to the active site, the result of the mutagenesis was interpreted as a partial blocking of oxygen entry to the active site. This observation further supports the location of the calculated and experimentally determined oxygen channels reported to date (Hofacker and Schulten 1998; Luna et al. 2008; Tiefenbrunn et al. 2011; Lyons et al. 2012).

Comparison of the oxygen channels in the Type A and B cytochrome *c* oxidases from *T. thermophilus* highlight a conserved bifurcated pathway with two channels leading to the apolar region of the membrane with entrances between helices II and III and between helices IV and V (Fig. 16.5c–d) (Luna et al. 2008; Lyons et al. 2012). Interestingly, the oxygen channel in the ba_3-type CcO is larger than that of its type A counterpart. The latter has a constriction where the two branches meet. This difference in channel architecture may be related to the fact that ba_3-type CcO needs to sequester oxygen under low oxygen conditions. Molecular dynamic simulations performed with the aa_3-type CcO of *R. sphaeroides* identified a bifurcated pathway similar to that observed for caa_3-type CcO, with the calculations indicating a role for gating via constriction and dilation within the oxygen channel, which has been postulated to regulate oxygen supply to the active site (Buhrow et al. 2012). A similar bifurcated channel has been described for cbb_3-type CcO (Fig. 16.5e) (Buschmann et al. 2010).

VII. The Catalytic Cycle

The catalytic cycle of cytochrome *c* oxidase is illustrated in Fig. 16.6. It can be divided into an oxidative ($\mathbf{R} \rightarrow \mathbf{O}$) and a reductive ($\mathbf{O} \rightarrow \mathbf{R}$) phase. The cycle involves several intermediate states of the active site, identified by one-letter code. The reaction cycle presented here describes the reduction of oxygen by A-type HCOs. As only limited information for the other two HCO types is available, they will not be described further.

The oxidative phase of the redox cycle begins with the binding of molecular oxygen to the fully reduced binuclear centre ($\mathbf{R}$ state) forming the $\mathbf{A}$ state (Fig. 16.6). In this step, molecular oxygen binds to heme a_3. The $\mathbf{A}$ state spontaneously transforms to the $\mathbf{P_M}$ state upon the transfer of four electrons to molecular oxygen. This causes immediate cleavage of the dioxygen bond which, in turn, prevents the formation of reactive oxygen species. Two of these electrons derive from heme a_3 and one from Cu_B. The last electron along with a proton comes from the cross-linked tyrosine, yielding the $\mathbf{P_M}$ intermediate with a ferryl iron, Fe[IV] = O, a cupric copper/hydroxyl species and a neutral
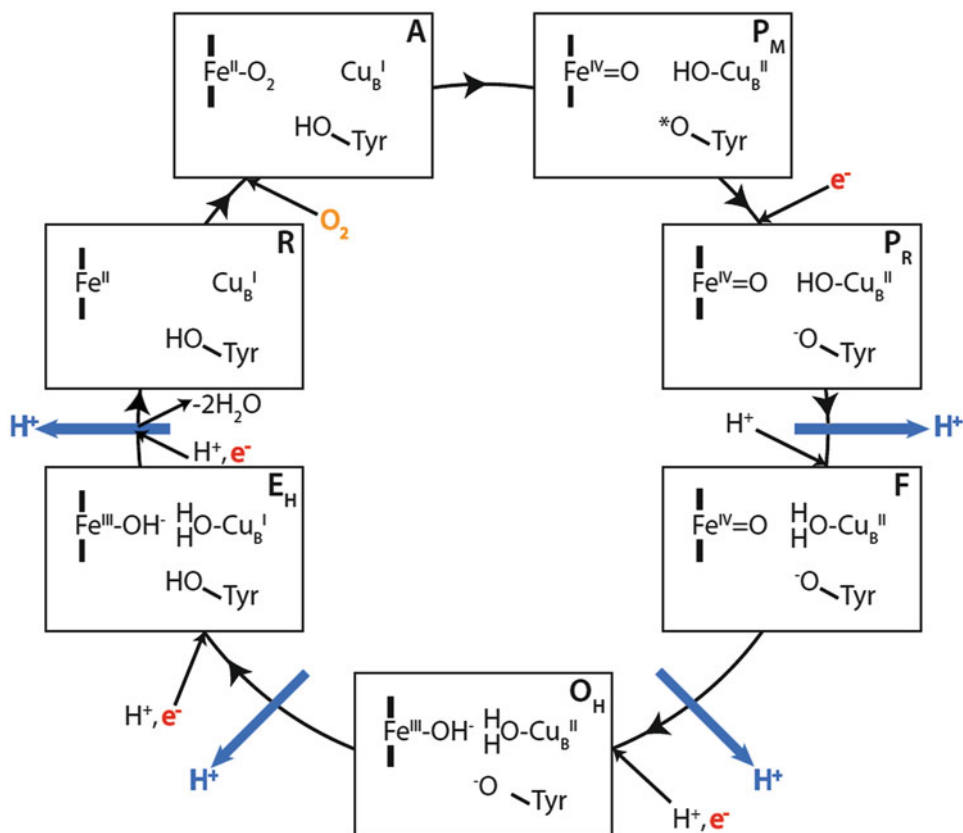
Fig. 16.6. The catalytic cycle of cytochrome *c* oxidase. The catalytic cycle for type A HCOs is drawn clockwise with the name of each intermediate state noted in the upper right corner of each rectangle. For simplicity, only the active site is shown which consists of heme a_3, Cu_B and the tyrosine of the cross-linked histidine-tyrosine pair. The oxidation states of Fe, Cu_B, and the cross-linked tyrosine in the binuclear center are indicated, along with protonation states of the cross-linked tyrosine and metal ligands. Proton pumping events are identified by *blue arrows*, while electrons and protons consumed during turnover are shown in *red* and *black*, respectively. Binding of O_2 to the binuclear center takes place in the **R** to **A** transition (Modified from (Sharma et al. 2013)).

tyrosine radical. The P_M state transforms to the P_R state accompanied by the addition of an electron to the active site with the tyrosine radical converting to a tyrosylate in the P_R state. The latter evolves into the **F** state through the addition of a proton to the active site with the only observable difference between these states being the ligand on Cu_B (OH^- vs H_2O). Experimentally, this has been seen by EPR and UV–VIS spectroscopy measurements on the transformation of the **F** state into the P_R state by the addition of ammonia at high pH, which subsequently ligates to Cu_B (as described in detail in von der Hocht et al. (2011)). The first proton pumping event in the catalytic cycle takes place during the Pr to F state transition. The oxidised ferric/cupric O_H state is formed by another transfer of an electron and a proton to the binuclear centre with the pumping of the second proton. Transition from the O_H to the E_H state proceeds with the pumping of the third proton and the uptake of an electron and proton to the active site, resulting in a cuprous Cu_B and reprotonation of the tyrosine. The reaction cycle is completed when the **R** state is regenerated from the E_H state during which an electron and a proton are donated to the binuclear centre with the two product waters released and the fourth and final proton pumped (Michel 1999; Kaila et al. 2009, 2011).

Wikström and coworkers observed at least two of these natural intermediates (**P** and **F** states) in mitochondria for the first time when the reaction catalyzed by the F_oF_1-ATP synthase was reversed (thereby hydrolyzing ATP and pumping protons back across the membrane (Wikstrom 1981)). This generates a positive redox-potential, resulting in the partial reversal of the cytochrome *c* oxidase reaction. Initially, it was believed that the structure of the active site in the P-intermediate resembled a peroxy state (hence P intermediate) and that of the F intermediate a ferryl (or oxoferryl) state. However, Resonance Raman spectroscopy was used to show that both states have an oxoferryl structure (Ogura and Kitagawa 2004). After Wikström's description of intermediates in the reaction cycle, it was found that at least some of these intermediates can easily be generated artificially and exploited to investigate detailed aspects of the reaction cycle. These states are only defined as intermediates with distinct absorption maxima in difference spectra. For example, the **P** and **F** intermediates have distinctive positive signals at 607 and 580 nm, respectively (Wikstrom 1981). The **P** intermediate can be generated artificially by adding 5 molar equivalents of hydrogen peroxide at high pH or by purging with CO. One recently discovered method to generate an artificial **P** intermediate involves adding ammonia at high pH after artificially generating the **F** intermediate by treating the oxidase with 500 molar equivalents of hydrogen peroxide (for a summary see (von der Hocht et al. 2011)). These intermediates were then further studied by a variety of different techniques. Resonanace Raman spectroscopy and FTIR were used to probe the exact structure of the active center in different (artificial) intermediate states of the reaction cycle (Hrabakova et al. 2006; Voicescu et al. 2009; Kozuch et al. 2013; Marechal et al. 2013).

To fully reduce dioxygen to water in a single step, four electrons are needed. However, as noted, the metal ions in the active center are only able to provide three electrons. Investigations on artificial intermediates helped identify possible sources of the fourth electron. The mystery was solved to some degree by applying EPR spectroscopy using a high concentration of hydrogen peroxide (500 molar equivalents) to generate the artificial **F**-state of the cytochrome *c* oxidase (for recent studies see (de Vries 2008; Sharpe et al. 2009; von der Hocht et al. 2011; Yu et al. 2012)). The source of the electron turned out to be a tyrosine or tryptophan radical close to the active site. However, the exact identity of the amino acid providing this electron is still under debate.

The groups of Adelroth and Brzezinski implemented stopped flow methods to monitor the various catalytic intermediates in the reaction cycle (Thornstrom et al. 1988). These groups used a combination of trapped components that deliver electrons upon excitation, together with flash photolysis of CO from C*c*O, to specifically target and observe different intermediates in the reaction cycle of C*c*O. Stopped flow measurements have been exploited to calculate rate constants for the generation of the different natural intermediates.

It has recently been shown, by the group of Konstantinov, that a fine tuning of the catalytic activity of cytochrome *c* oxidase happens via a specifically bound Ca^{2+} ion. This group also showed that a high affinity zinc binding site exists within the C*c*O and that zinc in this site inhibits the enzyme (Vygodina et al. 2008, 2013).

VIII. Side Reactions in CcO

Apart from their oxygen reductase activity, all HCOs show a variety of side reactions. C type HCOs, for example, exhibit nitric oxide (NO) reductase activity (Forte et al. 2001). This is not surprising, since NO reductases are closely related to HCOs. Similarly, members of the Type A and B HCO family from *T. thermophilus* have NO reductase activity (Giuffre et al. 1999).

Apart from its established oxidoreductase activity, Type A and C HCOs have been

reported to possess a background catalase-like activity (Orii and Okunuki 1963; Xie et al. 2014). This activity is attributed to the heme component of the enzymes as observed with several other heme-containing proteins such as methemoglobin (Dalziel and O'Brien 1954; Orii and Okunuki 1963; Paco et al. 2009; Bolshakov et al. 2010; Hilbers et al. 2013). Interestingly, aa_3-type CcO in *P. denitrificans* containing a recombinantly overexpressed SUI (all other subunits were chromosomally encoded) has been shown to exhibit increased catalase activity in comparison to the wild type enzyme. This activity was later linked to co-factor insertion during the biogenesis of recombinant CcO, where SU I was overexpressed on a low copy number plasmid. The catalase activity was shown to be diminished when the protein was co-expressed with a plasmid containing genes which encode chaperones previously reported to be required for correct insertion of Cu and heme *a*, thus increasing the chaperone levels in the cell (Kulajta et al. 2006; Bundschuh et al. 2008; Greiner et al. 2008).

IX. Conclusions

Although HCOs exhibit a wide diversity in subunit and heme type composition, the fundamental mode of action remains, for the most part, conserved across its various types. Questions relating to the pathways through which the pumped protons exit and the identity of the pump loading site still remain. Given that the majority of information has been obtained from extensive studies of the Type A, and to a lesser extent Type B HCOs, a similar focus on Type C HCOs is in order. Recent structures of the Type A2 and C HCOs, coupled with the available models of the Type A1 and B variants have allowed identification of conserved pathways and channels and provide a framework for further structure-function studies of this important enzyme.

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The Respiratory Terminal Oxidases (RTOs) of Cyanobacteria

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Summary

Cyanobacteria are unique in performing oxygenic photosynthesis and aerobic respiration in the same compartment. Therefore the bioenergetic situation of cyanobacteria can be considered to be the most complex among all organisms known. While the components of oxygenic photosynthesis are largely known, the components of cyanobacterial respiration still present a number of mysteries for two reasons: (1) Many cyanobacteria contain several respiratory electron transport chains (up to ten) and (2) there are at least eight types of respiratory terminal oxidases (RTOs) that catalyse the reduction of O_2 to water in cyanobacteria. Starting from a list of all RTOs in all currently sequenced cyanobacterial genomes, this review defines the eight different types of known cyanobacterial RTOs: namely, mitochondrial-type cytochrome *c* oxidase, four types of alternate respiratory terminal oxidases (ARTOs), cytochrome *c* oxidase of the *cbb₃* type, cytochrome *bd*-type quinol oxidases (cyanide sensitive or cyanide insensitive), and plastidic type terminal oxidase (Ptox). The goal of this chapter is to review current understanding of the function of these RTOs, especially in cyanbacteria that contain more than one RTO. The author starts from the hypothesis that in strains with more than one RTO, each must have a specific function, otherwise the genes encoding "superfluous" RTOs would quickly be lost by spontaneous mutation,

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because most RTOs consist of several polypeptide subunits that must be synthesized during every cell division cycle. Two conclusions can be drawn from the available data: (1) Every known cyanobacterium contains at least one RTO, and (2) no one type of RTO is present in all cyanobacteria. Furthermore, the available data probably allow the conclusion that all cyanobacteria contain at least one RTO that catalyzes the transfer of electrons from a soluble cytochrome c to O_2 (cytochrome c oxidase reaction).

I. Introduction

There are three primary bioenergetic process in nature: photosynthesis, respiration, and fermentation. Photosynthesis and respiration are membrane bound processes with light and chemical bond energy as primary energy sources, respectively, while fermentation operates with soluble components using chemical bond energy. Cyanobacteria are defined as prokaryotes (eubacteria) able to perform oxygenic photosynthesis. Therefore, the bioenergetic reactions of cyanbacteria are central to the properties of these organisms. All known cyanobacteria also perform aerobic respiration in the dark and many, perhaps all, also perform these reactions in the light. (Tchernov et al. 2003). Relatively little is known about fermentation in cyanobacteria, which is probably only performed under anaerobic dark conditions. Among cyanobacteria three known modes of growth are photo-litho-autotrophy, photo-organo-heterotrophy, and chemo-organo-heterotrophy.

All cyanobacteria are capable of photo-litho-autotrophy, some are able to grow also photo-organo-heterotrophically, and of these a few are known to possess the capability for chemo-organo-heterotrophy. For the latter strains, respiration is essential,

no cyanobacterium known is able to grow chemo-organo-heterotrophically under anaerobic conditions using fermentation processes for its energy metabolism. For respiration, the only known terminal electron acceptor in cyanobacteria is dioxygen. The reduction of dioxygen to water is the chemical reaction common to all cyanobacterial "Respiratory Terminal Oxidases" (RTOs):

$$O_2 + 4\,H^+ + 4\,e^- \rightarrow 2\,H_2O$$
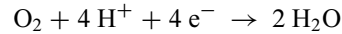
There exist several earlier reviews on the RTOs of cyanobacteria (e.g. Bernroitner et al. 2011; Hart et al. 2005; Schmetterer and Pils 2004; Schmetterer 1994). All RTOs are membrane-bound enzymes. The source of the electrons necessary for this reaction can be different for different RTOs. The RTO reaction, independent of which RTO catalyses it, is apparently of great importance for cyanobacteria. One example for this is the observation that in the absence of all RTOs – in *Synechocystis* sp. PCC 6803 – the photochemical reduction of external substrate (specifically ferricyanide) is enhanced 24 times (Bradley et al. 2013). This could be of potential commercial interest by using cyanobacteria that lack RTOs for photo-voltaic systems (Bombelli et al. 2011).

II. Types of RTOs Occurring in Cyanobacteria and Definitions

Table 17.1 lists all genes that encode putative subunits (SU) of RTOs in cyanobacteria in the Joint Genomics Institute, Integrated Microbial Genomes (IMG) database (https://img.jgi.doe.gov/cgi-bin/mer/main.cgi). In

Abbreviations: Aox – Mitochondrial alternative oxidase; ARTO – Alternate respiratory terminal oxidase; Cbb3 – cbb_3 type cytochrome c oxidase; CM – Cytoplasmic membrane; Cox – Mitrochondrial-type cytochrome c oxidase; Cyd – Homolog of cytochrome bd-type quinol oxidase; ICM – Intracytoplasmic membranes (thylakoids); Ptox – Plastidic type terminal oxidase; RTO – Respiratory terminal oxidase; SU – Subunit

Table 17.1. Genes encoding respiratory terminal oxidases of cyanobacteria (December 2013).

Gloeobacter violaceus PCC 7421

1 cox (gvip300, gvip299, gvip298)

1 cyd (gvip165, gvip164)

1 ptox (gll0601)

1 ARTO2a (incomplete, only gvip077 [CTQLCG], gvip078, no SU3)

Gloeobacter kilaueensis JS1

1 cox (GKIL_3886, GKIL_3887, GKIL_3888)

1 ARTO3 (GKIL_3027 [DSQFSG], GKIL_3026, GKIL_3025)

2 ARTO2a, both only SU2 and SU1, no SU3, (GKIL_2666 [CTQLCG], GKIL_2665 [WVHHMF]), (GKIL_3016 [CTQLCG], GKIL_3017 [WVHHMF])

Prochlorococcus marinus AS9601

1 cox (A9601_05011, A9601_05001, A9601_04991)

1 ptox (A9601_03651) (protein lacks 1/3 of normal sequence at the C-terminus, functional?)

Prochlorococcus sp. CC9311

1 cox (sync_2127, sync_2126, sync_2125)

1 ARTO3 (sync_1927 [DAMFSG], sync_1928, sync_1929)

Prochlorococcus sp. CC9605

1 cox (Syncc9605_0608, Syncc9605_0607, Syncc9605_0606)

1 ARTO1 (Syncc9605_0981 [DAMFSG] Syncc9605_0980 [WVHHMF], Syncc9605_0979)

Prochlorococcus sp. CC9902

1 cox (Syncc9902_1754, Syncc9902_1755, Syncc9902_1756)

1 ptox (Syncc9902_0439)

Prochlorococcus marinus marinus CCMP 1375

1 cox (Pro0442, Pro0441, Pro0440)

Prochlorococcus marinus pastoris CCMP 1986

1 cox (PMM0446, PMM0445, PMM0444)

1 ptox (PMM0336)

Prochlorococcus marinus MIT 9211

1 cox (P9211_04431, P9211_04421, P9211_04411)

Prochlorococcus marinus MIT 9215

1 cox (P9215_05251, P9215_05241, P9215_05231)

1 ARTO1 P9215_16631 [DAMFSG], P9215_16641 [WVHHMF], P9215_16651

1 ptox (P9215_03641)

Prochlorococcus marinus MIT 9301

1 cox (P9301_04701, P9301_04691, P9301_04681)

1 ptox (P9301_03641)

Prochlorococcus marinus MIT 9303

1 cox (P9303_06421, P9303_06411, P9303_06401)

Prochlorococcus marinus MIT 9312

1 cox (PMT9312_0445, PMT9312_0444, PMT9312_0443)

1 ptox (PMT9312_0341)

Prochlorococcus marinus MIT 9313

1 cox (PMT1341, PMT1342, PMT1343)

Prochlorococcus marinus MIT 9515

Table 17.1. (continued)

1 cox (P9515_05081, P9515_05071, P9515_05061)

1 ARTO1 (P9515_12631 [DAMFSG], P9515_12641 [WVHHMF], P9515_12651)

Prochlorococcus marinus NATL1A

1 cox (NATL1_05001, NATL1_04991, NATL1_04981)

1 ptox (NATL1_11341) (protein lacks 28% of normal sequence, functional?)

Prochlorococcus marinus NATL2A

1 cox (PMN2A_1777, PMN2A_1776, PMN2A_1775)

1 ptox (PMN2A_0429)

Prochlorococcus sp. WH 7803

1 cox (SynWH7803_1871, SynWH7803_1872, SynWH7803_1873)

1 ARTO1 (SynWH7803_0715 [DAMFSG], SynWH7803_0714 [WVHHMF], SynWH7803_0713)

Prochlorococcus sp. WH8102

1 cox (SYNW1861, SYNW1862, SYNW1863)

1 ARTO3 (SYNW1528 [DAMFSG], SYNW1529, SYNW1530)

1 ptox (SYNW0887)

Synechococcus elongatus PCC 6301

1 cox (syc1508_c, syc1507_c, syc1506_c)

1 cyd (syc2325_d, syc2326_d)

1 cbb3 (syc1309_d, syc1310_d)

Synechococcus sp. PCC 6312

1 ARTO2 (Syn6312_1832, Syn6312_1831 [WVHHMF], Syn6312_1830)

1 cyd (Syn6312_1302, Syn6312_1301)

Synechococcus sp. PCC 7002

1 cox (SYNPCC7002_A1162, SYNPCC7002_A1163, SYNPCC7002_A1164)

1 ARTO1 (SYNPCC7002_A0727 [DSQFSG], SYNPCC7002_A0726 [WVHHMF], SYNPCC7002_A0725)

Synechococcus sp. PCC 7336

1 ARTO2 (Syn7336_0128, Syn7336_0127 [WVHHMF], Syn7336_0126)

1 cyd (Syn7336_2230, Syn7336_2231)

Synechococcus sp. PCC 7502

1 cox (Syn7502_01300, Syn7502_01301, Syn7502_01302)

1 cyd (Syn7502_03463, Syn7502_03462)

Synechococcus elongatus PCC 7942

1 cox (Synpcc7942_2602, Synpcc7942_2603, Synpcc7942_2604)

1 cyd (Synpcc7942_1767, Synpcc7942_1766)

1 cbb3 (Synpcc7942_0202, Synpcc7942_0201)

Synechococcus sp. RCC 307

1 cox (SynRCC307_0672, SynRCC307_0671, SynRCC307_0670)

1 ARTO3 (SynRCC307_1937 [DAHFSG], SynRCC307_1936 + SynRCC307_1935: sequence complete, but split into 2 ORFs, functional?, SynRCC307_1934)

Synechococcus sp. WH 8016

1 cox (Syn8016_2863, Syn8016_2864, Syn8016_2865)

1 ARTO3 (Syn8016_0007 [DAMFSG], Syn8016_0006, Syn8016_0005)

Synechococcus sp. JA-2-3B'a(2–13)

1 cox (CYB_2697, CYB_2698, CYB_2700; genes are immediately adjacent)

1 cyd (CYB_1208, CYB_1209)

Table 17.1. (continued)

Synechococcus sp. JA-3-3Ab

1 ARTO2 (CYA_1514, CYA_1513 [WVHHMF], CYA_1512)

1 cyd (CYA_1764, CYA_1766; genes are immediately adjacent)

Synechococcus sp. KORDI-49

1 cox (KS49-00006190, KS49-00006180, KS49-00006170)

1 ARTO3 (KS49-00010360 [DAMFSG], KS49-00010350, KS49-00010340)

Synechococcus sp. KORDI-100

1 cox (KS100-00007580, KS100-00007570, KS100-00007560)

1 ARTO3 (KS100-00020350 [DAMFSG], KS100-00020360, KS100-00020370)

Synechocystis sp. PCC 6803

1 cox (SYNPCCP_0713, SYNPCCP_0714, SYNPCCP_0715)

1 ARTO1 (SYNPCCP_1544 [DSQFSG], SYNPCCP_1395 [WVHHMF], SYNPCCP_1396; gene for SU2 far away from putatively associated genes encoding SU1 and SU3!)

1 cyd (SYNPCCP_0610, SYNPCCP_0611)

Cyanobium gracile PCC 6307

1 cox (Cyagr_3213, Cyagr_3212, Cyagr_3211)

1 ARTO1 (Cyagr_2789 [DSQFSG], Cyagr_2788 [WVHHMF], Cyagr_2787)

Microcystis aeruginosa NIES-843

1 cox (MAE_22930, MAE_22940, MAE_22950)

Gloeocapsa sp. PCC 7428

1 cox (Glo7428_4450, Glo7428_4451, Glo7428_4452)

1 ARTO1 (Glo7428_4787 [DSMFSG], Glo7428_4786 [WVHHMF], Glo7428_4785)

Cyanothece sp. ATCC 51472

1 cox (Cy51472_2994, Cy51472_2993, Cy51472_2992)

2 ARTO1 (Cy51472_4951 [DSQFSG], Cy51472_4950 [WVHHMY], Cy51472_4949), (Cy51472_0426 [DSQFSG], Cy51472_0427 [WVHHMY], Cy51472_0428)

1 cyd (Cy51472_4388, Cy51472_4387)

Cyanothece sp. PCC 7424

1 cox (PCC7424_4071, PCC7424_4072, PCC7424_4073)

1 ARTO1 (PCC7424_3139 [DSQFSG], PCC7424_3140 [WVHHMF], PCC7424_3141)

1 cyd (PCC7424_0151, PCC7424_0150)

Cyanothece sp. PCC 7425

2 cox (Cyan7425_3958, Cyan7425_3957, Cyan7425_3956), (Cyan7425_1211, Cyan7425_1212, Cyan7425_1213)

Cyanothece sp. PCC 7822

1 cox (Cyan7822_4713, Cyan7822_4712, Cyan7822_4711)

1 ARTO1 (Cyan7822_4378 [DSQFSG], Cyan7822_4379 [WVHHMF], Cyan7822_4380)

1 ARTO3 (Cyan7822_5867 [DSQFSG], Cyan7822_5868, Cyan7822_5869)

1 cyd (Cyan7822_1642, Cyan7822_1641)

Cyanothece sp. PCC 8801

1 cox (PCC8801_0567, PCC8801_0568, PCC8801_0569)

Cyanothece sp. PCC 8802

1 cox (Cyan8802_0584, Cyan8802_0585, Cyan8802_0586)

Cyanothece sp. ATCC 51142

1 cox (cce_1977, cce_1976, cce_1975)

Table 17.1. (continued)

2 ARTO1 (cce_4109 [DSQFSG], cce_4110 [WVHHMF], cce_4111), (cce_4815 [DSQFSG], cce_4814 [WVHHMF], cce_4813)

1 cyd (cce_3415, cce_3414)

Cyanothece sp. ATCC 51472

1 cox (Cy51472_2994, Cy51472_2993, Cy51472_2992)

2 ARTO1 (Cy51472_0426 [DSQFSG], Cy51472_0427 [WVHHMF], Cy51472_0428), (Cy51472_4951 [DSQFSG], Cy51472_4950 [WVHHMF], Cy51472_4949)

1 cyd (Cy51472_4388, Cy51472_4387)

Halothece sp. PCC 7418

1 cox (PCC7418_3827, PCC7418_3826, PCC7418_3825)

Dactylococcopsis salina PCC 8305

1 cox (Dacsa_0127, Dacsa_0126, Dacsa_0125)

1 cyd (Dacsa_3582, Dacsa_3581)

Thermosynechococcus elongatus BP-1

1 ARTO2 (tll2011, tll2010 [WVHHMF], tll2009)

1 cyd (tll1602, tll1601)

Acaryochloris marina MBIC11017

1 cox (AM1_4621, AM1_4620, AM1_4619)

3 ptox (AM1_A0138), (AM1_0483), (AM1_1551)

Cyanobacterium aponinum PCC 10605

1 cox (Cyan10605_1220, Cyan10605_1221, Cyan10605_1222)

1 cyd (Cyan10605_1133, Cyan10605_1134)

Cyanobacterium stanieri PCC 7202

1 cox (Cyast_0400, Cyast_0401, Cyast_0402)

1 cyd (Cyast_1965, Cyast_1964)

Pleurocapsa sp. PCC 7327

1 cox (Ple7327_1598, Ple7327_1599, Ple7327_1600)

1 ARTO1 (Ple7327_1318 [DSQYSG], Ple7327_1317 [WVHHMF], Ple7327_1316)

1 incomplete ARTO (no SU2, SU1: Ple7327_3511 [WVHHMF] incomplete, large part of N-terminaql part is missing, SU3: Ple7327_3510 consists of 2 ORFs, probably non-functional)

1 cyd (Ple7327_3726, Ple7327_3727)

Stanieria cyanosphaera PCC 7437

1 cox (Sta7437_0429, Sta7437_0428, Sta7437_0427)

1 cyd (Sta7437_2309, Sta7437_2308)

Oscillatoria acuminata PCC 6304

1 cox (Oscil6304_4839, Oscil6304_4840, Oscil6304_4841)

1 cyd (Oscil6304_4617, Oscil6304_4616)

Oscillatoria nigro-viridis PCC 7112

1 cox (Osc7112_2626, Osc7112_2627, Osc7112_2628)

1 ARTO1 (Osc7112_1280 [DSQFSG], Osc7112_1279 [WVHHMF], Osc7112_1278)

1 cyd (Osc7112_1947, Osc7112_1946)

2 ptox (Osc7112_0370), (Osc7112_4313)

Planktothrix NIVA CYA 15

1 cox (NIVACYA15_00041000, NIVACYA15_00040990, NIVACYA15_00040980)

1 ARTO1 (NIVACYA15_00005730 [DSQYSG], NIVACYA15_00005740 [WVHHMF], NIVACYA15_00005750)

Table 17.1. (continued)

Planktothrix agardhii NIVA-CYA 34

1 cox (DRAFT34_00040540, DRAFT34_00040530, DRAFT34_00040520)

1 ARTO1 (DRAFT34_00025670 [DSQYSG], DRAFT34_00025660 [WVHHMF], DRAFT34_00025650)

Planktothrix agardhii NIVA-CYA 126/8

1 cox (P.agardhii_20090528_2646, P.agardhii_20090528_2645, P.agardhii_20090528_2644)

1 ARTO1 (P.agardhii_20090528_3646 [DSQYSG], P.agardhii_20090528_3647 [WVHHMF], P.agardhii_20090528_3648)

Planktothrix rubescens NIVA-CYA 98

1 cox (Draft98_00012580 + Draft98_00012610, Draft98_00012570, Draft98_00012560) (functional?, the *coxB* gene contains a putative intron and is therefore annotated as being split into 2 ORFs)

1 ARTO1 (Draft98_00045960 [DSQYSG], Draft98_00045950 [WVHHMF], Draft98_00045940)

1 cyd (Draft98_00045930, Draft98_00045920)

Planktothrix NIVA-CYA405

1 cox (CYA405DRAFT_0002.00012730, CYA405DRAFT_0002.00012720, CYA405DRAFT_0002.00012710)

1 ARTO1 (CYA405DRAFT_0003.00002480 [DSQYSG], CYA405DRAFT_0003.00002490 [WVHHMF], YA405DRAFT_0003.00002500)

Planktothrix NIVA-CYA406

1 cox (CYA406DRAFT_0001.00035910 + CYA406DRAFT_0001.00035930, CYA406DRAFT_0001.00035940, CYA406DRAFT_0001.00035950) (functional?, the *coxB* gene contains a putative intron and is therefore annotated as being split into 2 ORFs)

1 ARTO1 (CYA406DRAFT_0001.00002530 [DSQYSG], CYA406DRAFT_0001.00002540 [WVHHMF], CYA406DRAFT_0001.00002550)

1 cyd (CYA406DRAFT_0001.00002560, CYA406DRAFT_0001.00002570)

Geitlerinema sp. PCC 7407

1 cox (GEI7407_0253, GEI7407_0252, GEI7407_0251)

1 cyd (GEI7407_1752, GEI7407_1751)

1 ptox (GEI7407_0144)

Chroococcidiopsis sp. PCC 6712

1 cox (Chr6712_0562, Chr6712_0561, Chr6712_0560)

1 ARTO1 (Chr6712_1947 [DSQYSG], Chr6712_1946 [WVHHMF], Chr6712_1945)

1 cyd (Chr6712_1774, Chr6712_1773)

Chroococcidiopsis thermalis PCC 7203

1 cox (Chro_4049, Chro_4050, Chro_4051)

1 ARTO1 (Chro_0830 [DSMFSG], Chro_0829 [WVHHMF], Chro_0828)

1 cyd (Chro_2619, Chro_2620)

Crinalium epipsammum PCC 9333

1 cox (Cri9333_4097, Cri9333_4098, Cri9333_4099)

1 ptox (Cri9333_3623), (gene Cri9333_2513 is a truncated ptox and probably not functional?)

Calothrix sp. PCC 6303

1 cox (Cal6303_4202, Cal6303_4201, Cal6303_4200)

1 ARTO1 (Cal6303_0060 [DSQFSG], Cal6303_0061 [WVHHMY], Cal6303_0062)

1 ARTO2 (Cal6303_2019, Cal6303_2877 + Cal6303_2020 [WVHHMF], Cal6303_2878) (2 far apart loci containing an almost complete set of ARTO2 genes, the sequence for SU1 is split, probably not functional?)

1 cyd (Cal6303_2052, Cal6303_2051)

Calothrix sp. PCC 7507

1 cox (Cal7507_2608, Cal7507_2609, Cal7507_2610)

Table 17.1. (continued)

1 ARTO1 (Cal7507_4381 [DSQYSG], Cal7507_4380 [WVHHLY], Cal7507_4379)

1 ARTO2a (Cal7507_0173 [CTELCG], Cal7507_0172 [WVHHMF], Cal7507_0171) (1 additional gene for SU3 - Cal7507_2184 - contains a frameshift in the middle of its sequence and is therefore not functional?)

1 cyd (Cal7507_3535, Cal7507_3534)

Fremyella diplosiphon UTEX 481

1 cox (SU2: fdiDRAFT73520, SU1: fdiDRAFT73520, separately SU3: fdiDRAFT37390)

3 ARTO1: (fdiDRAFT79940 [DSQFSGV], fdiDRAFT79920 [WVHHMF], fdiDRAFT79910. 1 small ORF between SU2 and S1 on the opposite DNA strand, function?), (fdiDRAFT45260 [DALFSGV], fdiDRAFT45250 [WVHHMF], fdiDRAFT45240), (SU2: fdi DRAFT08180 [DSQYSG], SU1and SU3: fdiDRAFT07660 ([WVHHLY], fdiDRAFT0750)

1 ARTO2 (fdiDRAFT83790, fdiDRAFT83780 [WVHHMF], fdiDRAFT83770)

1 ARTO2a (fdiDRAFT37920 [CTELCG], fdiDRAFT37910 [WVHHMF], 37900)

2 cyd (fdiDRAFT40960, fdiDRAFT40970), (fdiDRAFT65210,fdiDRAFT65220)

Leptolyngbya sp. PCC 7376

1 cox (Lepto7376_1818, Lepto7376_1819, Lepto7376_1820)

1 ptox (Lepto7376_3160)

Microcoleus sp. PCC 7113

1 cox (Mic7113_3734, Mic7113_3735, Mic7113_3736)

1 ARTO1 (Mic7113_4334 [DSMFSG], Mic7113_4335 [WVHHMF], Mic7113_4336)

1 ARTO2 (Mic7113_0967, Mic7113_0966 [WVHHMF], Mic7113_0965)

1 ARTO (incomplete, only genes for SU1 and SU3, functional?) (Mic7113_3050 [WVHHMF], Mic7113_3051)

Rivularia sp. PCC 7116

1 cox (Riv7116_0273, Riv7116_0274, Riv7116_0275)

2 ARTO1 (Riv7116_3245 [DSQYSG], Riv7116_3244 [WVHHMF], Riv7116_3243), (Riv7116_3237 [DSQYSG], Riv7116_3236 [WVHHMF], Riv7116_3235) (the 2 ARTO1 loci are only ca. 6000bp apart!)

1 ARTO2a (Riv7116_6530 [CTELCG], Riv7116_6529 [WVHHMF], Riv7116_6528)

1 cyd (Riv7116_5466, Riv7116_5465)

Pseudanabaena sp. PCC 7367

1 cox (Pse7367_0191, Pse7367_0190, Pse7367_0189)

1 ptox (Pse7367_3413)

Spirulina major PCC 6313

1 ARTO2 (Spi6313_4172, Spi6313_4171 [WVHHMF], Spi6313_4170)

1 cyd (Spi6313_1329, Spi6313_1328)

Spirulina subsalsa PCC 9445

1 ARTO2 (Spi9445_0790, Spi9445_0789 [WVHHMF], Spi9445_0788)

1 cyd (Spi9445_3064, Spi9445_3063)

(1 incomplete gene for cyd SU1, encodes potentially only 32 amino acids, probably pseudogene)

Arthrospira platensis NIES-39

1 cox (NIES39_O03100, NIES39_O03090, NIES39_O03080)

1 cyd (NIES39_A01920, NIES39_A01910)

Trichodesmium erythraeum IMS101

1 ARTO1 (Tery_0278 [DSQYSG], Tery_0277 [WVHHMY], Tery_0276)

1 ARTO2 (Tery_1779, Tery_1778 [WVHHMF], Tery_1777)

Anabaena sp. PCC 7108

1 cox (Ana7108_3268, Ana7108_3269, Ana7108_3270)

Table 17.1. (continued)

2 ARTO1 (Ana7108_2154 [DSQYSG], Ana7108_2153 [WVHHMF], Ana7108_2152), (Ana7108_0877 [DSQFSG], Ana7108_0878 [WVHHMF], Ana7108_0879)

1 ARTO2 (Ana7108_0810, Ana7108_0809 [WVHHMF], Ana7108_0808)

1 cyd (Ana7108_2333, Ana7108_2334)

Anabaena cylindrica PCC 7122

1 cox (Anacy_4800, Anacy_4801, Anacy_4802)

1 ARTO1 (Anacy_5413 [DSQYSG], Anacy_5412 [WVHHMY], Anacy_5411)

1 ARTO2 (Anacy_0079, Anacy_0080 [WVHHMF], Anacy_0081)

1 cyd (Anacy_0048, Anacy_0049)

1 ptox (Anacy_4546)

Anabaena variabilis ATCC 29413

1 cox (Ava_0528, Ava_0529, Ava_0530) (gene for SU2 contains in the middle unusual "insert" of 31 amino acids, function?)

3 ARTO1 (Ava_B0179 [DSQFSG], Ava_B0178 [WVHHMF], Ava_B0177) (located on plasmid A), (Ava_4298 [DSQYSG], Ava_4299 [WVHHLY], Ava_4300), (Ava_3577 [DSLFSG], Ava_3576 [WVHHMF], Ava_3575)

1 ARTO2 (Ava_0446, Ava_0447 [WVHHMF], Ava_0448)

4 cyd (Ava_1674, Ava_1675), (Ava_4048, Ava_4047) (Ava_B0175, Ava_B0174) (located on plasmid A) (Ava_B0172, Ava_B0173) (located on plasmid A directly adjacent to the other cyd locus, but encoded on the complementary strand, functional?)

1 ptox (Ava_1055)

Anabaena sp. 90

1 ARTO 1 (ANA_C20658 [DSQYSG], ANA_C20657 [WVHHMY], ANA_C20656) (on plasmid)

2 ARTO2 (ANA_C13186, ANA_C13185 [WVHHMF], ANA_C13184), (ANA_C10492, ANA_C10491 [WVHHMF], ANA_C10490)

Nostoc azollae 0708

1 cox (Aazo_3573, Aazo_3574, Aazo_3575)

3 ARTO1 (Aazo_3660 [DSQYSG], Aazo_3659 [WVHHMY], Aazo_3658), (Aazo_3102 [DALFKG], Aazo_3101 [WVHHMF], Aazo_3100), (Aazo_5318 [DALFSG], Aazo_5317 [WVHHMF], Aazo_5316) (located on plasmid pAzo01)

1 ARTO2 (Aazo_2641, Aazo_2640 [WVHHMF], Aazo_2639) (3 putative pseudogenes with high sequence similarity to cox genes, containing only parts of the normal cox sequences: Aazo_1738 SU2, Aazo_1737 SU1, Aazo_3578 SU3)

Nostoc sp. PCC 7107

1 cox (Nos7107_1624, Nos7107_1625, Nos7107_1626)

1 ARTO1 (Nos7107_0351 [DSQYSG], Nos7107_0352 [WVHHMY], Nos7107_0353)

1 ARTO2 (Nos7107_0751, Nos7107_0750 [WVHHMF], Nos7107_0749)

1 cyd (Nos7107_5139, Nos7107_5138)

Nostoc sp. PCC 7120

1 cox (alr0950, alr0951, alr0952)

1 ARTO1 (alr2731 [DSQYSG], alr2732 [WVHHLY], alr2734) (locus is functional, although there is a small ORF - on the other strand - between genes for SU1 and SU3)

1 ARTO2 (alr2514, alr2515 [WVHHMF], alr2516)

1 cyd (all4024, all4023)

1 ptox (all2096)

Nostoc punctiforme PCC 73102

1 cox (Npun_F5532, Npun_F5533, Npun_F5534)

Table 17.1. (continued)

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2 ARTO1 (Npun_R3537 [DSQYSG], Npun_R3536 [WVHHMY], Npun_R3535) (Npun_R1789 [DSQFSG], Npun_R1788 [WVHHMF], Npun_R1787)

1 ARTO2 (Npun_F0336, Npun_F0337 [WVHHMF], Npun_F0338)

Nostoc sp. PCC 7524

1 cox (Nos7524_3669, Nos7524_3670, Nos7524_3671)

1 ARTO1 (Nos7524_3778 [DSQYSG], Nos7524_3779 [WVHHMY], Nos7524_3780)

1 ARTO2 (Nos7524_4668, Nos7524_4667 [WVHHMF], Nos7524_4666)

1 cyd (Nos7524_3691, Nos7524_3690)

1 ptox (Nos7524_1144)

Nodularia spumigena CCY9414

1 cox (NSP_4060, NSP_4050, NSP_4040)

1 ARTO1 (NSP_960 [DSQYSG], NSP_970 [WVHHMY], NSP_980)

1 ARTO2a (NSP_41420 [CTELCG], NSP_41410 [WVHHMF], NSP_41400)

Cyanobacterium sp. UCYN-A

1 cox (UCYN_12300, UCYN_12290, UCYN_12280)

The genes listed were identified by using the BLAST-P program (with a preset E-value of 1e-0) on all finished cyanobacterial genomic sequences in the JGI-IMG database https://img.jgi.doe.gov/cgi-bin/mer/main.cgi as of March 2014. The database contains four finished sequences for *Synechocystis* sp. PCC 6803 that were performed to study the development of spontaneous mutations in laboratory-grown cultures. They differ by only a few base pairs and only one of these genomes (that of *Synechocystis* sp. PCC 6803, PCC-P) was considered for this chapter. Partially sequenced genomes were not included, although many potential RTO seqences are present in the incomplete genomic sequences of this database. The BLAST-P program was performed with the following "master gene" query sequences (names as used in the "Cyanobase" database (http://genome.microbedb.jp/cyanobase): *coxA* (*Synechocystis* sp. PCC6803 slr 1137), *coxB* (*Synechocystis* sp. PCC6803 slr 1136) *coxC* (*Synechocystis* sp. PCC6803 slr 1138), ARTO-A (*Synechocystis* sp. PCC6803 slr 2082), ARTO-B (*Synechocystis* sp. PCC6803 slr 0813) ARTO-C(*Synechocystis* sp. PCC6803 slr 2038), *cydA* ((*Synechocystis* sp. PCC6803 slr 1379), *cydB* (*Synechocystis* sp. PCC6803 slr 1380), *ccoN* (*Synechococcus* sp. PCC 6301 syc1309_d), *ccoO* (*Synechococcus* sp. PCC 6301 syc1310_d), *ptox* (*Anabaena* sp. PCC 7120 all2096). Genes thought to encode polypeptides belonging to one protein complex are listed jointly in parenthesis in the following orders: for Cox: *coxBAC*, for ARTO: *ARTO-BAC*, for Cyd: *cydAB* and for *cbb*$_3$ oxidase *ccoNO*. These are the order in which the genes occur in most genomes. Definitions of the different types of ARTO genes are described in the text. The actual sequence present in the "marker region" of SU1 and SU2 of the ARTOs are given in brackets. The absence of such a bracket after an ARTO subunit implies that the actual "marker sequence" for this ARTO subunit is that of Cox: WAHHMF for SU1 and CAELCG for SU2. The list includes one organism (*Cyanobacterium* sp. UCYN-A) that does not formally satisfy the definition of a "cyanobacterium," see text for details. The numbers in front of the RTOs gives the numbers of RTOs for this particular RTO in the strain. For most genes in this list there is no experimental evidence beyond the sequence similarity found and the reader is therefore cautioned when interpreting these data

order to to present a complete picture for each strain, only finished genomes were considered for Table 17.1, although the many partially sequenced strains in this database contain a large number of additional genes encoding putative subunits of RTOs. The author is not a specialist in cyanobacterial nomenclature and the names of the strains are listed as they currently appear in the IMG database. In some cases, the names of strains are controversial, such as for strains PCC 7120 (*Anabaena* or *Nostoc*) and WH8102 (*Synechococcus* or *Prochlorococcus*). The names of genes are also listed exactly as they appear in the IMG database to allow unequivocal identification.

The RTOs present in cyanobacteria belong to three protein families:

(A) homologs of heme-copper oxidases
(B) homologs of cytochrome *bd* quinol oxidases
(C) homologs of alternative oxidases as found in chloroplasts (Ptox) and mitochondria (Aox)

The homologs of heme-copper oxidases can be further divided into three subgroups

(A1) mitochondrial-type cytochrome *c* oxidases
(A2) alternate respiratory terminal oxidases (ARTOs)
(A3) homologs of cytochrome cbb_3 cytochrome *c* oxidases

The homologs of cytochrome *bd* quinol oxidases are either

(B1) cyanide sensitive or
(B2) cyanide insensitive

In all mitochondria, there exists a cytochrome *c* oxidase (Cox) that oxidizes reduced soluble cytochrome *c* from the Fe^{2+} form to Fe^{3+} and transfers the electrons to O_2 with H_2O as the final product. This chemical reaction is strongly exergonic and part of the liberated energy can be used to generate a proton gradient across the membrane to be used for ATP synthesis and transport processes. Mitochondrial Cox consists of 13 subunits (see e.g. Tsukihara et al. 1996) while related enzymes found in many, but not all (see e.g. Saraste et al. 1991) bacteria have only the three largest subunits found in the mitochondrial complex (see e.g. Iwata et al. 2002). For the purpose of this chapter these three subunits are called CoxA (SU1), CoxB (SU2), and CoxC (SU3), although largely for historical reasons the genes encoding these subunits do not have the same names in all cyanobacteria.

In many cyanobacteria there exist genes encoding homologs of *coxB*, *coxA*, and *coxC* that do not encode cytochrome *c* oxidases. These were first discovered in *Synechocystis* sp. PCC 6803 (Howitt and Vermaas 1998). In this chapter the difference between Cox and ARTO is defined by short characteristic marker sequences present in SU1 and/or SU2. Mitochondrial-type cytochrome *c* oxidases contain the amino acid sequence WAHHMF in SU1 that is involved in binding the heme a_3/ Cu_B catalytic center. Saraste et al. (1996) first discovered that this sequence can vary. In *Bacillus subtilis* it is WVHHMF. Similarly, there is a marker sequence in SU2, CAELCG, that binds the Cu_A ion (Yoshikawa et al. 2012),

which is involved in the primary electron transfer from soluble cytochrome *c* to the cytochrome *c* oxidase complex. This sequence has also been characterized in the SU2 of cyanobacterial cytochrome *c* oxidase (Paumann et al. 2004b). All cyanobacterial homologs that have the sequence WAHHMF in their SU1 and CAELCG in their SU2, and only such protein complexes, are considered mitochondrial-type cytochrome *c* oxidases in this chapter. All other homologs of mitochondrial cytochrome *c* oxidase that have a different sequence in at least SU1 or SU2 (or both) will be called Alternate Respiratory Terminal Oxidases (ARTOs, a name introduced by Pils et al. 1997).

III. Genes Encoding Putative Subunits of Cyanobacterial RTOs

Several interesting general conclusions can be inferred from Table 17.1. First, each cyanobacterium contains at least one RTO, meaning that all known wild type cyanobacteria should be capable of aerobic respiration. It is also evident from Table 17.1 that no single type of RTO is present in every cyanobacterium. The number of different RTO genes in one strain varies widely, from one (in several strains) to ten (in *Anabaena variabilis* ATCC 29413). The latter is currently the organism with the largest known number of putative RTO genes among all living organisms. Even among closely related strains, the types and number of RTOs present can vary widely. For example, strains of *Nostoc* (also known as *Anabaena*) sp. PCC 7120 and *Anabaena variabilis* ATCC 29413 show high sequence similarity for many corresponding genes, but strain PCC 7120 contains only five RTOs. Similarly, the several different, but closely related *Prochlorococcus marinus* strains do not all have the same set of RTOs. To evaluate the function of the several RTOs present in many cyanobacteria, mutants that lack all but one RTO are extremely helpful. Unfortunately, such a set of mutants is currently only available for a single cyanobacterial strain,

Synechocystis sp. PCC 6803 (Howitt and Vermaas 1998).

Although there is no experimental evidence for this in cyanobacteria, ARTO type 2 and ARTO type 2a (see below, Sect. V) may actually catalyze the same cytochrome *c* – dioxygen oxidoreductase reaction as mitochondrial-type cytochrome *c* oxidase. This is suggested by the presence of the two cysteine residues that form a key part of the Cu$_A$ site of SU2, which is essential for the electron transfer reaction from soluble cytochrome *c* to the oxidase complex. The well characterized cytochrome *c* oxidase of *Paracoccus denitrificnas* indeed contains the marker sequence CSELCG – rather than CAELCG – in its SU2 (Larsson et al. 1995). All strains listed in Table 17.1 contain at least one of the following three putative cytochrome *c* oxidase complexes: Cox, ARTO type 2, or ARTO type 2a. Thus it can probably be inferred from Table 17.1 that every cyanobacterium contains at least one enzyme that catalyzes the cytochrome *c* oxidase reaction. At least one gene for a soluble cytochrome *c* is present in the genomes of all cyanobacteria analyzed so far. This suggests that the cytochrome *c* oxidase reaction is of central importance for cyanobacteria, probably for generating ATP during dark periods.

The cyanobacterium UCYN-A does not contain a functional Photosystem II (Zehr et al. 2001) and is therefore unable to perform oxygenic photosynthesis. This quite common organism is therefore not a cyanobacterium according to the classical definition (prokaryote capable of oxýgenic photosynthesis), but otherwise is very similar to typical cyanobacteria such that its inclusion in the list is warranted. UCYN-A is able to fix dinitrogen (Moisander et al. 2010) and lives as a symbiont of a marine eukaryotic alga (Thompson et al. 2012).

Not listed in Table 17.1, but possibly of biological importance is the occurrence of genes encoding cyanobacterial RTOs in cyanophages. Ignacio-Espinoza and Sullivan (2012) found *ptox* genes in the genomes of 10 cyanophages.

IV. Mitochondrial-Type Cytochrome *c* Oxidases (Cox)

Undoubtedly these enyzme are the most important RTOs in cyanobacteria, and no other RTO occurs more often in cyanobacterial genomes. Cyanobacterial Cox always contain three subunits whose genes are arranged in the order *coxBAC*, as in other prokaryotes. No known cyanobacterium contains a gene encoding a polypeptide with sequence similarity to any of the other ten subunits of mitochondrial cytochrome *c* oxidase. It can hardly be a coincidence that these ten subunits are all nucleus-encoded in eukaryotes, while the three largest, SU1, SU2, and SU3, are encoded in the genomes of the mitochondria. There is experimental evidence that Cox is of central importance for dark metabolism in cyanobacteria. For three strains that are capable of chemo-organo-heterotrophic growth, it has been demonstrated that Cox is essential for this growth mode. Cox deletion mutants of *Synechocystis* sp. PCC 6803 (Pils et al. 1997), *Anabaena variabilis* ATCC 29143 (Schmetterer et al. 2001), and *Nostoc* sp. PCC 7120 (Stebegg et al. 2012) have lost the capacity for chemo-organo-heterotrophic growth. Similar experiments on strains that do not encode a Cox in the wild type genome but have ARTO type 2 or ARTO type 2a instead have not yet been performed. The Cox SU2 of *Anabaena variabilis* ATCC 29143 has an "insertion" of 31 amino acids that is not present in any other known Cox SU2. Its origin and significance are unknown. In *Fremyella diplosiphon* UTEX 481, the three genes *coxB*, *coxA*, and *coxC* are localized in two widely separated loci, *coxBA* and *coxC*.

Since cyanobacteria are the only organisms in which oxygenic photosynthesis and aerobic respiration occur in the same compartment, there is interaction between these two electron transport processes. Pils et al. (1997) constructed several mutants of *Synechocystis* sp. PCC 6803 that demonstrate this interaction in vivo. This cyanobacterium contains three small soluble redox proteins

that are possible electron donors to Cox: cytochrome c_6, cytochrome c_M, and plastocyanin (for reviews see Ho et al. 2011 and the Chaps. 30, 31 and 34 by Bendall and Howe, Diaz-Moreno et al., and Bialek et al., in this volume). Both cytochrome c_6 and plastocyanin can act as electron donors to Photosystem I (see e.g. Hervás et al. 2005). *Synechocystis* 6803 strain PDJ lacks cytochrome c_6, strain PDM lacks cytochrome c_M, and strain PDJM lacks both cytochromes; it is not possible to construct a homozygous double mutant lacking cytochrome c_6 and plastocyanin. All three PD mutant strains are capable of chemo-organo-heterotrophic growth. Since a functional Cox is necessary for this growth mode, the double mutant PDJM must have another electron carrier that allows delivery of electrons to Cox. This is most likely plastocyanin. Similar experiments were performed by Durán et al. (2004). Paumann et al. (2004a) have demonstrated in vitro that electron transfer from plastocyanin to the Cu_A binding region of cytochrome c oxidase SU2 of *Synechocystis* sp. PCC 6803 is possible.

One strain in Table 17.1 (*Cyanothece* sp. PCC 7425, also known as *Aphanocapsa*) contains two complete sets of *coxBAC* genes. There are currently no data showing what the specific function of the two gene sets might be, but it is interesting to note that this strain is a unicellular obligate photo-litho-autotroph that is able to fix dinitrogen under microaerobic conditions (Rippka et al. 1979). In this strain the Cox proteins may have an additional function besides the bioenergetic one; for example, in protecting nitrogenase from dioxygen by consuming O_2 via the RTO reaction. On the other hand, Schmetterer et al. (2001) found, in the heterocyst forming strain *Anabaena variabilis* ATCC 29413, that deletion of the *cox* genes leads to a mutant that is still capable of growth on dinitrogen. Two related strains (*Planktothrix rubescens* NIVA-CYA 98, *Planktothrix* NIVA-CYA406) have a *cox* locus that may contain an intron. Although interesting, there are currently no expermental data that corroborate the functionality of these

introns and *cox* genes. However, the presence and function of introns in cyanobacterial genomes has been verified in tRNA genes (Paquin et al. 1997).

Nomura et al. (2006a) constructed a Cox mutant of *Synechococcus* sp. PCC 7002 and discovered a remarkable phenotype (Nomura et al. 2006b). This mutant strain is very sensitive to high light; but, curiously, a double mutant that lacks both the Cox and the ARTO type 1 (see Table 17.1) is much less sensitive to high light. It is currently not possible to explain this unusual phenotype.

V. Alternate Respiratory Terminal Oxidases (ARTOs)

For the purpose of this chapter, four types of ARTO are defined based on marker sequences in SU1 and SU2:

(i) ARTO type 1: has no WAHHMF in SU1, and has no CAELCG in SU2

(ii) ARTO type 2: has no WAHHMF in SU1, but has CAELCG in SU2

(iii) ARTO type 2a: has no WAHHMF in SU1, but has CxxxCx in SU2

(iv) ARTO type 3: has WAHHMF in SU1, but has no CAELCG in SU2

Subunits are defined as belonging to the same protein complex if they are encoded in the genome in the order SU2 → SU1 → SU3, which is by far the most common arrangement. If there is more than one heme-copper RTO in an organism, it would be interesting to know whether a polypeptide encoded by one RTO gene locus can form a functional protein complex with polypeptide(s) encoded by another locus. There is currently no information to answer this question. A few strains listed in Table 17.1 have unusual ARTO gene loci. *Gloeobacter violaceus* PCC 7421 has one locus and *Gloeobacter kilaueensis* JS1 has two loci with two genes each encoding SU2 and SU1 of an ARTO type 2a complex. However, these genomes contain no recognizable gene for a SU3 of this protein complex. Especially in these strains, it would

be interesting to test whether the SU3 of the mitochondrial-type Cox (present in both strains) can substitute to form a functional ARTO type 2a complex. No such experiments have thus far been performed, largely because these interesting organisms are apparently not yet amenable to genetic manipulation. *Synechococcus* sp. RCC 307 has an interrupted SU3 gene that could either be non-functional or "repaired" in a secondary reaction, possibly at the post-transcriptional stage.

Unusual locations for ARTO type 1 genes are observed in *Synechocystis* sp. PCC 6803 and *Fremyella diplosiphon* UTEX 481. The latter strain additionally has two "normal" gene loci for ARTO type 1 enzymes. In both strains, ARTO genes are located in two loci that contain SU1 and SU3 genes, with the SU2 gene in a separate distant locus. In both strains, the ARTO SU2 has high sequence similarity to all other ARTO-type SU2, significantly higher than the sequence similarity to Cox-type SU2. *Synechocystis* sp. PCC 6803 is one the few strains for which a function of its ARTO has been determined (see below). Thus its genome clearly encodes a functional enzyme. However, it is not known, what polypeptides make up this ARTO enzyme.. Similarly, *Microcoleus* sp. PCC 7113 contains a locus with genes for only SU1 and SU3, with no evidence for a gene encoding an ARTO SU2. *Pleurocapsa* sp. PCC 7327 contains an imcomplete locus for an ARTO that does not seem to encode a functional polypeptide. Similarly, *Nostoc azollae* 0708 contains three incomplete genes with sequence similarity to ARTO genes that are probably pseudo-genes. *Calothrix* sp. PCC 6303 contains an unusual set of ARTO type 2 genes, with two segments for SU1 that are more than 900,000 bp apart. While these genes may be non-functional, cross-formation of protein complexes from polypeptides encoded by different loci may be possible and thus the synthesis of a biologically functioning enzyme cannot be excluded. For example, the *dnaE* gene of *Synechocystis* sp. PCC 6803 is trans-spliced by an intein dependent mechanism (Wu et al.

1998). *Nostoc* sp. PCC 7120 contains a very unusual ARTO locus, with a small gene located between the coding sequences for SU1 and SU3. Even so, this is one of the few ARTOs for which a function has been established (see below).

The details of the reaction catalyzed by cyanobacterial ARTOs are still unclear. The electron acceptor seems to be dioxygen. A mutant strain of *Synechocystis* sp. PCC 6803 that lacks all RTOs except the ARTO consumes O_2 in a KCN-dependent manner (Pils et al. 1997). This reaction is, however, very slow, compared to the O_2 consumption rate of the other two RTOs (Cox and Cyd) in this strain. Recently, Kranzler et al. (2013) provided evidence that the ARTO type 1 can have another electron acceptor besides O_2, namely inorganic Fe^{3+}, with Fe^{2+} as the reaction product. Because many cyanobacterial strains (Table 17.1) contain neither an ARTO type 1 nor any ARTO, the role of an ARTO in iron metabolism cannot be a general one for all cyanobacteria. Even so, this is the first evidence for a cyanobacterial RTO that uses an electron acceptor other than O_2.

A completely different function for ARTOs has been found in the heterocyst-forming strain *Nostoc* (*Anabaena*) sp. PCC 7120. Heterocysts are diffentiated cells in filamentous cyanobacteria that are specialized for N_2 fixation (for reviews see e.g. Flores and Herrero 2010; Ehira 2013). The nitrogenase enzyme that catalyzes the reduction of N_2 to NH_3, is one of the most O_2 sensitive proteins known. Valladares et al. (2003) constructed three mutants of *Nostoc* (*Anabaena*) PCC 7120 lacking either the ARTO type 1 or the ARTO type 2 loci, or both. These were designated *cox3* and *cox2*, respectively, but are now classified as ARTOs. Both enzymes were shown to be expressed only in heterocysts. Only the double mutant was unable to grow on dinitrogen. This was interpreted as these enzymes having the function of removing O_2 from the heterocyst and thus contributing critically to an anaerobic environment for nitrogenase function. Interestingly, the two single mutants lacking only one of the

two ARTOs were able to support growth on N_2, showing that these ARTOs can, in this respect, fulfill the same function, despite belonging to different ARTO groups.

The molecule donating electrons to ARTO type 1 and type 3 is often assumed to be plastoquinol (e.g. Bernroitner et al. 2011). To my knowledge this has not yet been demonstrated experimentally. Table 17.2 compares all marker sequences for SU2 of ARTO type 1, ARTO type 2a, and ARTO type 3 sequences that appear in Table 17.1. These amino acid sequences are compared with the corresponding sequences present in SU2 of the so-called cytochrome *bo* quinol oxidases present in *Escherichia coli* and their relatives (see e.g. Gennis and Stewart 1996). These cytochrome *bo* enzymes are well characterized and consist of three polypeptides that have significant sequence similarity to the three subunits of prokaryotic (and therefore also cyanobacterial) mitochondrial-type cytochrome *c* oxidase and ARTO. Table 17.2 shows that the "marker sequences" of SU2 cyanbacterial ARTO type 1 and type 3 are very similar to each other, and less similar to the corresponding sequences in cytochrome *bo*. From Table 17.2, the possibility that ARTO type 1 and type 3 are quinol oxidases cannot be inferred but also not excluded, and further sudies are necessary to clarify this important point. A search for ARTOs type 1 in other organisms showed that this enzyme seems to occur strictly in cyanobacteria.

VI. *cbb₃* Type Cytochrome *c* Oxidases

Two closely related strains (*Synechococcus* sp. PCC 6301 and *Synechococcus* sp. PCC 7942) contain two genes with high sequence similarity to the two largest subunits of the *cbb₃* type cytochrome c oxdase, *ccoN* and *ccoO*. The CcoN protein is clearly related to subunit 1 of mitochondrial-type cytochrome *c* oxidase The CcoO protein is a

Table 17.2. Marker sequences for subunits of ARTOs in cyanobacteria.

(a) Subunit 2:	
ARTO type 2a:	CTQLCG 3
	CTELCG 3
ARTO type 1 and ARTO type 2	DAMFSG 10
	DSQFSG 18
	DAHFSG 1
	DSMFSG 2
	DSQYSG 21
	DSLFSG 1
	DALFKG 1
	DALFSG 2
Escherichia	SASYSG 30
Salmonella	SASYSG 29
Shigella	SASYSG 7
Mitochondrial cytochrome *c* oxidase subunit 2: CAELCG	
(b) Subunit 1	
ARTO type 1, ARTO type 2, ARTO type 2a:	
WVHHMF 59	
WVHHMY 11	
WVHHLY 3	
Escherichia	WLHHFF 33
Salmonella	WLHHFF 22
Shigella	WLHHFF 0
Mitochondrial cytochrome *c* oxidase subunit 1: WAHHMF	

The table is derived from the data presented in Table 17.1 and shows, the number of times a certain amino acid marker sequence is present in SU2 and SU1, respectively, in the ARTOs of cyanobacterial strains listed in Table 17.1. For comparison, the occurrence of marker sequences for the cytochrome *bo* oxidases of totally sequenced *Escherichia*, *Salmonella*, *Shigella* strains and also mitochondrial Cox are shown. The mitochondrial marker sequences are also present in every cyanobacterial Cox SU2 and SU1 sequence

c-type cytochrome and probably acts as the acceptor subunit for electron transfer from soluble cystochrome *c*. A Cu_A center like that in SU2 of mitochondrial cytochrome *c* oxidase is not present in *cbb₃* oxidases. *Cbb₃* type cytochrome c oxdases are quite common among bacteria (see e.g. the review by Pitcher and Watmough 2004) and are especially well characterized in purple

bacteria (Oh and Kaplan 2004). The X-ray structure of the cbb_3 oxidase from *Rhodobacter spaeroides* shows that the enzyme consists of four subunits encoded by the *ccoNOPQ* genes (Oh and Kaplan 1999). In the related strain, *Rhodobacter capsulatus,* there are five subunits, CcoNOPQH (Ekici et al. 2012). *Synechococcus* strains PCC 6301 and 7942, mentioned above, do not contain any recognizable homologs beyond *ccoN* and *ccoO*. This led Ducluzeau et al. (2008) to suggest that the two genes encoding putative membrane proteins of "unknown function," immediately downstream of *ccoN* and *ccoO* in these strains, may actually be subunits of a cyanobacterial cbb_3 oxidase. While this cannot be ruled out, it is striking that both of these genes for putative membrane proteins have homologs in many other cyanobacteria that clearly do not contain cbb_3 type cytochrome c oxidases.

We were recently able to show that the cbb_3 type oxidase of *Synechococcus* sp. PCC 7942 is an active cytochrome *c* oxidase and is present in this strain in the ICM (thylakoids) and not in the cytoplasmic membrane (CM) (Burgstaller 2012; Schmetterer et al. 2010). Homozygous knock-out mutations in the cbb_3 oxidase and, independently, in the mitochondrial-type cytochrome *c* oxidase genes were constructed and the resulting strains tested for in vitro cytochrome *c* oxidase activity in purified membranes (CM or ICM). The Cbb3 mutant exhibited cytochrome c oxidation only in the CM, and the Cox mutant only in the ICM, which implies that the cbb_3 oxidase is an active cytochrome *c* oxidase and is located only in the ICM of this strain. This is all the more remarkable, since cbb_3 oxidases tend to be expressed only under microaerobic conditions in many bacteria (Pitcher and Watmough 2004). This was certainly not the case in the above mentioned studies, since the *Synechoccus* strains were grown under conditions of oxygenic photosynthesis. Indeed both of these *Synechococcus* strains, which carry *ccoN* and *ccoO* genes, are obligate photo-litho-autotrophs.

VII. Homologs of Cytochrome *bd* Quinol Oxidases

A review by Borisov et al. (2011) lists the categories of enzymes that are collectively called "homologs of cytochrome *bd* quinol oxidase" (here abbreviated Cyd). Such Cyd enzymes are widely distributed among prokaryotes and are always quinol-dioxygen oxidoreductases. Cyd oxidases are not known to occur in any eukaryote. These enzymes contains two subunits, encoded by the *cydAB* genes that are found in this order in genomes. The sequences of CydA proteins can be either "long Q loop" (as e.g. in *E. coli*) or "short Q loop" (as e.g. in *Bacillus stearothermophilus*), the Q loop being the part of the protein where quinol binds. The significance of the additional amino acids in the "long Q loop" enzymes is unknown. All cyanobacteria that contain a Cyd have "short Q-loop" enzymes. It has been shown in *Synechocystis* sp. PCC 6803 that the Cyd enzyme indeed uses plastoquinol as the electron donor (Berry et al. 2002). However, Mogi and Miyoshi (2009) determined that the Cyd from *Synechocystis* sp. PCC 6803 *cydAB* genes expressed in *E. coli* preferred ubiquinol over plastoquinol.

The "*bd*-type" oxidase nomenclature originates from *Escherichia coli*, where the enzyme was found to contain two hemes, a heme *b* and a heme *d*. Many Cyd enzymes, however, contain either no heme *d* or only a small proportion of these proteins present in one cell contain heme *d*. These enzymes are insensitive to KCN. Unfortunately, these KCN-insensitive Cyd enzymes can currently not be recognized from their amino acid sequences. The analysis of the heme content of Cyd enzymes turned out to be tricky because heme *d* is very unstable (Sotiriou and Chang 1988). Indeed, Timkovich et al. (1985) found that extraction of heme *d* from cells or isolated membranes of *E. coli* is not possible because of its severe instability. The chemical structure of heme *d* could be determined only from the isolated enzyme complex. There is a report that heme *d* is present in several cyanobacteria (Fromwald et al. 1996). These

data may be unreliable, however, because the hemes were extracted from isolated membranes in an acidic medium. Recently, it was determined that a mutant form of the *Nostoc* (*Anabaena*) sp. PCC 7120 Cyd enzyme is quite resistant to KCN at 0.67 mM (A. Valladares, unpublished; Mikulic 2013). In this strain, this mutant Cyd enzyme is the only RTO that is rather resistant to KCN. This is remarkable because earlier experiments showed that all RTOs of *Synechocystis* sp. PCC 6803 – and thus also the Cyd oxidase – are sensitive to KCN (Pils et al. 1997). This sensitivity was also found in a *Synechocystis* mutant, in which the Cox and ARTO had been inactivated, and the Cyd was the only remaining RTO (Howitt and Vermaas 1998). In contrast, Mogi and Miyoshi (2009) expressed the *cydAB* genes of *Synechocystis* sp. PCC 6803 in *E. coli* (lacking its wild type RTOs) and found that the expressed *Synechocystis* enzyme behaved like a cyanide-insensitive cyd. The discrepancy may have to do with the content of hemes in the Cyd oxdases in *Synechocystis* relative to *Escherichia coli*. In *E. coli* the heme *d* content of the *Synechocystis* Cyd was found to be much lower than in the *E. coli* wild type Cyd (Mogi and Miyoshi 2009).

Two inhibitors of *Synechocystis* sp. PCC 6803 Cyd are known: 2-heptyl-4-hydroxyquinoline-N-oxide (HQNO) and pentachlorophenol (PCP) (see e.g. Pils et al. 1997). These are specific inhibitors that act only on the Cyd, but not on either the Cox or ARTO enzymes. Note, however, that HQNO and the related NQNO are also partially effective as inhibitors of the quinone-binding Q_i (or Q_n) site of cytochrome b_6f complexes (see the Chap. 15 by Zito and Alric in this volume).

In *E. coli,* the Cyd oxidase can support growth by respiration (Gennis and Stewart 1996). In contrast, in three cyanobacteria (*Anabaena variabilis* ATCC 29413, *Nostoc* (*Anabaena*) sp. PCC 7120, and *Synechocystis* sp. PCC6803), Cox is the enzyme essential for chemo-organo-heterotrophic growth (see Sect. IV). All three strains contain a Cyd (see Table 17.1), but apparently – with respect to chemo-organo-heterotrophic growth – the Cyd cannot substitute for Cox. The function of the Cyd in cyanobacteria seems therefore not to be a bioenergetic one. Berry et al. (2002) found that the Cyd in *Synchocystis* sp. PCC 6803 seems to function mainly in preventing overreduction of the quinone pool, which could otherwise lead to oxygen radical formation and damage to Photosystem 2 and other proteins. This protective function of the Cyd enzyme in *Synechocystis* sp. PCC 6803 implies that the Cyd must be located in the same membrane as the photosystems, namely the ICM. However, as suggested by Schmetterer (1994), this implies that under normal conditions of photosynthesis, electron flow from the quinone pool to the Cyd must be blocked, because otherwise electrons would be "lost" for transfer to Photosystem 1. The mechanism for this is still not known, but the discovery that expression of the Cyd in *Synechocystis* sp. PCC 6803 is strongly dependent on growth conditions, and especially active under high light (Berry et al. 2002; Gendrullis et al. 2008), implies that the Cyd is an important factor in regulating overall electron transport. The higher activity of Cyd under high light conditions does not result from a higher mRNA level (Gendrullis et al. 2008). Another function for Cyd in *Nostoc* sp. PCC 7120 was discovered recently by Mikulic (2013). A mutant of this heterocyst-forming strain that lacks Cyd was not able to grow diazotrophically. The reason for this is unknown, but it may be the same as that for the ARTOs (see above); i.e. removal of O_2 from the cytoplasm of heterocysts to protect the nitrogenase enzyme from oxidative damage.

VIII. Plastidic-Type Terminal Oxidases (Ptox)

Ptox and the related Aox (alternative terminal oxidases) are members of the di-iron carboxylate group of proteins. They are quinol-dioxygen oxidoreductases and contain no heme. They were originally found in plant mitochondria (Aox, alternative

oxidase) and chloroplasts (plastidic oxidase, Ptox). Both Aox (Ito et al. 1997) and Ptox from chloroplasts (Josse et al. 2003) are cyanide resistant and function as a scavengers for dioxygen to prevent overreduction of the quinone pool and formation of reactive oxygen species (McDonald et al. 2011). Ptox in cyanobacteria was discovered by genome sequencing (McDonald et al. 2003; McDonald and Vanlerberghe 2004; Atteia et al. 2004). McDonald et al. (2003) showed that the gene encoding Ptox is expressed. Recently, two homozygous mutants lacking the *ptox* gene were constructed in *Nostoc* (*Anabaena*) sp. PCC 7120 (Mikulic 2013). One was in a PCC 7120 wild type background strain and the other in a Cox minus mutant (Stebegg et al. 2012). In both cases, the loss of the *ptox* gene made dark respiration less resistant to KCN. This means that either the Ptox is not solely responsible for KCN resistance or the removal of the Ptox protein induces the biosynthesis of an RTO that is insensitive to KCN, or both. Bailey et al. (2008) studied electron flow from the quinone pool to RTOs in *Synechococcus* (*Prochlorococcus*) sp. WH 8102 and found spectroscopic evidence that the Ptox protein is used by this strain to prevent overreduction of the quinone pool under iron-limiting conditions. In their experiments, the oxidase was only slightly influenced by KCN, azide or salicylhydroxamic acid, but strongly inhibited by propyl gallate.

Genes with significant sequence similarity to cyanobacterial *ptox* genes were also found in several cyanophages (Sullivan et al. 2010; Ignacio-Espinoza and Sullivan 2012), but the biological significance of this is unclear.

IX. Cellular Location of Cyanobacterial RTOs

Except for *Gloeobacter* (Rippka et al. 1974), which has only a cytoplasmic membrane (CM), all cyanobacteria have at least two bioenergetically active membranes, the CM and the intracytoplasmic membranes or thylakoids (ICM). The ICM contains all photosystems and other chlorophyll containing proteins. Some filamentous cyanobacteria are able to differentiate some cells into specialized cells for specific functions: heterocysts for N_2 fixation, akinetes (spores) for surviving nutritionally adverse conditions (e.g. low phosphate, low water) and/or hormogonia with motility for infecting other organisms to form symbioses (for reviews see e.g. Flores and Herrero 2010; Meeks et al. (2002)). In cyanobacteria without cell differentiation, each RTO is – as far as is currently known – located in one membrane only (either CM or ICM). This has important implications for their function, because in the ICM the electron transport chains for respiration and photosynthesis are linked and share common components. Therefore these RTOs must be considered as part of a larger electron transport system that can transfer electrons to O_2 as the terminal acceptor. For the Cyd and Ptox enzymes, this is apparently their major function in cyanobacteria and they may be exclusively found in the ICM. For the heme-copper oxidases the situation is more complex. In *Synechocystis* sp. PCC 6803 it is well established that the Cox and Cyd enzymes are localized in the ICM and the ARTO type 1 in the CM (Schmetterer and Pils 2004). Especially striking are the results of Pisareva et al. (2011) who found in a proteomic study of *Synechocystis* sp. PCC 6803 the two largest ARTO subunits (SU1 and SU2) only in the CM and not in the ICM. Similarly Berry et al. (2002) found no evidence for ARTO in the ICM of *Synechocystis* sp. PCC 6803. The Cyd in this strain was detected only in the ICM (Berry et al. 2002). Ardelean and Peschek (2011) summarize evidence for Cox in the ICM of *Synechocystis* sp. PCC 6803. In contrast, in *Synechococcus* sp. PCC 7942, Schmetterer et al. (2010) found Cox located exclusively in the CM. In this cyanobacterium, the cytochrome *c* oxidase activity of the ICM is due to the cbb_3 oxidase (see Sect. VI above). The main conclusion from these studies is that it is not possible to predict the cellular location of an RTO in any cyanobacterium, especially since it

is not yet possible to predict from the amino acid sequence, to which membrane the leader sequence directs the protein. In *Arthrospira* (*Spirulina*) *platensis,* a filamentous strain that does not undergo cell differentiation, purified CM did not show cytochrome *c* oxidase activity (Xu et al. 1994). This implies that in this strain, the single Cox (see Table 17.1) is localized exclusively in the ICM, as in *Synechocystis* sp. PCC 6803.

In heterocysts, there exist three different types of bioenergetically active membranes: CM and two forms of ICM, the photosynthetic thylakoids and the so-called honeycomb membranes that derive from the ICM and are localized close to the heterocyst junctions with adjacent vegetative cells. In heterocysts of *Anabaena cylindrica* strain 629, RTOs were found to be localized in the honeycomb membranes by oxidation of diaminobenzidine, a reaction typical for heme-containing oxidoreductases (Murry et al. 1981). Similar results were obtained (Valladares et al. 2007) when heterocysts of a mutant of *Nostoc* (*Anabaena*) sp. PCC 7120 lacking both ARTO type 2 and ARTO type 1 were subjected to electron microscopy: in the mutant the honeycomb membranes were almost absent. This suggests that these two ARTO proteins are actively involved in the cell differentiation process from vegetative cell to heterocyst that occurs after removal of combined nitrogen from the medium.

X. Regulation of Expression of Cyanobacterial RTOs

It is clear, even from the comparatively few data on expression of cyanobacterial RTOs, that the expression of these enzymes is highly regulated by multiple external factors. Cyanobacteria are the only prokaryotes that contain an internal circadian clock (for reviews see e.g. Golden et al. 1997; Terauchi and Kondo 2008; Johnson et al. 2011). Liu et al. (1995) first noted that the

number of genes regulated by the circadian clock is very high in *Synechococcus* sp. PCC 7942, especially compared to the number of circadian clock-controlled genes in eukaryotes. Since then, systematic investigations of certain cyanobacterial strains have revealed that some but not all RTOs are subject to regulation by the circadian clock. Specifically, in *Synechococcus* sp. PCC 7942 (Ito et al. 2009), the *coxBAC* genes encoding the Cox enzyme and the *ccoN* and *ccoO* genes encoding the two large subunits of the cbb_3 cytochrome *c* oxidase are subject to circadian clock regulation. In contrast, the *cydAB* genes encoding the Cyd quinol oxidase are not regulated by this system. As pointed out above (Sect. VI), only the CcoN and CcoO polypeptides (encoded by adjacent genes) are recognized as probable subunits of cyanobacterial cbb3, although no other cbb_3 cytochrome *c* oxidases with only two subunits are known. The study by Ito et al. (2009) revealed that the two genes downstream of *ccoN* and *ccoO* are regulated by the circadian clock in coordination with *ccoN* and *ccoO*. Nothing is known about these two downstream genes, and they are currently annotated as "hypothetical" (both in the *Synechococcus* sp. PCC 6301 and *Synechococcus* sp. PCC 7942 genomes), but one might speculate that these genes may encode functional subunits of the cyanobacterial cbb3 not present in any cbb3 protein complex from other bacteria. In *Synechocystis* sp. PCC 6803, the expression of the three RTOs is controlled differently by the circadian clock. Whereas the Cox shows a clear rhythmic behavior, the Cyd and the ARTO type 1 do not (Kucho et al. 2005). Experiments that use light/dark growth cycles do not necessarily reflect only circadian gene regulation, but may also reflect the influences of light intensity or quality. This was shown by Lea-Smith et al. (2013) who demonstrated that a *Synechocystis* sp. PCC 6803 mutant lacking both Cox and Cyd is especially sensitive to photooxidative damage in cycles of 12 h high light/12 h darkness. In *Nostoc* (*Anabaena*)

sp. PCC 7120 the Cox and ARTO type 2 are circadian-controlled, whereas the ARTO type 1, Cyd, and Ptox are not (Kushige et al. 2013). In this study, it was also reported that the respiratory activity during cycles of 12 h light/12 h dark was highest from before darkness to about the middle of the dark period. Light regulation has also been found for the Cyd in *Synechocystis* sp. PCC 6803 (Berry et al. 2002), but in this case and also in *Thermosynechococcus elongatus*, a higher Cyd activity in high light did not result from higher *cydAB* mRNA levels in the cells (Gendrullis et al. 2008).

While it is likely that many cyanobacterial RTO-encoding genes are in operons, because many occur adjacent to each other, few cyanoabacterial RTO-encoding gene loci have been systematically studied at the mRNA level. Schmetterer et al. (2001) found in the heterocyst-forming strain *Anabaena variabilis* that the Cox locus (*coxBAC* genes) produces three transcripts of different lengths, containing only the *coxB* gene, *coxA* plus *coxC* genes, and all three genes, respectively. Surprisingly the first of these was by far the most abundant, which may have to do with the stability of the primary transcript(s). Although respiration is dramatically higher in heterocysts than in vegetative cells (see e.g. Wolk et al. 1994), the transcripts of the *coxBAC* locus are not very different in filaments containing heterocysts (grown on N_2) versus filaments without heterocysts (grown on combined nitrogen sources) (Schmetterer et al. 2001). The largest influence on mRNA abundance was found in cells grown with fructose (a heterotrophic growth substrate) relative to cells grown without fructose. Of the two transcriptional start sites identified for this *coxBAC* operon, only one (tsp2) was induced by fructose. In the related strain *Nostoc* (*Anabaena*) sp. PCC 7120, both the *coxBAC* genes and the three homologous genes encoding the ARTO type 2 were found to be within operons encompassing all three genes (Jones and Haselkorn 2002).

The *cydA* gene is expressed at a higher level under microaerobic conditions in *Nostoc* (*Anabaena*) sp. PCC 7120 (Nicolaisen et al. 2010). This is interesting, because in *E. coli*, the well studied Cyd (cytochrome *bd* quinol oxidase) is also activated under low O_2 conditions and has a higher affinity for O_2 than the other RTO, cytochrome *bo* quinol oxidase (Borisov et al. 2011).

Adaption to changing concentrations of NaCl is very important for cyanobacteria in natural environments (for a review see Hagemann 2011). It has long been known that respiration in cyanobacteria is enhanced in the presence of high NaCl (Fry et al. 1986; Nitschmann and Packer 1992). These studies were performed with *Synechococcus* sp. PCC 6311, for which no genome sequence data are currently available, so the discovery of in vitro cytochrome *c* oxidase activity in this strain in response to high salt (Nitschmann and Packer 1992) cannot be specifically attributed to any Cox, cbb3, or ARTO type 2 or 2a enzyme complex. Curiously, an extensive analysis of genes in *Synechocystis* sp. PCC 6803 induced by different stresses, including high NaCl (500 mM), did not identify any gene encoding an RTO subunit (Los et al. 2008), although enhanced, in vitro cytochrome *c* oxidase activity had been detected in high NaCl (550 mM) in this strain (Jeanjean et al. 1993).

Two examples exist of regulation of RTO expression by inorganic ions. Ni deprivation causes upregulation of Cox and Ptox in *Synechococcus* (*Prochlorococcus*) WH 8102 and of Cox and ARTO type 1 in *Synechococcus* sp. PCC 7002, when these strains are grown with NH_4^+ as the nitrogen source (Dupont et al. 2012). The FurA transcriptional regulator of *Anabaena* (*Nostoc*) sp. PCC 7120 (González et al. 2012), that was discovered as a regulator of Fe uptake (hence the name), is now known to be a much more general transcriptional regulator. The *coxBAC* operon contains a characteristic DNA sequence, that binds FurA, in front of both the *coxB* and *coxA* genes. For *coxB*, the binding was experimentally verified by

vitro by electrophoretic mobility shift assay (EMSA) experiments (González et al. 2014). Hakkila et al. (2013) discovered that in a *Synechocystis* sp. PCC 6803 mutant that lacks the sigma factors sigC, sigD, and sigE, the RTO genes *coxB*, *coxA*, *coxC*, *cydA* and *cydB* are all downregulated.

Cyanobacteria that are capable of cell differentiation must have complex gene regulation. With respect to RTOs there are few data concerning expression in heterocysts, akinetes, or hormogonia. As mentioned above (Sect. V), transcripts for the genes encoding the ARTO type 1 and ARTO type 2 proteins in *Anabaena* (*Nostoc*) sp. PCC 7120 (see Table 17.1) could only be found in heterocysts (Valladares et al. 2003).

Campbell et al. (2007) performed A unique study with *Nostoc punctiforme* PCC 73102. This filamentous strain is among the most complex cyanobacteria, because it can form all four types of cells that occur in cyanobacteria: vegetative cells, heterocysts, akinetes, and hormogonia. In heterocysts, the ARTO type 2 genes and one of the the ARTO type 1 loci (genes Npun_R3537, Npun_R3536, Npun_R3535) are up-regulated compared to their expression in vegetative cells grown in NH_4^+. This is similar to the situation in *Anabaena* (*Nostoc*) sp. PCC 7120 (see above), except that *Nostoc punctiforme* PCC 73102 contains a second ARTO type 1 (see Table 17.1), whose expression is not affected by the cell differentiation process from vegetative cell to heterocyst. The two ARTO 1 enzymes may therefore have different functions despite their high sequence similarity. The differentiation from vegetative cells to akinetes is not accompanied by a change in expression of any of the RTO genes in *Nostoc punctiforme* PCC 73102. In hormogonia, the ARTO type 2 genes and one of the ARTO type 1 gene sets (genes Npun_R3537, and Npun_R3535) are upregulated relative to vegetative cells grown in NH_4^+, while the *coxC* gene is down regulated.

Clearly, much remains to be learned about the expression and function of RTOs in cyanobacteria.

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XFEL Studies on Bovine Heart Cytochrome *c* Oxidase

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Summary

Cytochrome *c* oxidase (CcO) has four redox-active metal sites, Fe_a, Fe_{a3}, Cu_A and Cu_B. Each site reversibly receives one electron equivalent. The four electron equivalents required to reduce the bound O_2 are sequentially transferred from cytochrome *c* and each is coupled to the pumping of one proton equivalent. The purified enzyme fraction as prepared (the resting oxidized form) is not involved in catalytic turnover since one electron reduction of the resting oxidized form is not coupled to proton pumping. Redox and resonance Raman data suggest that a peroxide molecule bridges Fe_{a3} and Cu_B in the O_2 reduction site. X-ray structural analyses show that the O-O bond length is 1.7 Å, which is significantly longer than that of the typical peroxide bridge between two metals (1.5 Å), suggesting an activated state of the bound peroxide. On the other hand, investigation of a bacterial CcO produced the proposal that the bound peroxide is not the intrinsic ligand; instead the peroxide is formed from hydroxyl radicals created by the strong X-ray radiation. Establishment of the ligand structure in the O_2 reduction site is a prerequisite for elucidation of the proton-pumping mechanism.

The damage-free X-ray structure of CcO determed using the recently developed femtosecond pulse of an X-ray free-electron laser (XFEL) system shows the existence of a typical peroxide ligand with an O-O distance of 1.55 Å. Thus, the negatively-charged peroxide in the O_2 reduction site blocks the proton-pump upon initial two electron reduction by suppressing the electron transfer from Fe_a to Fe_{a3} (with Fe_a oxidation driving the proton pump).

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This successful determination of a high resolution X-ray structure using femtosecond XFEL pulse validates the system for analysis of changes in X-ray structures over a physiologically-relevant time scale. This chapter includes a discussion for the new field, "Picobiology", which is made possible by XFEL.

I. Introduction

Cytochrome c oxidase (CcO) is the terminal oxidase of cellular respiration, which reduces O_2 to H_2O coupled with proton pumping (Yoshikawa et al. 2011). Bovine heart CcO contains 13 different subunits which hold hemes a (Fe_a) and a_3 (Fe_{a3}), Cu_A and Cu_B, each of which reversibly receives one electron equivalent (Tsukihara et al. 1996). Heme a_3, the O_2 binding site, and Cu_B form the O_2 reduction site (Yoshikawa et al. 2011). The electron equivalents used to reduce the bound O_2 are sequentially transferred to the O_2 reduction site from cytochrome c at the positive side phase (P-phase) of the mitochondrial inner membrane via Cu_A and heme a. The protons in the synthesis of water molecules are transferred from the negative side phase (N-phase) via the two proton conducting pathways (K and D pathways) (Yoshikawa et al. 2011). Each electron transfer from cytochrome c is coupled to proton pumping in A type CcOs (Pereira et al. 2008). It is well established that protons are pumped through a proton-conducting pathway (H-pathway) which connects the N-phase to the P-phase in most of animal CcOs (Yoshikawa et al. 2011). On the other hand, either the D or the K pathway has been proposed to function as the proton pumping pathway for eukaryotic (except for most animals) and prokaryotic CcOs (Konstantinov et al. 1997). The structure of the O_2 reduction site which is composed of a high-spin Fe_{a3}^{2+} and a Cu_B^{1+} coordinated with three histidine imidazole groups in a trigonal planar conformation in the reduced state, is completely conserved in all CcOs thus far (Chang et al. 2009). The conservation strongly suggests that there is a common mechanism for O_2 reduction. The completely conserved low spin heme suggests that the heme plays crucial roles in the proton pumping function (Artzatbanov et al. 1978).

It has been established that the O_2 reduction site receives O_2 only when the two metals are in the reduced state (R form) (Dodson et al. 1996) to form the oxygenated form (A form) closely similar to the oxygenated forms of myoglobins (Tsubaki et al. 1980; van Wart and Zimmer 1985)

Abbreviations: A form – The O_2-bound form of CcO closely similar to oxymyoglobin, which appears in the catalytic turnover of CcO; A type CcO – Group of CcOs containing three proton conducting pathways D, K, and H; B type CcO – A group of CcOs containing only one proton conducting pathways corresponding to the K pathway of A type CcO; CcO – Cytochrome c oxidase; Cu_A – One of the copper sites of cytochrome c oxidase which is the initial electron acceptor from cytochrome c; Cu_B – One of the copper sites of cytochrome c oxidase which forms the O_2 reduction site with Fe_{a3}; D-pathway – One of the proton conducting pathways connecting N-phase and the O_2 reduction site for transfer of protons for making water molecules; Fe_a – The low spin iron ion of one of the two heme As designated as heme a, contained in cytochrome c oxidase; Fe_{a3} – The high spin iron ion of one of the two heme. As designated as heme a_3, contained in cytochrome c oxidase; fs – Femtosecond (10^{-15} s); H-pathway – The proton conducting pathway connecting the N-phase with the P-phase for proton transfer and pumping; K-pathway – One of the proton conducting pathways connecting the N-phase and the O_2 reduction site for transfer of protons to make water molecules; N-phase – Aqueous phase located in the inside of mitochondrial inner (or bacterial plasma) membrane; O form – The fully oxidized form which appears under turnover conditions; P-phase – Aqueous phase located in the outside of mitochondrial inner (or bacterial plasma) membrane; R form – One of the intermediate species of the catalytic turnover of CcO in which both metals in the O_2 reduction site are in the reduced state and free from any external ligand; SACLA – The nick name of the XFEL facility constructed in Sayo Japan; XFEL – X-ray free electron laser

and of picket-fence heme model compounds (Burke et al. 1978). The transiently bound O_2 at Fe_{a3} is spontaneously (without any electron donation from the low spin heme (heme *a*)) reduced to two oxides (O^{2-}) upon receipt of four electron equivalents from Fe_{a3}, Cu_B and possibly the nearby OH group of Y244 to provide the P form. Then, four electron equivalents are transferred to the O_2 reduction site from cytochrome *c* via Cu_A and heme *a* to regenerate the R form giving three intermediate species, F, O, E (Yoshikawa et al. 2011). In the O form, all of the redox-active metal sites are in the oxidized form with OH^- ligands bound to Fe_{a3}^{3+} and Cu_B^{2+} (Ogura et al. 1993).

The method for isolation of bovine heart CcO has been well established and yields single crystals which diffract X-rays to 1.3 Å or better resolution. Because the isolation is performed under aerobic conditions, the isolated sample as prepared is in the fully oxidized state. However, although quite stable, the sample as prepared has significantly lower reactivity than that of the fully oxidized state which exists under turnover conditions (the O form described above) in terms of the functions of electron receiving, cyanide binding, and proton pumping (Yoshikawa et al. 2011). Thus, the CcO sample as prepared is known as the "resting oxidized form." Reductive titration analyses under strict anaerobic conditions indicate that six electrons are required for complete reduction of the resting oxidized form (Mochizuki et al. 1999). Furthermore, a resonance Raman analysis indicates the presence of peroxide bound to the O_2 reduction site (Sakaguchi et al. 2010). These results strongly suggest that peroxide bound to the O_2 reduction site in the resting oxidized CcO blocks the proton pump. The physiological significance of this form has not been well established. However, a detailed structural analysis of resting oxidized CcO would provide various insights into the proton pump mechanism and the coupling between the proton pump and O_2 reduction. Thus, the X-ray structure of the resting oxidized form was examined at SPring-8, a third-generation synchrotron radiation facil-

ity. Significant X-ray damage was detected at the O_2 reduction site, resulting in decreased electron density between the two metals in the O_2 reduction site, with increased X-ray exposure time, which is required to obtain high resolution X-ray diffraction (Aoyama et al. 2009). The X-ray structure without X-ray irradiation, determined by extrapolating the time dependence of the decrease in the electron density, indicates that the O-O bond distance is significantly longer than that of a typical O-O bridge between the two metal atoms (Aoyama et al. 2009). Although the structure may indicate the existence of an activated peroxide molecule bound to the O_2 reduction site, the possibility that the extrapolation was unsuccessful cannot be excluded.

Thus, a damage-free X-ray structural analysis using a recently developed X-ray free electron laser facility (XFEL) was performed for resting oxidized CcO. This investigation concluded that the O-O bond distance is 1.55 Å, a value consistent with the resonance Raman results (Hirata et al. 2014). Thus, binding of peroxide (in the ground state) blocks the proton pump. Careful structural comparison of the resting oxidized form with the O-form (when it is available) would provide various insights in the proton-pump mechanism.

It is noteworthy that the damage-free structural analyses using XFEL pulses of 20 fs validates the feasibility of time resolved X-ray structural analysis, which is a prerequisite for elucidation of the protein function as the chemical reaction (i.e. description of the reaction mechanism in terms of the action of individual atoms).

II. Properties of the Resting Oxidized Form of Bovine Heart CcO

Evaluation of a method of purifying a protein from its cell is most appropriately and sensitively evaluated by the resolution of the X-ray diffraction obtained from single crystals of the sample isolated by the purification method. In this sense,

the purification method, developed by Okunuki, Yonetani and Yoshikawa groups (Yonetani 1961; Tsukihara et al. 1995; Mochizuki et al. 1999), is the best one since it produces crystals with the highest X-ray diffraction resolution of bovine CcO. Resolution of up to 1.3 Å has been obtained for crystals purified by this method. The CcO sample as prepared is in the fully oxidized state since the purification of the protein with extremely high sensitivity to O_2 is performed under aerobic conditions. However, the sample as prepared by the best method has a much slower cyanide binding compared with that of the fully oxidized form under turnover conditions. This suggests that the O_2-reduction site of the sample as prepared is not in the same state as the fully oxidized state involved in the catalytic turnover (Mochizuki et al. 1999). Furthermore, some heterogeneity in the cyanide-binding rate has been reported, depending on the purification procedure. Some preparations react with cyanide at a rate of $1–2\ M^{-1}s^{-1}$ (Naqui et al. 1984; Baker et al. 1987; Mitchell et al. 1992), which is measurable by a standard absorbance spectrophotometer. Other preparations have much slower cyanide binding rates. These preparations are designated as "fast" and "slow" preparations (or forms) (Mitchell et al. 1992). These preparations are also characterized by the Soret band position, at 424 and 418 nm, respectively (Moody 1996). Furthermore, in the dithionite reduction process, the slow form has a much slower reduction rate of heme a_3 (time scale of minutes) compared with that of heme a (Wrigglesworth et al. 1988). This property also suggests that the slow form is not involved in catalytic turnover. The slow form can be transformed to the fast form by O_2-pulsing (i.e. complete reduction of the sample followed by exposure of excess O_2) (Antonini et al. 1977). It is well known that most bovine CcO preparations have variable heterogeneity with respect to the ratio of fast/slow forms (Moody 1996), although purification methods for pure fast and slow forms have been reported (Baker et al. 1987;

Brandt et al. 1989; Cooper and Salerno 1992). Prokaryotic CcOs also demonstrate the slow and fast forms (Cheesman et al. 2004). These results strongly suggest that the slow and fast forms are not due to denatured enzyme preparations, although the physiological functions of the two forms have not been established. The preparation of CcO purified with the method of Okunuki, Yonetani and Yoshikawa groups (Yonetani 1961; Tsukihara et al. 1995; Mochizuki et al. 1999) has the cyanide reactivity, Soret band position and the dithionite reduction rate of the "fast" form. Most of the structural and functional studies on bovine heart CcO have been performed using the "fast" preparation. Thus, in this article, the resting oxidized form is designated as the resting oxidized "fast" form.

III. Redox Properties of the Resting Oxidized Bovine Heart CcO

Various special precautions are required to obtain an accurate redox titration of CcO, since CcO has the strongest O_2 affinity of the O_2-reactive proteins in the cell. Repeated evacuation/pure nitrogen-gas equilibration cycles are necessary to completely remove O_2 from the purified sample, which is stabilized by detergents attached to the transmembrane surface. Thus, the stability of the purified sample is crucial. Further, complete removal of contaminating proteins which contain redox-active metals or functional groups is also necessary. In the case of bovine heart CcO, some CcO preparations have been reported to contain contaminant iron proteins which contribute a 30 % higher iron content than that due to heme A. The contaminant proteins are removed only with repeated crystallization (Mochizuki et al. 1999). Thus, a CcO preparation purified by crystallization must be used for redox titration analyses of CcO. A detailed examination of the purity of various bacterial CcO preparations has not been reported thus far.

The most reliable redox titration of bovine heart CcO thus far indicates that six electron equivalents supplied by dithionite are required for complete reduction of the resting oxidized CcO purified by recrystallization (Mochizuki et al. 1999). The slope of the titration curve (generated from the absorbance spectral changes vs the electron equivalents added) during addition of the initial two electron equivalents is significantly less steep than the slope generated by addition of the subsequent four electron equivalents. The oxidative titration of the dithionite-reduced CcO with O_2 indicates that four oxidation equivalents supplied by O_2 are sufficient for re-oxidation of the dithionite-reduced CcO. However, the absorbance spectrum of the O_2-re-oxidized sample is slightly but significantly different from that of the resting oxidized form existing before the initiation of the dithionite titration. The O_2-re-oxidized sample after aeration for 30 min requires six electron equivalents from dithionite for complete reduction (Mochizuki et al. 1999). These results strongly suggest that peroxide (O_2^{2-}) is bound to the O_2-reduction site. The shallow titration curve observed during the initial two electron reduction suggests that the electron equivalents are mostly used to reduce the bound O_2^{2-} to water. The O_2-re-oxidized sample is likely to correspond to the oxidized form under turnover conditions. The O_2-re-oxidized sample obtained under strict anaerobic conditions without addition of any external reducing system undergoes slow reduction of both hemes (Mochizuki et al. 1999). These results suggest the presence of slow electron donors in the protein moiety at the O_2 reduction site. Such slow electron donors could reduce O_2 to peroxide when the O_2-re-oxidized sample is exposed to excess O_2. Certain amino acid residues such as tryptophan, tyrosine and lysine could serve as two-electron donors and provide stable non-radical products which are not reducible with dithionite (Mochizuki et al. 1999).

It is well known that H_2O_2 reacts with resting oxidized CcO to generate the P or F form in a manner which depends on the amount of H_2O_2 added (Wrigglesworth 1984; Weng and Baker 1991). Although the mechanism is still unknown, the generation of these forms is well established by absorbance and resonance Raman analyses (Proshlyakov et al. 1994, 1996). Thus, it has been claimed that the peroxide-bound form is unlikely to be stable enough to allow the resting oxidized CcO to be stored in the crystalline state for several months at 4 °C without any significant absorbance spectral change (Kim et al. 2004; Kaila et al. 2010; Wikström 2012). However, as described above, the resting oxidized CcO is formed from the fully oxidized form corresponding to the oxidized form under turnover conditions (Fe_{a3}^{3+}-OH^-, Cu_B^{2+}-OH^-) by slow spontaneous electron transfer from the protein moiety in the presence of O_2 (Mochizuki et al. 1999). On the other hand, H_2O_2 reacts with the resting oxidized CcO which has a peroxide molecule bound to the O_2 reduction site. Thus, the high reactivity of resting oxidized CcO with H_2O_2 does not provide evidence for the absence of peroxide in the O_2 reduction site of the resting oxidized CcO. It is possible that H_2O_2 functions as a two electron acceptor from the bound peroxide in the resting oxidized CcO to provide the fully oxidized state as follows; one equivalent of H_2O_2 added oxidizes the bound peroxide in [Fe_{a3}^{3+}-O-O-Cu_B^{2+}] to provide transiently [Fe_{a3}^{3+}, Cu_B^{2+}], O_2 and $2H_2O$. The O_2 molecule produced in this step is unlikely to interact with the O_2 reduction site. Then the second H_2O_2 is trapped at the O_2 reduction site and generated the P-form, which is characterized by $Fe_{a3}^{4+} = O^{2-}$, Cu_B^{2+}-OH^- and a Y244 OH radical (Y244 is cross-linked covalently to one of the three histidine imidazole groups coordinated to Cu_B (Yoshikawa et al. 1998)). An excess amount of H_2O_2 would provide another electron donor to the P-form to generate the F-form. The coupling of the single electron injection with the pumping of one proton equivalent has been well established for all of the intermediate species which appear after the A form (the O_2-bound form), namely, the

P, F, O and E forms (Verkhovsky et al. 1999; Bloch et al. 2004; Siletsky and Konstantinov 2012). Although single electron reduction of the resting oxidized CcO initiates rapid electron transfer from Cu_A to heme a, no further electron transfer to the O_2 reduction site from heme a is detectable (Verkhovsky et al. 2001; Bloch et al. 2004). It appears that the negative charges of the bound peroxide on the O_2 reduction site prevent the redox-potential of heme a_3 from increasing in heme a_3. The increased redox potential of heme a_3 is responsible for the electron extraction from heme a. It is well known that oxidation of heme a drives the proton pump (Yoshikawa et al. 2011). These results suggest that the structure of the O_2 reduction site is crucial for proton pump. In other words, the O_2 reduction site is also involved in proton-pumping. Further, the second single electron reduction flash to the single-electron reduced resting oxidized CcO induces a slower electrogenic phase (135 μs). However, the intensity is too weak to drive the pumping of a single proton (Verkhovsky et al. 2001).

IV. Resonance Raman Analysis of the Resting Oxidized CcO

The peroxide binding is confirmed by resonance Raman analysis excited at 647.1 nm from a Kr^+ laser. This laser excites the charge transfer band (detectable as a shoulder near 650 nm) characteristic of the resting oxidized CcO to provide a clear band at 755 cm^{-1}. Cyanide eliminates both the resonance Raman and the absorption bands. A linear relationship between the O-O stretching frequency and the corresponding O-O bond length suggest that the 755 cm^{-1} band corresponds to the frequency due to the O-O stretch of the bond length of 1.50 Å (Sakaguchi et al. 2010). The bond length is consistent with the O-O bond length detectable in the X-ray structures of various peroxide-bridged inorganic compounds deposited in the Cambridge Database. The absence of the Fe-O stretch mode in the

resonance Raman spectrum of the resting oxidized CcO also supports this assignment (Collman et al. 2003).

V. X-ray Structural Analyses of the Resting Oxidized CcO Using Third Generation Synchrotron Radiation Facilities

Although the above redox and resonance Raman characteristics strongly provide strong evidence that peroxide is bound to the O_2 reduction site of the resting oxidized CcO, X-ray structure at high resolution is indispensable for elucidation of the mechanism of blockage of the proton pump. Thus, an X-ray structural analysis of the resting oxidized CcO has been conducted at SPring-8. As expected, significant X-ray radiation effects were detected in the absorbance spectra of the frozen single crystals as shown in Fig. 18.1 (Aoyama et al. 2009). The visible spectral changes induced by 30 s irradiation of a single crystal of the resting oxidized CcO at 100 K show a significant increase in the α-band region at 604 and 582 nm (Fig. 18.1). The third peak is detectable between the two peaks near the α-band peak before X-ray irradiation. The time course of the absorbance changes shows that the maximal changes for both peaks are attained after 20 s of irradiation. No further spectral change is detectable with longer exposure, although the spectrum of CcO in the crystal is clearly different from that of the fully reduced CcO (see Fig. 18.1b). The appearance of the residual peak at 595 nm near the α-band peak of the oxidized CcO and the residual charge transfer band near 650 nm strongly suggest that reduction of the CcO in the crystals is incomplete. In fact, warming the crystals induces the fully reduced CcO spectrum (Aoyama et al. 2009). The sharp bands at 582 and 604 nm suggest that two different low spin ferrous hemes are induced. The 604 nm band is reasonably assignable to heme a. Essentially identical absorbance spectral changes are detectable in the bacte-
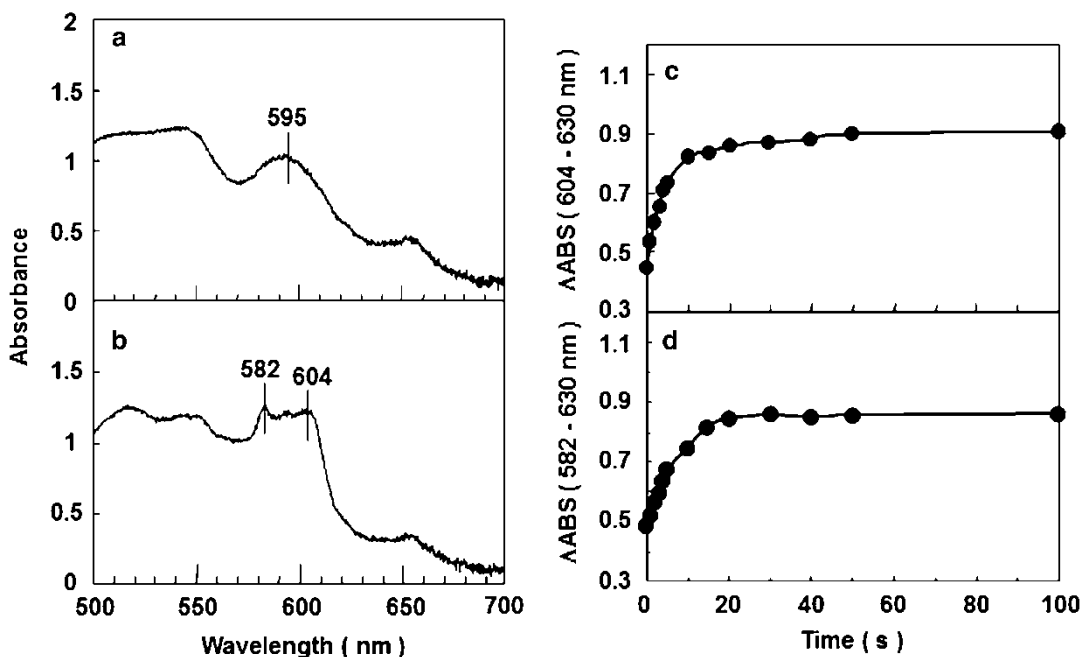
Fig. 18.1. Effects of X-ray irradiation on the visible spectra of the single crystals of the resting oxidized bovine heart cytochrome *c* oxidase. The measuring light beam focused to about 50 μm in diameter was injected perpendicular to the most-extended plane of (010). Visible spectra are shown for the sample before X-ray irradiation (**a**) and after a 30 s exposure (**b**). Time course of the increases in the absorbance difference are shown between 604 and 630 nm (**c**) and between 582 and 630 nm (**d**) (Reprinted with permission from "Aoyama et al. 2009").

rial (*R. sheroides*) CcO, which has electron density closely similar to that of bovine heart CcO (Qin et al. 2006).

One of the simplest interpretations of the irradiated spectrum (Fig. 18.1b) is as follows: heme *a* is fully reduced while heme a_3 is partially reduced to give a low spin ferrous heme a_3 with the 582 nm band and the residual resting oxidized heme a_3 exhibiting absorbance bands at 595 and 650 nm. The partial reduction of heme a_3 could be induced by uneven crystal packing of CcO in the dimer state. One of the monomers in the dimer is subject to significantly stronger constraints from the adjacent CcO molecules in the crystal, compared with the other monomer. On the other hand, the electron density between the two metals in the O_2 reduction site is weakened by the longer exposure of the crystals to X-ray irradiation. The electron density decrease, which is accompanied by an increase in the O-O bond distance, proceeds af-

ter the maximal absorbance spectral changes are attained following the 20 s irradiation period. Careful refinement of X-ray structural data obtained at various exposure times indicates that electron density assignable to a water molecule hydrogen-bonded to the OH group of Y244 increases as the electron density of the peroxide molecule decreases. The X-ray-irradiation-induced appearance of the water molecule also suggests that the bridging peroxide is reduced by the hydrated electrons which are generated by X-ray irradiation (Aoyama et al. 2009).

In order to obtain an X-ray structure with minimal radiation effects, an X-ray structure was extrapolated to 0 s using X-ray data from a 1 s exposure to a 400 s exposure. The resulting structure with 2. 1 Å resolution indicates that the O-O bond distance is 1.7 Å, as given in Fig. 18.2 (Aoyama et al. 2009). It is noteworthy that the Fe_{a3}-O distance is significantly longer than that of the typical
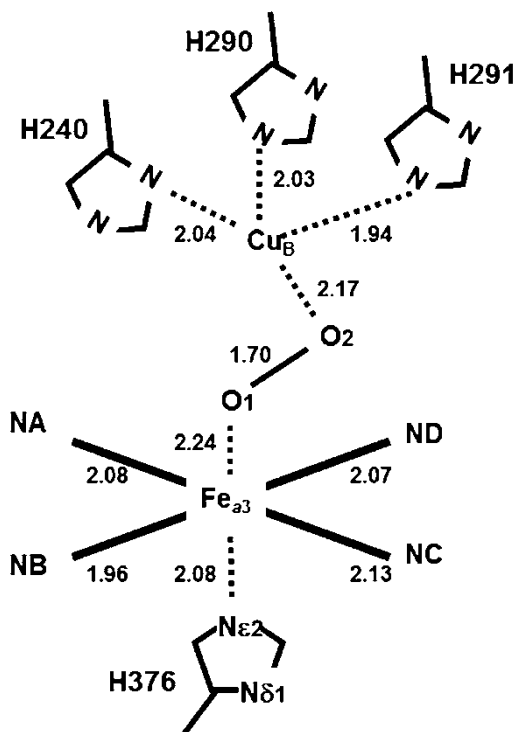
Fig. 18.2. Coordination geometries of the O_2 reduction site of the resting oxidized bovine heart CcO at 2.1 Å resolution, extrapolated to 0 s X-ray exposure. The interatomic distances are given in Å. Other distances and angles are Fe_{a3}-Cu_B, 4.87 Å; $N_{\varepsilon 2}$ (H376)-Fe_{a3}-O_1, 168.5°; Fe_{a3}-O_1-O_2, 144.1° and O_1-O_2-Cu_B, 90.5°. These geometries are fully consistent with those of the X-ray structure determined from the 103.5 s exposures at 1.95 Å resolution (Reprinted with permission from "Aoyama et al. 2009").

low spin coordination, which is consistent to the proposal that heme a_3 is in the high spin state in the resting oxidized form (Day et al. 1993; Cheesman et al. 2004). On the other hand, the O-O bond distance of the bound peroxide is significantly longer than that of typical bridging peroxides reported thus far. Also, the distance is inconsistent with the resonance Raman results.

One of the possible interpretations of these results is that the extrapolation to 0 s of exposure was unsuccessful and that the X-ray data is contaminated by X-ray-damaged structures. The other interpretation is that the peroxide in the O_2 reduction site is in an activated state which is stable only in the protein. The O-O bond length of 1.5 Å, indicated by the resonance Raman results is based on an empirical relationship between the bond length and the position of the O-

O stretch mode from various inorganic compounds (Sakaguchi et al. 2010). Thus, the resonance Raman result is not conclusive evidence against a bond length of 1.7 Å. On the other hand, the activated state of the peroxide in the O_2 reduction site is inconsistent with the high stability of the resting oxidized CcO, especially in the crystalline state.

The X-ray structure of the fully oxidized state under turnover conditions (the O form) has not been determined. However, the X-ray structures of the fully oxidized azide and cyanide bound forms indicate that significant structural differences between these fully oxidized CcOs are detectable only in the O_2 reduction site. Thus, the structural differences between the O form and the resting oxidized form are likely to be small. An accurate determination of the resting oxidized CcO and comparison of this form with the structure

of the O-form would provide various crucial insights which will contribute to elucidation of the proton-pump mechanism. Thus, an experimental evaluation of the two interpretations described above is highly desirable.

The O_2 reduction site of *R. sphaeroides* CcO has an electron density similar to that of bovine heart CcO (Qin et al. 2006). However, it has been proposed that two equivalents of OH^- or H_2O are present instead of one equivalent of peroxide. On the other hand, Michel and coworkers have proposed that the O_2 reduction site in the oxidized *P. denitrificans* CcO has a peroxide bridging between the two metals, i.e. the same assignment as bovine CcO (Koepke et al. 2009). However, it was proposed that the peroxide-bound species is involved in catalytic turnover. A B-type CcO, cytochrome ba_3 from *T. thermophiles*, a thermophilic bacteria, provides a clear electron density peak in the O_2 reduction site (1.8 Å resolution) which indicates an O-O distance of 1.50 Å. A peroxide molecule was assigned as the bridging ligand to the fully reduced O_2 reduction site (Tiefenbrunn et al. 2011). It has been proposed that the bridging peroxide is a product generated from two equivalents of hydroxyl radical (•OH) created by the synchrotron radiation beam. The H_2O_2 ligand is bound to the fully reduced O_2 reduction site (Fe_{a3}^{2+}, Cu_B^{1+}) which is formed by reduction of the oxidized CcO by hydrated electrons produced by the strong X-ray beam. This interpretation is consistent with the absorption spectral change induced by the X-ray irradiation which produces a hemochrome-type spectrum characterized by a sharp band in the α-band region (Tiefenbrunn et al. 2011). However, a decrease in electron density induced by long exposure to the X-ray beam, as in the case of bovine CcO, has not been reported for the ba_3 type CcO. A plausible interpretation for these results is that ba_3 CcO has a weaker sensitivity to X-ray irradiation, compared with that of bovine CcO, and as a result, no further significant radiation effect on the bridging peroxide is detectable. On the other hand, bovine heart CcO is more significantly affected by the

X-ray beam and, therefore, there is a decrease in the electron density due to the bound peroxide coupled with increase in O-O bond length.

However, this interpretation is inconsistent with the observation that the electron density peak between the two metals in the O_2 reduction site after 1 s exposure indicates a clear O-O bond with an intensity consistent with two oxygen atoms linked by a covalent bond, while the 1 s exposure induces less than 10 % of the maximal absorption spectral change (Aoyama et al. 2009). Because the X-ray-irradiation effect is detectable most sensitively by the absorbance spectral change, the 1 s exposure is unlikely to cause significant damage into the conformation of CcO. The hemochrome-type absorbance spectrum showing its maximum intensity at 20 s X-ray exposure more likely arises from a radiation-damaged product originating from Fe^{3+}-O-O-Cu^{2+} moiety. In the product, the O-O bond has been broken and the heme iron is reduced. This enables binding of a strong ligand of unknown origin which gives rise to the hemochrome spectrum. Furthermore, the stability of the hemochrome spectrum induced by the X-ray irradiation is inconsistent with the proposed structure of Fe^{2+}-O-O-Cu^{1+}, since the metal site has two electron equivalents which are able to readily reduce the peroxide completely to the oxide (O^{2-}) level. Although a theoretical (DFT) calculation indicating the possibility of this structure has been reported (Du and Noodleman 2013), experimental confirmation such as a damage-free X-ray structural analysis is needed for ba_3 CcO. Furthermore, the redox titration results (which indicate a requirement of six electron equivalents for complete reduction of the resting oxidized bovine CcO which has not been exposed to any strong X-ray beam) are also inconsistent with the above interpretation. Careful redox titration experiments similar to the bovine heart CcO titrations are desirable for these bacterial CcOs since significant diversity in the function of the O_2 reduction site between bacterial CcOs and bovine CcO cannot be excluded at this stage.

VI. X-ray Structural Analyses of the Resting Oxidized CcO Obtained at an XFEL Facility

In order to eliminate the radiation damage effect, a newly constructed X-ray free electron laser facility (XFEL, SACLA) has been employed in the X-ray structural analysis of the resting oxidized CcO (Hirata et al. 2014). The facility generates extremely strong and short (20 fs) X-ray pulses which lead to complete and instantaneous destruction of protein molecules in the crystalline array would be destroyed completely by the X-ray pulse. However, before inducing such serious damages the strong X-ray pulse produces X-ray diffraction with sufficient intensity to enable X-ray structural analyses. In the X-ray diffraction experiments using SACLA, the X-ray pulse is sufficiently strong to destroy completely the protein structure and the crystalline array so that partially damaged protein molecules in the crystals which diffract X-rays are not produced during the X-ray diffraction experiments. Thus, only X-ray diffraction from the damage-free protein molecules in the regular array can be collected by this system. However, the pulse beam is too strong and too short to enable the acquisition of oscillation photographs. The position of a single shot of the pulse on a single crystal must be sufficiently separated from any position of the previous shots in order to avoid the X-ray damage effects induced by the previous shots. The extent of propagation of the X-ray irradiation damage was determined for the resting oxidized CcO crystals by evaluating the change in the integrity of the X-ray diffraction pattern at various positions within the crystal, depending on the proximity to the point of an individual XFEL shot. The integrity of the diffraction pattern was determined by an appropriately weakened X-ray beam. In the case of the resting oxidized CcO, a distance 50 μm from neighboring beam foot prints has been found to be sufficient for X-ray diffraction experiments which avoid the influence of X-ray damage propagation from

previous shots (Hirata et al. 2014). However, this distance is likely to be applicable for most protein crystals, since CcO, which has one of the highest redox potentials among the proteins in the cell, is likely to be very sensitive to X-ray radiation damage, which leads to production of hydrated electrons. The newly developed method for X-ray structural determination which does not require oscillation photographs has been reported (Hirata et al. 2014).

The damage-free structure of the resting oxidized form at 1.9 Å resolution has been published and is shown in Fig. 18.3. As described above, an electron density peak assignable to a water molecule hydrogen-bonded to Y244 OH group increases with decreasing electron density of the bound peroxide by X-ray irradiation. The X-ray structure of the cyanide-bound oxidized CcO shows a weak electron density peak (37 % of the peak intensity due to a fixed water molecule with a low B-factor), indicating the presence of a partially occupied water molecule-hydrogen bonded to Y244 before X-ray irradiation, since cyanide replaces the bound peroxide in the resting oxidized form completely. As shown in the upper panel of Fig. 18.3, a region of weak electron density (38 % of the peak intensity due to a fixed water molecule with low B-factor) hydrogen-bonded to Y244 is detectable. This region of intensity confirms the absence of any significant radiation damage to the bound peroxide.

The damage free structure (Fig. 18.3, the lower panel) includes contribution from a pair of conformations with 95 % and 5 % occupancies. Both conformers indicate an O-O bond distance of 1.55 Å, which indicates that the bound peroxide is not in the activated state as previously proposed. The Fe-O distance is significantly longer than the Cu-O distance. The structure is consistent with the resonance Raman results which indicate an O-O bond length of 1.50 Å, although the minor conformer is not detectable in the resonance Raman spectrum. The resonance Raman band position suggests that the species is O_2^{2-} rather than ^-OOH (Nakamoto 1997).
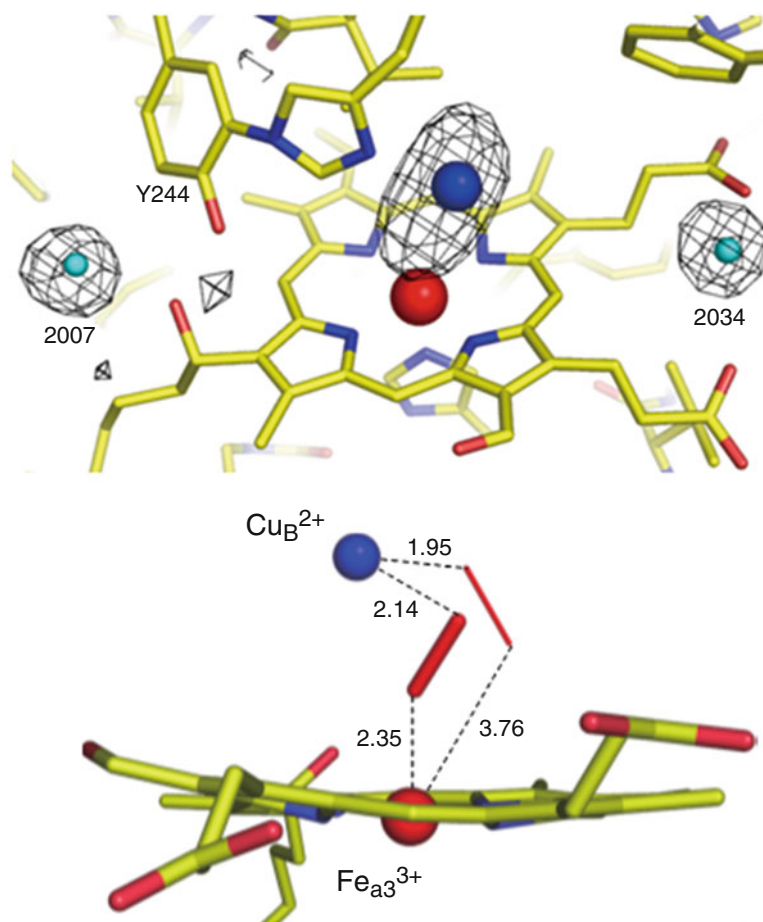
Fig. 18.3. X-ray structure of the O_2 reduction site of the resting oxidized bovine heart CcO, determined at an XFEL facility (SACLA). *Upper panel*: F_o-F_c difference map at 1.90 Å resolution for the XFEL data of CcO. Electron density cages at the 3σ level (σ/0.041 e/Å³) are depicted with the structural model including Fe_{a3} (*red*), Cu_B (*blue*), and oxygen atoms (*light blue*) of two water molecules, 2007 and 2034, which were not included in the structural refinements. The peak heights of both water molecules are 9.0σ, while the peak height near Tyr244 is 3.4σ. *Lower panel*: a stick model of the O_2 reduction site with a major component (95 %) and a minor component (5 %) of peroxide anions both with an O-O distance of 1.55 Å. The colors for designating atom species are the same as indicated for the upper panel. The *numerical values* indicated between the metal atoms and the oxygen atoms of the peroxide anions are their distances in Å (Reprinted with permission from "Hirata et al. 2014").

Thus, in spite of the long Fe^{3+}-O distance as a coordination bond, O_2^{2-} is bridged between the two metals. This is consistent with the long-standing proposal that Fe_{a3}^{3+} in the resting oxidized state is in the high spin state (Day et al. 1993; Cheesman et al. 2004).

The negative charges on the bound peroxide in the resting oxidized state are likely to suppress the electron transfer from heme *a* to heme a_3 to block the proton pump. (In bovine heart CcO, oxidation of heme *a* to heme

a_3 drives proton active transport through the hydrogen bond network of the H pathway, which connects the N-phase and the P-phase (Yoshikawa et al. 2011).) Resonance Raman analyses of the O_2 reduction process by CcO have shown that the O state has OH^- bound to the Fe_{a3}^{3+} site (Ogura et al. 1993). The structural difference of the O_2 reduction site between the resting oxidized CcO and the O form confirms the above proposal for the mechanism of blockage of the proton pump

by the bound O_2^{2-}. It is desirable to obtain a high resolution and damage free X-ray structure of the O form for comparison with the resting oxidized form. Such a comparison is expected to reveal various insights into the mechanism of the proton pump.

It is noteworthy that the successful determination of the damage-free X-ray structure of the resting oxidized CcO by the femtosecond level pulse (20 fs) from the XFEL facility indicates that X-ray structure of any protein can, in principle, be determined at high resolution with a time resolution of 20 fs, a much shorter time resolution than that of any physiological process driven by proteins in the cell. This finding is likely to induce a revolution in the Life Sciences as described in the following chapter.

VII. Picobiology

Any life phenomenon is comprised of chemical reactions driven by proteins. Thus, elucidation of the mechanism of the protein function as the chemical reaction (namely as the behaviors of the atoms comprised in the functional site of the protein) is indispensable for complete understanding of in vivo phenomena. For elucidation of the mechanism of a protein function, the three dimensional location and the chemical reactivity of each atom in the functional site of the protein must be determined accurately by crystallographic (mainly X-ray crystallographic) and vibrational (mainly infrared) spectroscopic analyses, respectively. The chemical reactivity of an individual atom is determined by the characteristic of the chemical bonds to that atom. Vibrational spectroscopy is the best method for examination of the chemical reactivity of any atom. The location and the chemical reactivity of each atom in the functional site undergo significant changes concomitant with the progression of the physiological (or chemical) reaction process driven by the protein. Thus, time-resolved techniques are required for both crystallography and vibrational spectroscopy.

X-ray strutures provide atomic coordinates of all the atoms of the protein at a resolution of 0.1 Å at the highest. However, the chemical reactivity of an individual atom is sensitively influenced by chemical bond length differences in the picometer range (in the vicinity of 0.01 Å or 10^{-12} m or shorter). Furthermore, crystallographically-determined electron density of proteins generally does not provide information regarding the chemical reactivity of atoms. The chemical bond length can be determined accurately by infrared spectroscopy at a resolution better than 1 picometer. However, infrared spectroscopy does not provide information regarding the three-dimensional arrangement of atoms. In other words, infrared spectroscopy determines bond length of a chemical bond regardless of the location of the bond in the protein. Thus, both vibrational and crystallographic techniques are indispensable. Therefore, we suggest the term, "picobiology", as a descriptive term referring to a scientific field which has the objective of understanding the mechanism of protein function in terms of the behavior of atoms (i.e. as a set of chemical reactions) based on structural analyses at the picometer level by using a combination of vibrational and crystallographic techniques.

It should be noted that the functional site of a protein is in most cases buried inside the protein moiety. As a result, the functional site may have a specialized microenvironment for a given chemical reaction which is definitely different from the environment outside of the protein. The chemical reactivity of any atom is sensitively influenced by its environment. For example, the proton affinity of an O atom of a terminal carboxyl group of acetate is highly sensitive to the polarity of the medium. In fact, the pKa value the terminal carboxyl group increases from 5 to 10 upon exchange of the medium from water to methanol (Isaacs 1995). In the functional site, various amino acid residues and cofactor molecules are arranged in three dimensional space to provide various unique microenvironments which cannot be artifi-

cially duplicated accurately in a non-protein model system. Thus, it is impossible to accurately predict the chemical reactivity of atoms (or functional groups) of a functional site based on an analysis conducted outside of the protein. Therefore, direct measurement of the chemical reactivity of the atoms in the functional site by infrared spectroscopy as well as a determination of the three dimensional location of atoms in the active site of the protein is absolutely necessary in order to elucidate the reaction mechanism. However, infrared spectroscopy is not appropriate for samples in aqueous solution, because water molecules have strong infrared absorption. While most proteins function in aqueous solution, the integral membrane proteins which are embedded in the biological membrane have hydrophilic surfaces at their ends which extend into both aqueous phases. In fact, it is essentially impossible to transfer membrane proteins into organic solvent without denaturation. Thus, even the integral membrane proteins cannot function without aqueous phases.

As described above, X-ray structural information intrinsically does not provide direct information regarding the chemical reactivity of atoms within a protein. Thus, in the past, the mechanisms that produce protein functions have been estimated by X-ray structures determined at various intermediate states experimentally stabilized in its crystals and by assuming that the chemical reactivity of atoms in the functional site is identical or similar to that determined in homogeneous system in the outside of the protein. However, this assumption is not practical as described above. In other words, without the direct infrared information on the chemical reactivity of the atoms, it is essentially impossible to discover the process (or the chemical reaction) which can be driven only by the protein microenvironment.

A time-resolved infrared system applicable for investigation of aqueous protein systems has been recently constructed. This system is equipped with a strong infrared light source created by a femtosecond pulse laser system and a highly sensitive infrared multichannel detector, and provides time resolution at the picosecond level and sensitivity sufficient for detection of a single $C=O$ stretch band of an α-helix in the amide I and II region with accuracy of ± 10 % at the peak position in H_2O (Kubo et al. 2013).

This system has been used to examine the process of CO release after flash photolysis of CO-bound CcO which is analogous to the reverse reaction of O_2 binding to Fe_{a3}^{2+}. Figure 18.4 shows the infrared spectral change after CO release in the amide I and II region between 1750 and 1600 cm^{-1} and in the time range from 50 ns to 50 μs. There is a prominent spectral change that generates peak/trough positions at 1655 and 1666 cm^{-1}, respectively. The band positions and intensities suggest that a single main chain $C=O$ group in a bulge conformation (or not involved in α-helix) becomes incorporated into the α-helix within the dead time (50 ns, the duration between photolysis and the time when measurement of the spectrum is initiated). The infrared spectral changes in the C-O triple bond stretch region indicate stoichiometric migration of CO from Fe_{a3}^{2+} to Cu_B^{1+} within the dead time, followed by release of the CO bound at Cu_B at $t_{1/2}$ at about 2 μs without rebinding of CO to Fe_{a3} (Kubo et al. 2013). Concomitant with the CO release, the 1655/1666 cm^{-1} band disappears as shown in Fig. 18.4, indicating formation of a bulge conformation. In order to identify the position of the main chain $C=O$ group, examination of the site-directed isotope shift effect is necessary. However, a cell-free synthesis system for site-directed isotope labeling has not been constructed for bovine heart CcO. On the other hand, X-ray structural results reported thus far indicate that a bulge/α-helix conformational transition coupled with a α-helix/bulge conformational transition upon CO binding to the fully reduced CcO is detectable only in the segment of helix X including S382 and V380 (Kubo et al. 2013). Thus, the spectral changes in the 1655/1666 cm^{-1} bands are reasonably assignable to the movement (or conformational change) of the S382/V380 segment.
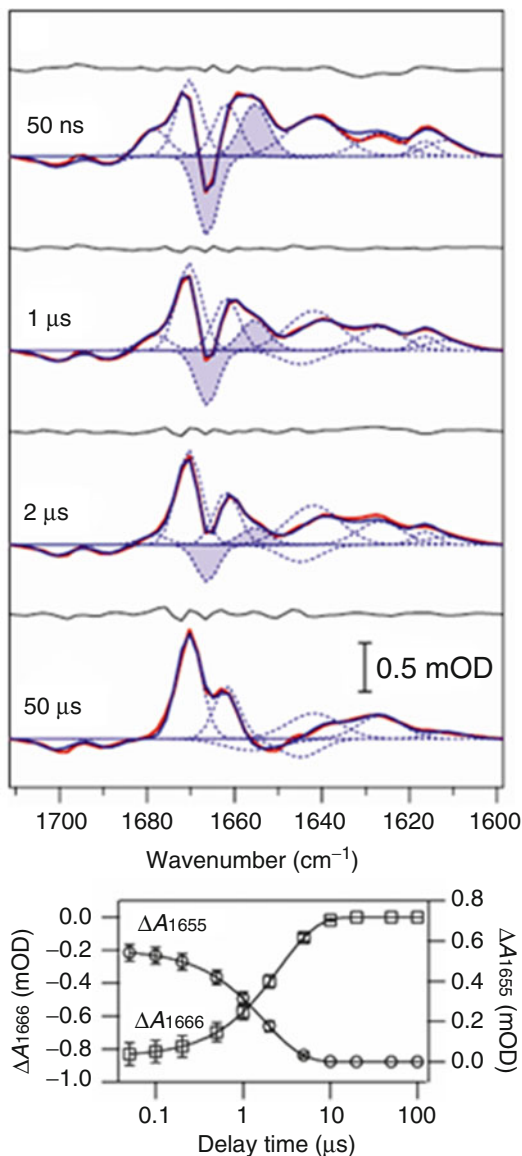
Fig. 18.4. Time-resolved infrared difference spectra of CcO in H_2O buffer. *Upper panel*: Difference of infrared spectrum as a function of time after flash photolysis relative to the spectrum obtained before photolysis (i.e. spectrum after photolysis minus the spectrum of CO-bond CcO). *Red*: measured spectra, *Blue*: Fitted spectra, with each Gaussian component shown by a dotted curve. The fitting residual is also shown for each spectrum. *Lower panel*: Changes in intensities of the peak (1655 cm^{-1}) and trough (1666 cm^{-1}) of the *shadowed band pair* showing the most prominent spectral changes. *Error bars* represent the standard deviation of five independent experiments performed on different days using different batches of CcO preparation (Reprinted with permission from "Kubo et al. 2013").

The conformational change plays a crucial role in proton pump. As mentioned above, in bovine heart CcO, protons are pumped through the H-pathway (a proton conducting pathway) which is composed of a hydrogen-bond network and a water channel operating in tandem (Yoshikawa et al. 2011). Protons for pumping are transferred as hydronium ion

from the N-phase through the water channel to the bottom end of the hydrogen bond network. The protons arriving at the hydrogen-bond network are actively transported to the P-phase by electrostatic repulsion between the protons and the positive charge of heme *a* which is attached to the hydrogen bond network via hydrogen bonds connected to the peripheral groups of heme *a*. The positive charge of heme *a* is created upon electron transfer from Fe_a to the O_2 reduction site. In the fully reduced and ligand-free state, S382, located near the water channel, is included in helix X, while the main chain $C=O$ of V380 is included in the bulge conformation. The bulge has no significant influence on the conformation of the water channel of the H-pathway. The X-ray structural analyses indicate that upon CO binding to Fe_{a3}^{2+}, S382 migrates to form a bulge conformation accompanied by incorporation of V380 into helix X (Muramoto et al. 2010). Similar conformational changes are likely to occur also upon O_2 binding to Fe_{a3}^{2+}. This bulge blocks water molecule (or hydronium ion) access through the water channel to the bottom end of the hydrogen-bond network in the H-pathway. Furthermore, the blockage prevents backward leakages of protons from the hydrogen-bond network. Thus, protons for pumping must be drawn into the hydrogen-bond network before O_2 is bound to Fe_{a3}. The intermediate conformation formed within the dead time as shown in Fig. 18.4 is likely to be a conformation which promotes proton collection from the N-phase. Formation of the intermediate conformation is coupled with CO binding to Cu_B. This suggests the existence of a relay system between Cu_B and S382. The coupling suggests that O_2 is transiently bound to Cu_B before binding to Fe_{a3}. This CO binding to Cu_B promotes proton collection for pumping before the initiation of O_2 reduction, which is critical for effective proton pump. Determination of the X-ray structure of the intermediate species induced by the ligand binding to Cu_B, as detected by the time-resolved infrared analysis, is necessary to determine the significance of the infrared data with respect to the intermediate species.

The above time-resolved infrared results show that, using this system, the infrared absorption spectral changes due to a single $C=O$ stretch can be accurately observed even in the frequency region which includes intense absorption by H_2O molecules. This system is applicable for any aqueous protein solution. Thus, one of the two most significant problems encountered in picobiology investigations has now been solved.

The second of the two most significant problems, time-resolved X-ray structural analysis, has been solved recently (Hirata et al. 2014). As described in the preceding chapter, a damage free X-ray structure of the resting oxidized CcO has been determined at 1.9 Å resolution using a femtosecond X-ray pulse generated at an XFEL facility. The results indicate that, in principle, an X-ray structure of any protein can be determined at a time resolution of 20 fs at high resolution. Although a number of issues relating specific analyses of various class of proteins must be solved before practical use of these facilities become widespread, it is now believed that the investigations in new field of "picobiology" are now possible as a result of the significant advances in these time-resolved X-ray and infrared techniques.

Acknowledgments

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Structure and Mechanism of Action of the Alternative Quinol Oxidases

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Summary

In addition to heme-copper oxidases, all higher plants, some algae, yeasts, molds, metazoans, and pathogenic microorganisms such as *Trypanosoma brucei* contain an additional terminal oxidase, the cyanide- and antimycin-insensitive alternative oxidase (AOX). It is a di-iron carboxylate protein that catalyzes the four-electron reduction of dioxygen to water by ubiquinol, short-circuiting the mitochondrial electron-transport chain prior to proton

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translocation by complexes III and IV, thereby dramatically reducing ATP formation. In plants, it plays a key role in cellular metabolism, thermogenesis, and energy homeostasis and is generally considered to be a major stress-induced protein. In *T. brucei*, a parasite that causes human African sleeping sickness, AOX plays a critical role in the survival of the parasite in its bloodstream form. Because AOX is absent from mammals, this protein represents a unique and promising therapeutic target. Despite its bioenergetic and medical importance, however, until recently structural features of any AOX were unknown. In this review we describe recent advances in our understanding of this protein's structure following the recent successful crystallization of the alternative oxidase from T. *brucei*. We focus on the nature of the active site and ubiquinol-binding channels and describe a mechanism for the reduction of oxygen to water based on these structural insights.

I. General Introduction

The alternative oxidase (AOX) is a monotopic protein with a diverse profile of postulated roles within cells, with each role being dependent upon the cell in which it is located. The protein is ubiquitous within the plant kingdom, in which it predominantly plays an energy homeostatic role. Readers are referred to recent reviews on this topic (Moore et al. 2013; Vanlerberghe 2013).

The most well documented role of AOX is in thermogenic plants, particularly those from the *Araceae* family. Thermogenic plants generate heat well above ambient temperatures through engagement of the alternative pathway (Meeuse 1975; Seymour et al. 2003). Energy released as a result of the direct reduction of oxygen through to water during the operation of this pathway results in heat generation since AOX is non-protonmotive (Moore et al. 1978). In thermogenic plants heat is used to either volatilize primary amines to attract insects for pollination purposes, as is the case in the voodoo lily (*Sauromatum guttatum)* and *Arum maculatum* (Lords & Ladies), or to melt the

surrounding ice, as occurs in skunk cabbage (*Symplocarpus foetidus)* (Seymour 2010).

In trypanosomes, the protozoa responsible for diseases such as Human African Trypanosomiasis (HAT) and nagana in livestock, AOX plays a more pivotal role within the electron transport chain. In its bloodstream form within the host, trypanosomes down regulate the expression of respiratory chain complexes III and IV (Grant et al. 1961), leaving the parasite dependent solely upon AOX as the terminal oxidase. Since oxidative phosphorylation can now no longer occur within the bloodstream form of the parasite, ATP is generated via glycolysis (Bienen et al. 1993) and the principal role of AOX is to regenerate cytosolic NAD^+ via a glycerol-3-phosphate re-oxidation system. Such a critical metabolic role for AOX, coupled with the lack of the protein in mammalian systems, has led to AOX being considered as a potential chemotherapeutic target for the treatment of HAT (Nihei et al. 2002).

The recent success in crystallisation of the AOX (Shiba et al. 2013) from *Trypanosoma brucei brucei* (TAO) allows a much more detailed investigation of the active site and highly conserved residues then has hitherto been possible. Previous structural predictions that the protein contains a non-heme di-iron center surrounded by a four-helix bundle in which the metal core is ligated by four glutamatic acids and two histidines (Moore et al. 1995; Siedow et al. 1995; Andersson and Nordlund 1999; Berthold et al. 2000) have proven essentially correct and, alongside the

Abbreviations: AOX – Alternative oxidase; Asco – Ascofuranone; CB – Colletochlorin B; HAT – Human African Trypanasomiasis; MMO – Methane monooxygenase; OG – Octyl gallate; PCET – Proton-coupled electron transport; Q(H$_2$) – (Reduced) ubiquinone; RBR – Rubrerythrin; RNR – Ribonucleotide reductase; rSgAOX' – Recombinant *Sauromatum guttatum* alternative oxidase; SHAM – Salicylic hydroxamic acid; TAO – Trypanosomal alternative oxidase

crystal structure, confirm that the AOX is indeed a legitimate member of the di-iron carboxylate superfamily, which includes other oxygen reductive species such as methane monooxygenase (MMO) and ribonucleotide reductase (RNR) (Lipscomb 1994; Kolberg et al. 2004).

While the active-sites of these proteins are similar, they undergo very different enzymatic reactions. MMO uses the oxygen to generate a high valent Fe(IV) (Shu et al. 1997) state for the conversion of methane to methanol, a reaction that has resulted in considerable scientific interest due to its catalytic conversion of a volatile gas into a transportable liquid at room temperature. RNR on the other hand uses the di-iron core to reduce ribonucleotides through to deoxyribonucleotides for use in DNA synthesis (Kolberg et al. 2004).

In this review we will consider in detail the crystallographic structure of the trypanosomal enzyme since it provides a useful framework in which to examine the mechanism of oxygen reduction by this family of proteins. Unlike the plant variant, the parasitic AOX, however, does not appear to be regulated either by a disulfide/sulfydryl system (Umbach and Siedow 1993; Rhoads et al. 1998) nor activated by pyruvate (Millar et al. 1993; Umbach et al. 2002), effectively existing as a 'stripped-down' more simplistic version of the protein which is found in plant and fungi. We discuss salient structural features that are common to all AOX proteins and suggest a mechanism for the four-electron reduction of oxygen to water. For a discussion of terminal oxidases of cyanobacteria, including alternative respiratory terminal oxidases (ARTOs), see the Chap. 17 by Schmetterer in this volume.

## II. Overall Structure of the Alternative Oxidases

TAO was crystallised as a homodimer (Fig. 19.1a), with each monomer being comprised of six α-helices arranged in an anti-parallel fashion, four of which form a characteristic four helix bundle which acts as a scaffold to bind the di-ion core. The two remaining helices (helices 1 and 4) are membrane binding, anchoring the protein to the phospholipid bilayer. Since there are in excess of 850 AOX sequences deposited in the database it is possible to determine the identity and location of those residues that are either partially or fully conserved across the AOX protein family. Conservation of amino acids within a protein family suggests that specific amino acids are important to the overall function of the protein, and can usually be attributed to either fulfilling a structural or mechanistic role, with some undertaking both functions. Sequence alignment of a cross section of AOX sequences from fungal, pathogenic and plant species reveals that there are a total of 72 conserved residues, 46 of which appear to be fully conserved across the entire currently sequenced AOX family. In this section we attempt to ascribe a function to a number of what we consider to be the most important of these conserved residues.

A. Role and Location of Universally Conserved Residues

In order to understand the possible role of conserved residues or regions it is important to consider their structural location. Table 19.1 summarizes the location of these residues in addition to suggesting a predicted function of the residue based on an analysis of the crystal structure. It is readily apparent from Table 19.1 that in addition to a number of single residues that are conserved throughout the structure, there are also regions of highly conserved amino-acids.

The larger conserved regions are predominantly residues surrounding the metal binding ligands, namely in the LETVA, NERMHL, GYLEEEA and RADEAxHR regions (where red letters are the metal ligands). A high level of conservation in this region is not surprising, given the crucial nature of the metallic core to the overall function of the protein, since perturbations caused by mutation would likely render the protein
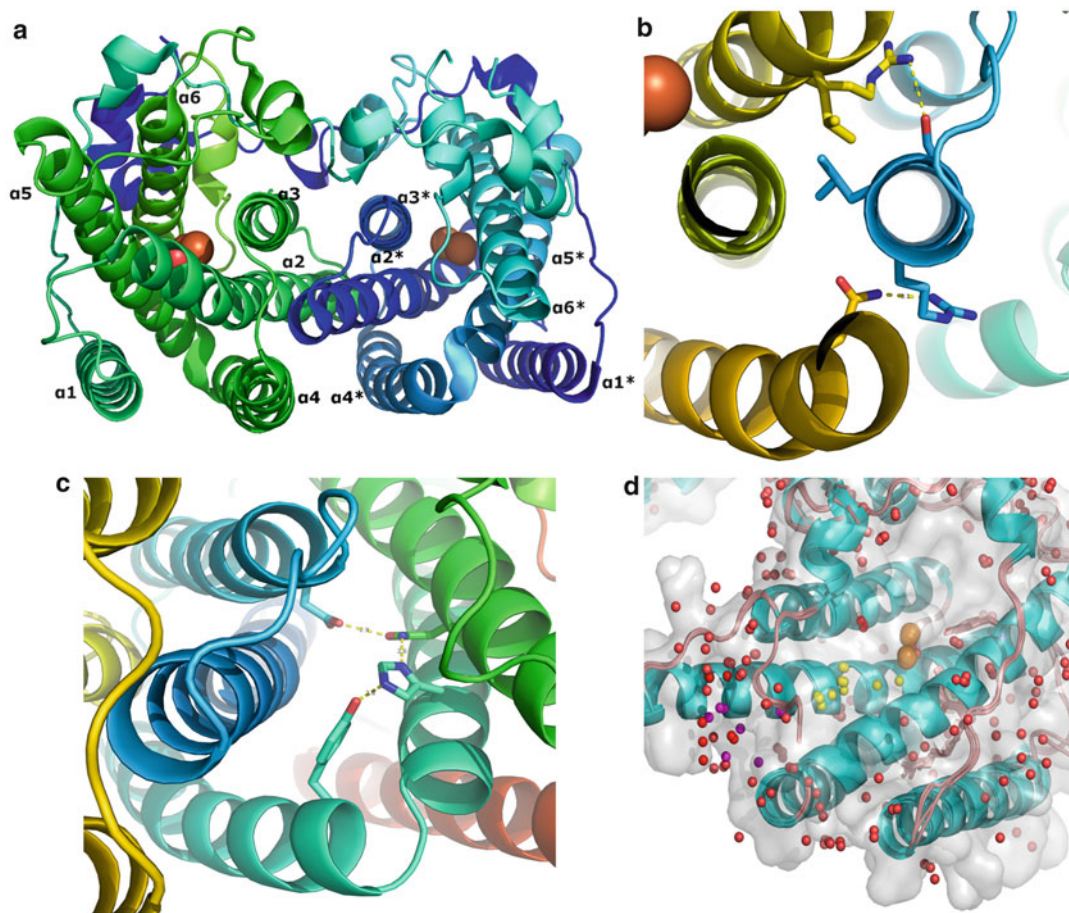
Fig. 19.1. (**a**) Crystal structure of TAO dimer (PDB accession code 3VV9). Di-iron center shown as *brown spheres*, with helical numbering added for reference. (**b**) Cross section of the SLR region between dimers, showing hydrogen bonds between S141 and R163*, and potentially between R143 and Q187*. L142 and L166* also shown, capping the interdimer gap with hydrophobic residues. (**c**) Hydrogen bond network linking four helices, consisting of Y198, H206, N273 and E158, non hydrogen bonding amino acids removed for clarity. (**d**) Water distribution throughout the crystal structure, shown with the four monomers of AOX inhibited with colletochlorin B overlaid (PDB: 3W54). *Yellow spheres* denote water within the structure, *red spheres* are water on the surface, and iron is shown as *orange spheres* in the centre. Helix 6 removed for clarity.

inactive. As expected, mutation of the iron binding residues in both the TAO (Ajayi et al. 2002; Nakamura et al. 2005) and plant AOX (Albury et al. 2002; Berthold et al. 2002) leads to a complete loss of enzymatic activity.

The SLR region (Fig. 19.1b) is located at the C-terminal end of helix 2 and is largely responsible for capping the dimer interface to either end of the protein. S141 forms a hydrogen bond with R163 (of NERMHL) to maintain the dimer formation between helices α2 and the neighbouring monomer α3*. A similar bond is also found at the opposite end of these two helices, with identical bond formation between helices α2* and α3 thereby effectively locking the helices together. The role of the R143 is less defined, given its location on the membrane face of helix α2. There is potential for hydrogen bond formation between R143 and Q187 on helix α4*, but given the variability on the rotamic positions between monomers it is more likely that both amino acids play a role in anchoring the protein to the membrane. L142

Table 19.1. Highly conserved amino acids throughout the sequence alignment, and their predicted roles within the structure based upon crystal structure analysis.

AA sequence	TAO numbering	Region	Role
W	65	N-terminal	
H	77	N-terminal	
D	85	Helix 1	
R	96	Helix 1	Substrate binding
D	100	Helix 1	Substrate bind
R	118	Helix 2	Substrate binding
LETVA	122-126	Helix 2	Iron ligation
VPGMV	128-132	Helix 2	Varied
H	138	Helix 2	
SLR	141-143	Helix 2	Varied
GWI	150-152	Helix 3	Varied
L	155	Helix 3	
EA	158-159	Helix 3	
E	160	Helix 3	
NERMHL	161-166	Helix 3	iron ligation
F	169	Helix 3	
P	175	Between 3 + 4	Interhelix bend
R	180	Helix 4	
Q	187	Helix 4	
Y	198	Helix 4	Interhelical binding
L	199	Between 4 and 5	
SPR	201-203	Between 4 and 5	Interhelix bend
HR	206-207	Helix 5	
VGYLEEEA	209-216	Helix 5	Varied
YT	220-221	Helix 5	Redox active tyr
D	228	Helix 5	
G	230	Helix 5	
P	240	Between 5 and 6	
A	243	Between 5 and 6	
YW	246-247	Short helix	Structural/electron transport
D	257	Helix 6	Structural
RADEA	263-267	Helix 6	Iron ligation
HR	269-270	Helix 6	Iron ligation
VNH	272-274	Helix 6	Interhelical
P	290	C terminal	

Numbering taken from the *Sauromatum guttatum* sequence. Residues in red correspond to universally conserved amino-acids

probably also plays a dual role, effectively capping the dimer interface with a hydrophobic residue and locking the helix into place through interaction with L166 located in the NERMHL region.

The membrane face of helix 4 is coated with hydrophobic residues which provide additional interaction with the phospholipid bilayer. While the conserved residues on helix 1 and 4 are mainly polar in nature and will bind directly to anchor points on the membrane surface, valine residues provide repulsion from the water layer on the membrane, thereby preventing the protein from embedding too far into the phospholipid bilayer.

The totally conserved tyrosine residue (Y198), which is located towards the C-terminal end of helix 4, is part of a hydrogen

bond network, which spans 4 helices. It appears that one of its main functions is to provide structural stability (Fig. 19.1c). It maintains the helical turn caused by P202 via formation of a hydrogen bond to H206 (Helix 5), which itself is further stabilised by N273 (helix 6) and E158 (Helix 3). While a majority of amino acids have a highly conserved rotamic position, the E158 appears to be fixed in one of two positions perpendicular from one-another dependent upon the location of the proximal crystallized water which co-crystallized with TAO (Shiba et al. 2013). CAVER (Chovancova et al. 2012) analysis reveals that a channel runs directly through these residues (E158, Y198, H206 and N273) from the protein exterior to the di-iron core. One of the features of the protein is the use of an aqueous channel, either for the transport of water away from the active site after the reaction is complete, or for the transport of protons into the active site. This notion is supported by the structures depicted in Fig. 19.1d which show an overlay of the four monomers from a single TAO crystal, revealing a pathway of internal water molecules (shown as yellow spheres) co-crystallised within the structure.

The hydrophobic channel itself is reasonably well conserved, consisting of a combination of hydrophobic and polar residues. Analysis of the pKa values of these polar residues, namely E158, Y198 and H206, using PropKa (Li et al. 2005), reveals a directional increase in pKa from the core to Y198 at the entrance of the tunnel, suggesting that if this channel is indeed used to transfer protons, the residues are aligned in such a manner that the route of proton transfer is towards the active site. While mutation of Y198 or H206 to alanine results in an inactive protein (Albury et al. 2010), this is most probably due to disruption of the tertiary structure rather than to a loss of the aqueous channel. Interestingly, a Y198F mutant retains approximately 50 % of its activity (Albury et al. 2002), suggesting that it is the presence of a hydrophobic residue in this pathway, rather than disruption of

the hydrogen bond network, that reduces the overall function of the protein.

H274 is the main focal point of interhelical interaction between helices 5 and 6, forming hydrogen bonds to both R207 and E214, effectively locking the helices together. While Y211 is found within this region, we suggest its role is to lock H274 in place, effectively preventing movement towards helix 1. At the C terminal end of helix 5, a highly conserved H-bond is formed between T221 and R263. The high conservation of these residues tends to suggest that their role is to stabilise the position of Y220, the redox-active tyrosine residue, in the region of the active site. The terminus of the interface is capped by a semi-conserved D228-R256 interaction, which also interacts with E71 in short helix α2. While the amino-acid sequence of TAO is the only one within the AOX family to contain a carboxylate at this position, alcohol containing amino acids are also found to occupy this location in all other AOX containing species and it is therefore likely that they undertake similar functions.

The YW pair (246 and 247) on short helix 3 is most likely used to stabilise the two histidines at the active site (Fig. 19.2). Hydrogen bonding between Y246 and N161 orientates helix 3 in such a way to facilitate a H-bond network between W247 and the iron-ligating residue H165 via D265. D265 is held in position by W65, a semi-conserved residue. It is important to note that this region is identical to that found in RNR, in which it has been suggested to be part of a proton-coupled electron transport (PCET) network (Stubbe et al. 2003). Mutation of Y246 to alanine results in 90 % inhibition of activity (Nakamura et al. 2005), demonstrating that this region is important to the overall function of the protein.

B. The Hydrophobic Nature of the Protein

Helices 1 and 4 are predicted to be the membrane binding helices. As previously indicated the surface of the membrane side of the protein is largely hydrophobic, with the
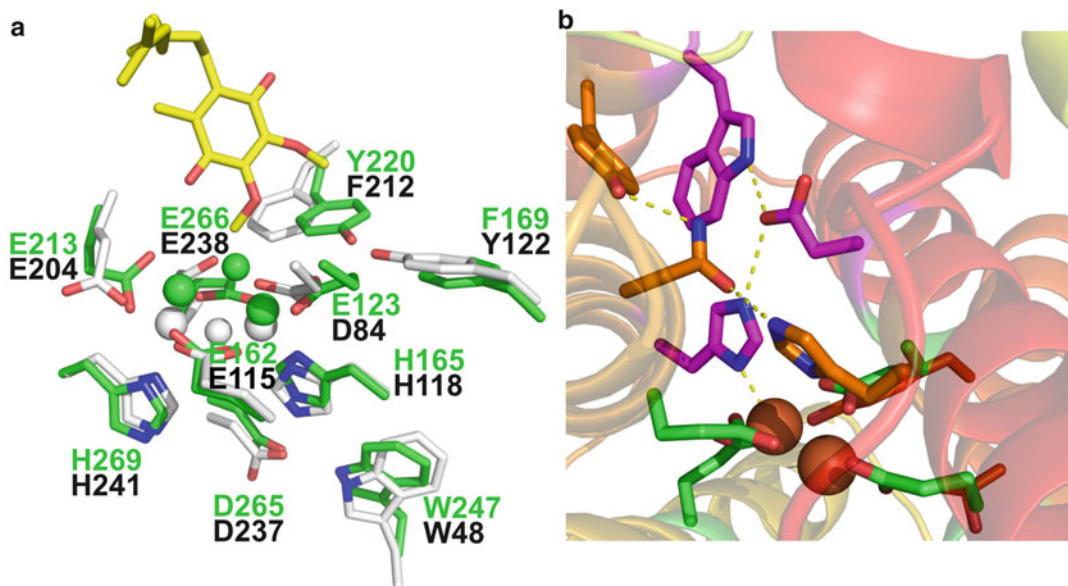
Fig. 19.2. The proton coupled electron transport (PCET) system within AOX. Figure (**a**) is an overlay of AOX (*green* – PDB:3VV9) and RNR (*white* – PDB:1RIB), showing good spatial alignment of the histidine, aspartic acid and tryptophan. (**b**) shows the PCET network in *purple*, with the proposed support network of tyrosine, asparagine and histidine shown in *orange*.

hydrophobicity extending to slightly above helices 1 & 4, indicating that this region resides below the polar head groups of the phospholipid bilayer and hence extends into the membrane (Fig. 19.3). Highly conserved arginine residues (R106, R143, R180 and R203) are present on the outside surface of the protein, probably acting as anchor points to the membrane itself.

The opening that is visible in the membrane facing-side of the protein illustrated in Fig. 19.3 is the entrance to a larger hydrophobic cavity that leads directly to the di-iron core of the protein. CAVER (Chovancova et al. 2012) analysis of the crystal structure produced an outline of the cavity as indicated in Fig. 19.4. It is more than likely that the cavity leads to the quinol binding region. The hydrophobic nature of the cavity is an ideal binding environment for the isoprenoid tail of the quinol, and the cavity allows direct access from the quinone pool within the membrane into the di-iron core.

Of particular note is the location of highly conserved leucine residues flanking the cavity (L177 and L267). These residues form

a bottleneck for the cavity (approximately 6.3 Å wide), probably serving to correctly orientate the substrate as it enters the hydrophobic pocket in order to reach the active site. Recent site-directed mutagenesis studies on these residues indicate that mutation to alanine markedly decreases the sensitivity of the AOX protein to AOX inhibitors (Young et al. 2014). Inhibitory efficacy was considerably decreased in the mutants suggesting that these residues are crucial ligands for inhibitor binding, which in turn would also suggest that they play a critical role in quinol binding. Whether this is a direct effect of losing *Van der Waals* binding, or a consequential effect of incorrect orientation of the inhibitor into the binding pocket remains to be elucidated.

C. Structure of the Di-iron Core

As with the other di-iron carboxylate proteins, the central core consists of two iron atoms ligated by four carboxylates. Figure 19.5 depicts the typical arrangement of ligands around the metal centres. Of

Front

Dimer interface

Membrane interface
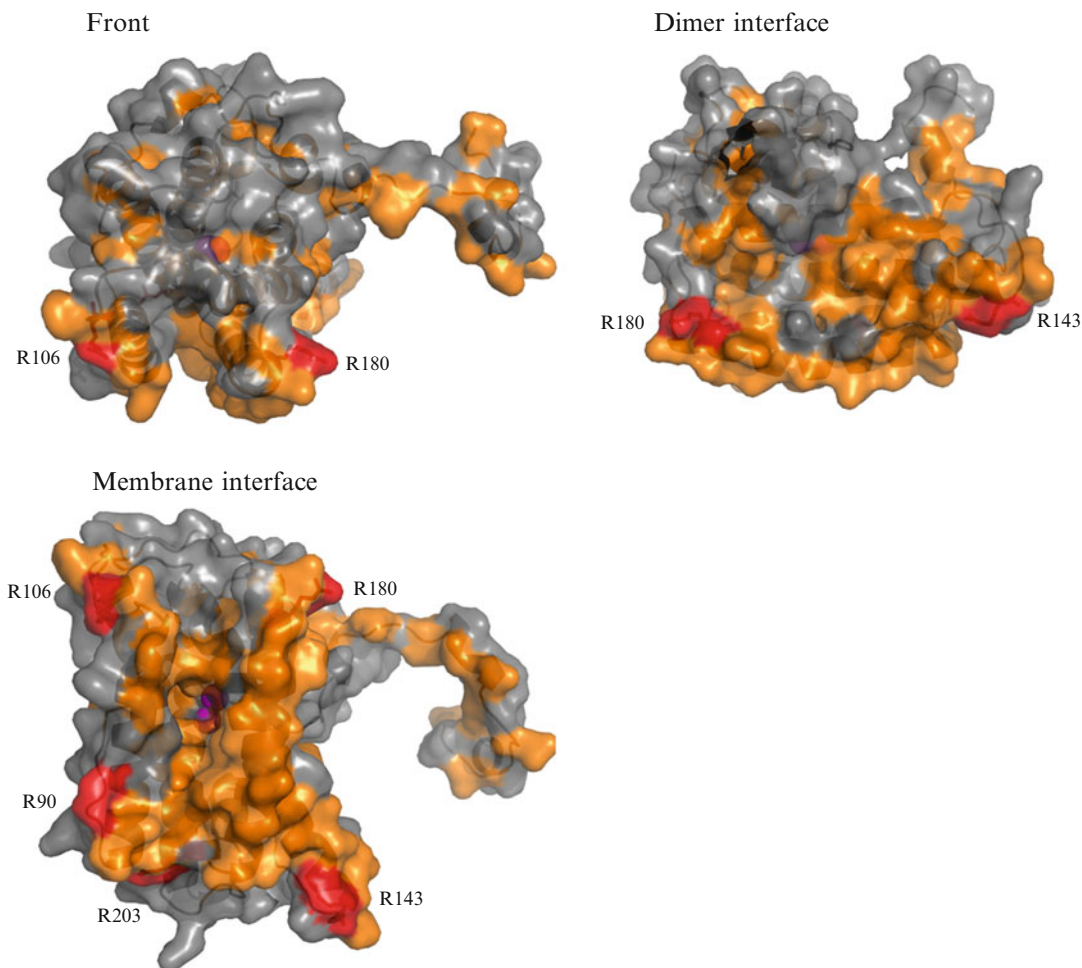
Fig. 19.3. Surface representation of a monomer from the TAO crystal structure, depicting the hydrophobic residues in *orange*. Membrane interface depicts the underside of the protein, and the di-iron core (*magenta spheres*) can be seen via the cavity. Residues coloured *red* are proposed to be responsible for membrane binding. N-terminal arm removed from dimer interface diagram for clarity.

particular note is the observation that the orientation of E123 appears to be dependent upon the location of the ligating oxygen species. While E123 is consistently observed to be in a bidentate binding mode, the closer the oxygen atom is located to E123 the more it induces rotational movement of E123, thereby resulting in hydrogen bond formation between the amino acid and the oxygen species.

A particular uniqueness about the ligation sphere of AOX is that although two histidines are universally conserved within the primary ligation sphere of all AOXs, neither one appears to be within co-ordination distance when the protein is in its oxidized form (Table 19.2). Interestingly when overlaid on top of the oxidised forms of MMO and RNR, as depicted in Fig. 19.6, it is apparent that the lack of ligation is not due to movement of the histidine ligands, but rather due to migration of the iron atoms. Furthermore if the PCET network within AOX does indeed include these histidines, it would suggest that in its oxidized form there is a disconnection of the iron atoms from
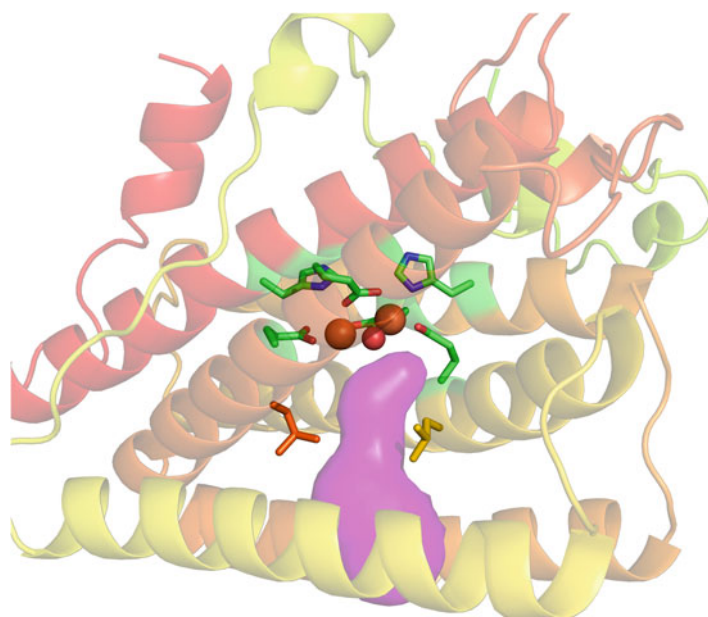
Fig. 19.4. Hydrophobic cavity (shown in *magenta*) leading towards the di-iron core between helices 1 and 4. Iron shown as *orange spheres*, oxygen as *red*, and the primary ligation sphere is shown as *green* sticks. Also shown are L177 and L267, the cause of the bottleneck in the cavity.
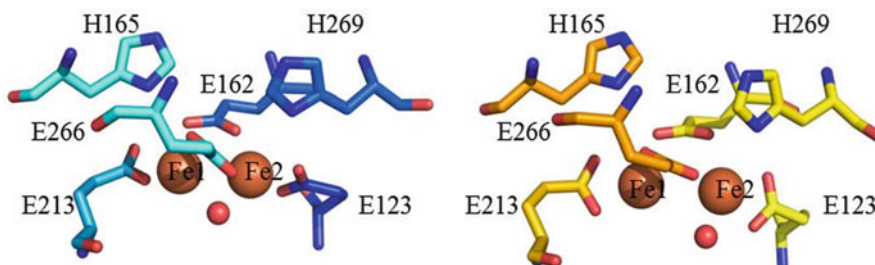
Fig. 19.5. Di-iron core of TAO (PDB:3VV9) from different monomers. *Cyan* figure on the *left* is monomer A, *yellow* on the *right* is monomer B. *Large orange sphere* corresponds to iron, with the *small red sphere* corresponding to oxygen.

Table 19.2. Bond distances in Angstroms for iron ligation sphere relative to the TAO numbering system.

Monomer	E123 (Fe2/Fe2)	E162 (Fe1/Fe2)	E213 (Fe1/Fe1)	E266 (Fe1/Fe2)	H165	H269
AOX	2.2/2.0	2.5/2.3	2.3/2.5	2.0/2.1	3.3	4.4
RNR	2.2/2.5	2.0/1.8	2.1/NB	2.1/NB	2.1	2.3
RNR D84E	2.2/2.9	2.2/2.1	2.1/NB	2.0/NB	2.4	2.1
MMO	2.0/NB	2.6/2.2	2.1/NB	2.0/NB	2.2	2.1
RBR	2.1/2.3	2.0/2.2	2.2/2.2	2.1/2.0	4.1	2.2

NB signifies no second bond towards to metal centre for that ligand
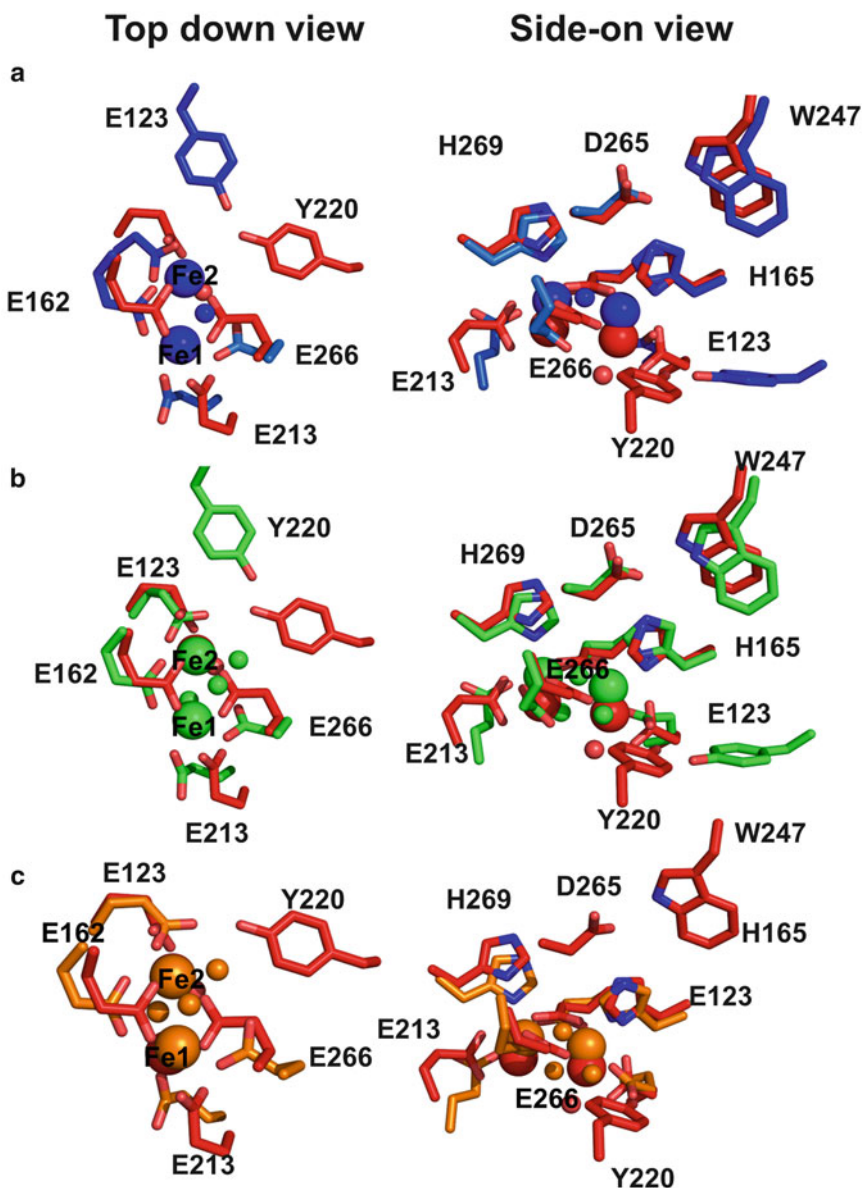
Fig. 19.6. Oxidized di-iron core of AOX aligned to; (**a**): RNR (PDB:1RIB), (**b**): RNR D84E (PBD:1PIU)and (**c**): MMO (PDB: 1MTY). AOX is shown in *red*, RNR wild type in *blue*, RNR D84E in *green* and MMO in *orange*. Alignment performed according to best fit with respect to the iron binding ligands. Atoms colored according to element, with oxygen shown in *red* and nitrogen in *blue*, with the exception of the di-iron core (*large spheres*) and ligating oxygens (*smaller spheres*), which were coloured according to crystal structure for visibility. Histidines removed from the top-down view for clarity. TAO numbering added for orientation purposes.

this network. It is conceivable that this may be necessary during the oxidative part of the catalytic cycle and not during the reaction with quinol.

The location of the proximal tyrosine (Y220) within the active site has also altered in comparison to RNR. Mutation of this tyrosine to phenylalanine results in 100 %

inhibition of activity (Nakamura et al. 2005; Albury et al. 2010), confirming the importance of the hydroxide moiety. The difference in location of Y220 in AOX in comparison to the position of Y122 in RNR is important since such a location, directly on top of the substrate binding cavity, in all probability allows the amino acid to interact directly with bound substrate.

III. Mechanism of Di-oxygen Reduction by the Alternative Oxidases

A. General Characteristics of Oxygen Reduction

While the full reduction of oxygen through to water requires four electrons, several mechanistic possibilities exist with respect to how this may occur. For instance, when oxygen binds to the protein it becomes fully reduced, leaving the protein in an overall 4^+ oxidation state, or alternatively it could get reduced to a peroxo-type species, thereby leaving the protein in an overall 2^+ oxidation state. It is worth emphasising that the overall oxidation state refers to that of the protein and does not necessarily imply that it is the oxidation state of the di-iron center. Both tryptophan and tyrosine residues have been well documented for their ability to retain radical charges within RNR (Saleh and Bollinger 2006). A lower oxidation state around the metal center would reduce the reduction potential of the metal itself, allowing an effective tuning of the protein to specific substrates.

While there is no direct spectroscopic evidence with respect to the mechanism by which AOX reduces oxygen to water, inferences can be drawn from EPR (Berthold et al. 2002; Moore et al. 2008) and FTIR (Marechal et al. 2009) studies. Other di-iron proteins, such as MMO and RNR, have been studied in depth with respect to their catalytic cycles and are a reasonable starting point for examining the reactivity

of AOX with oxygen. While outside the scope of this review, readers are referred to some recent articles on the mechanistic nature of soluble methane monooxygenase (MMO (Tinberg and Lippard 2009, 2011; Lee et al. 2013)) ribonulceotide reductase (RNR (Tomter et al. 2013)) and cryo-crystallographic time course studies of RBR (Dillard et al. 2011).

B. Proposed Reaction Mechanism

The suggested reaction mechanism depicted in Fig. 19.7 is similar to those proposed for MMO and RNR with some subtle differences. One of the key differences between AOX and MMO is the lack of visible absorbance band at 720 nm. Such a band can be observed in MMO and has been attributed to a ligand to metal charge transfer event (Valentine et al. 1999). Given that this event is likely due to energy transfer from the superoxo species to a bridging peroxo species, we suggest that an alternative route probably exists in AOX involving the quenching of the radical superoxide species by the proximal tyrosine prior to rearrangement of the core. The 'diamond core' formed after the peroxodi-iron species is a common intermediate found in di-iron enzymes (Shu et al. 1997) and reduction of this highly reactive core either by the PCET network or via protons derived from the solvent would result in a di-iron center which, in redox potential terms, is closer to that of bound ubiquinol.

1. Structure of the Fully Reduced Di-iron Core

EPR studies of the fully reduced protein resulted in a signal of $g = 16.9$ in parallel mode (Moore et al. 2008), which is characteristic signature of di-iron proteins (Solomon et al. 2000). This suggests that the protein in its fully reduced form is an Fe^{2+}/Fe^{2+} state, and coupled with the lack of an EPR signal in the perpendicular mode (Moore et al. 2008), suggests the irons are paramagnetically linked,
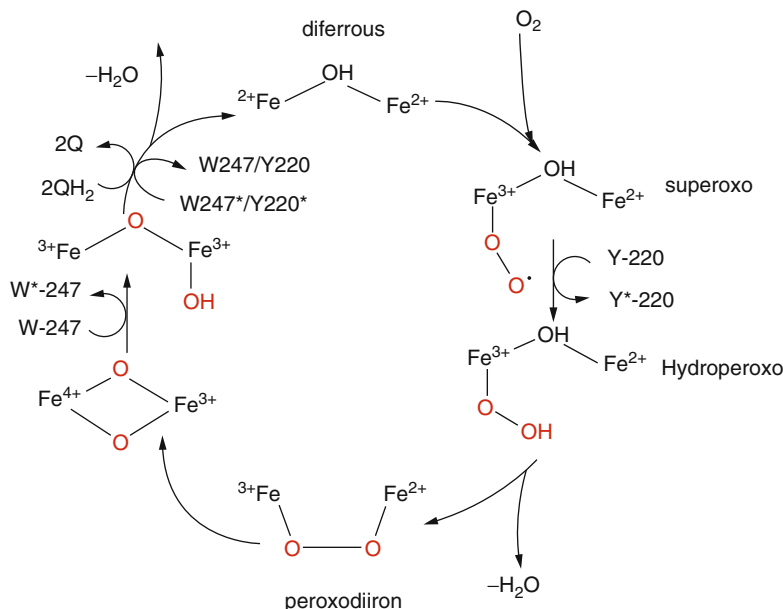
Fig. 19.7. Proposed mechanism for the oxygen reduction cycle within AOX.

either via a bridging hydroxide ion or one of the carboxylate ligands.

While a fully reduced species of AOX has yet to be crystallised, inferences as to the electronic state of the core can be made from an examination of the reduced structures of other di-iron proteins depicted in Fig. 19.8. Scrutiny of the wild type RNR and D84E mutant core (Nordlund and Eklund 1993; Voegtli et al. 2000; Voegtli et al. 2003) reveals that the effects of such a mutation are translated through the active site to the other terminal carboxylate, with movement of the carboxylate from a bidentate binding mode within the wild type to monodentate within the mutant. With the apparent lack of induction of movement caused by the proximal tyrosine in the RBR structure that occupies the same special region as Y220 in AOX, it would suggest that AOX would likely conform to an arrangement similar to that observed in the RNR-D84E mutant.

2. Initial Reaction of the Di-iron Core with Oxygen

EPR analysis with *Arabidopsis thaliana* AOX1a expressed in *E. coli* membranes provides some further insight into the possible stepwise nature of the oxidation mechanism. Berthold and colleagues detected a stable Fe^{3+}/Fe^{2+} species (Berthold et al. 2002) under oxidizing conditions suggesting that the initial binding of oxygen occurs via an 'end-on' binding mode, generating a superoxo species. It is worth noting that this mixed valence species was stable enough to detect by EPR suggesting, given the high reactivity of the superoxo species, that the terminal oxygen was reduced in a manner that does not affect the oxidation state of the metal centres.

Of particular importance in a consideration of the initial steps in the oxidation mechanism is the role of the PCET network. It has previously been demonstrated in RNR that a tryptophan radical is generated prior to the generation of the tyrosine radical (Saleh and Bollinger 2006). It is likely that this would buffer the change in oxidation state that would accompany the reaction with oxygen, spreading the otherwise highly reactive radical between the iron and the tryptophan. If such a reaction occurred it could provide the di-iron core with some degree of stereochemical control thereby allowing oxygen to distinguish which of the two irons it will react with. Mutational studies of residues flanking
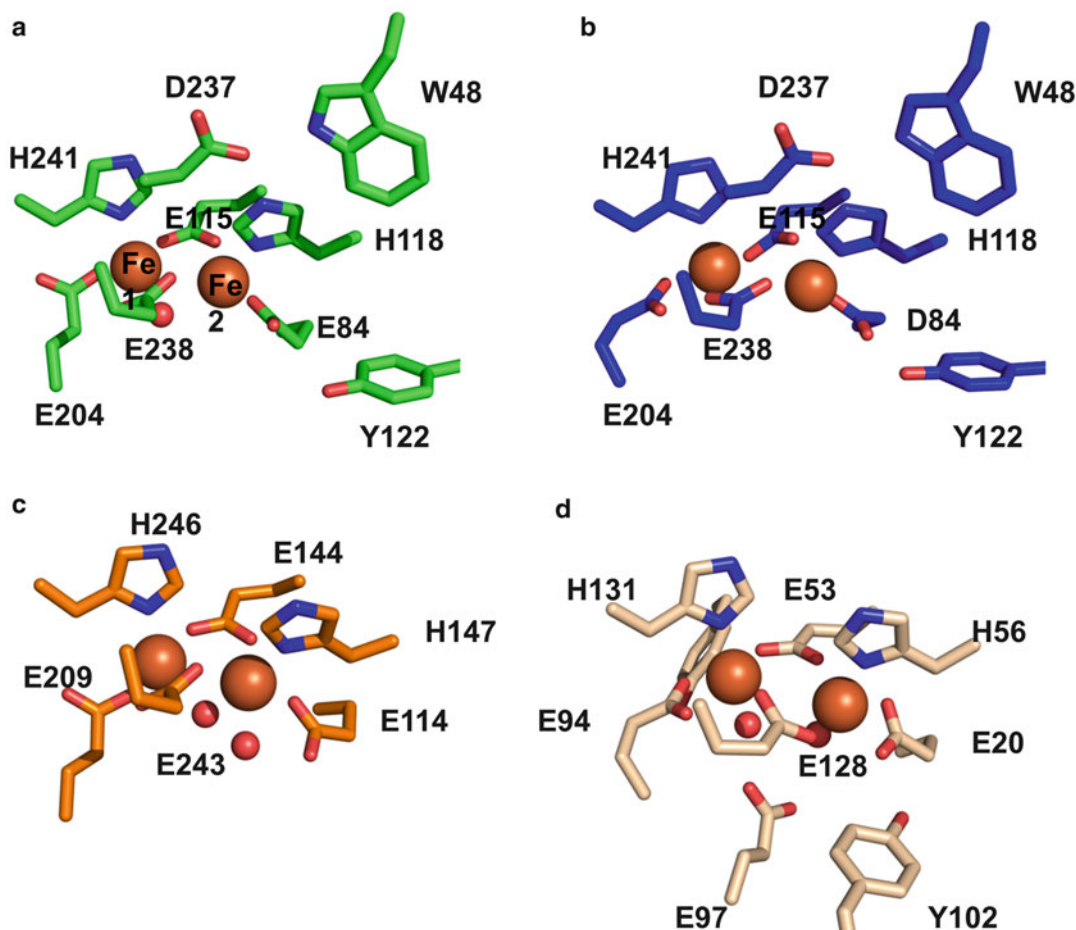
Fig. 19.8. Di-iron core of fully reduced species. (**a**): RNR-D84E mutant (PDB: 1PIM) in *green* (**b**): RNR wild type (1R56) in *blue*, (**c**): MMO (PDB:1FYZ) in *orange* and (**d**): RBR (PDB:1LKO) in wheat. Heteroatoms coloured with *blue* being nitrogen, oxygen being *red*, and iron as *orange spheres*. Numbering indicates amino acid position in respective protein.

the glutamate residue (L122A and T124A) on the PCET network side of the active site revealed that alterations within this region causes significant changes to the oxygen Km, further implicating a selective initial binding of oxygen to one of the irons within the active site (Young et al. 2014).

It is noteworthy that an Fe(III)-O-O* superoxo species has been crystallised in dioxygenase, with bond distances of 1.99 Å (Fe-O) and 1.47 Å (O-O*) (Kovaleva and Lipscomb 2007). Given an Fe(1)-Tyr-O distance of 6 Å in AOX, ($\sim$5 Å to the proton), a reactive distance of 1.5 Å is sufficient for the required orbital overlap

necessary for hydrogen atom extraction. Fe(III)O$_2\bullet^-$ has also been demonstrated to be much more reactive then its Fe(IV) = O counterpart that is part of the 'diamond core' (Cho et al. 2012) existing in the singlet state thereby permitting for spin allowed hydrogen atom abstraction from the tyrosine, as depicted in Fig. 19.9a. In RNR (Atkin et al. 1973) repeated reduction by ferricyanide and reintroduction of oxygen into the sample gave rise to a characteristic signal for a tyrosine radical at 410 nm, and similar observations have been made with AOX (A. Maréchal, P. R. Rich, and A.L. Moore, unpublished observations). While
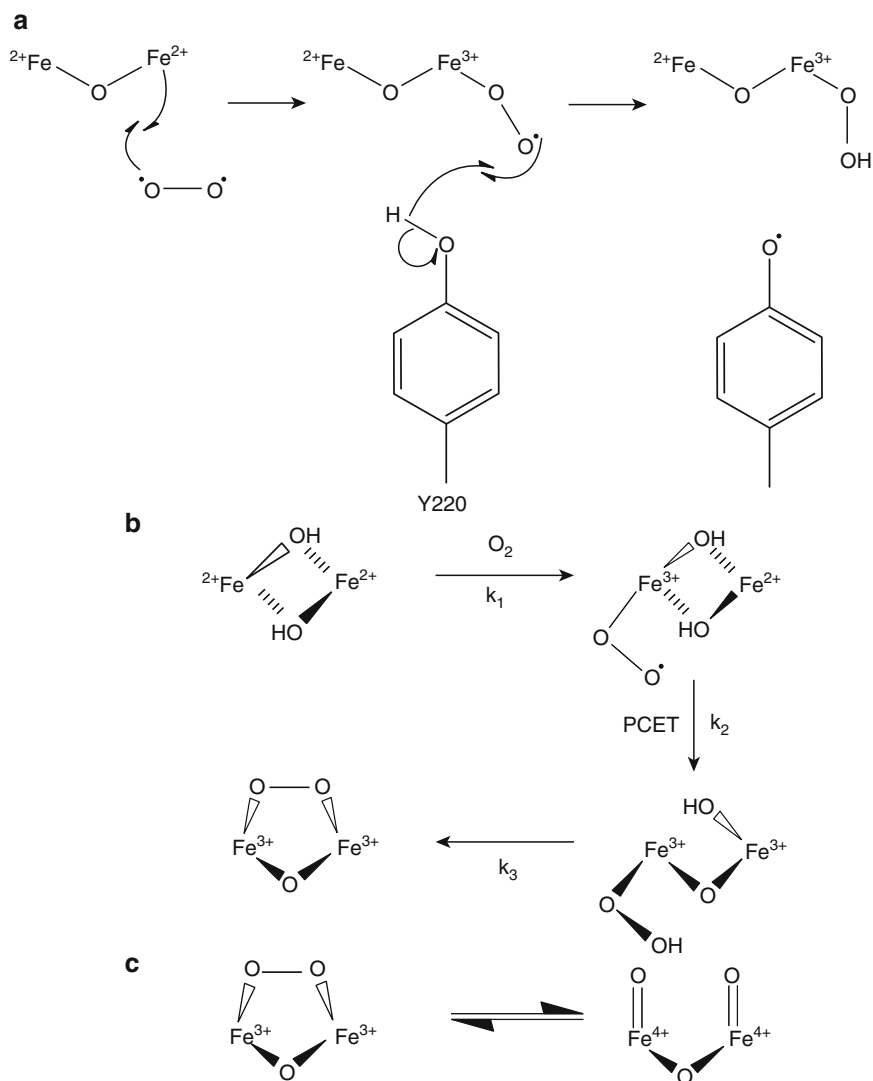
Fig. 19.9. (**a**) Initial reaction of oxygen with the di-iron core, followed by hydrogen abstraction from the neighbouring tyrosine to generate a tyrosyl radical. (**b**) Proposed oxygen binding reaction in [Fe2(μ-OH)2(6-Me3-TPA)2], adapted from (Shan and Que 2005). (**c**) Scission of the [Fe2(μ-O)(μ O-O)] unit, adapted from (Kodera et al. 2012).

such observations would suggest that the tyrosine radical is generated upon oxidation of the di-iron center, it does not necessarily imply that it occurs this early in the catalytic cycle and may indeed occur at a later point.

3. Rearrangement of the Core

Synthetic chemical systems that mimic the di-iron core present an alternative source of

information. One such system is the [Fe$_2$(μ -OH)$_2$(6-Me$_3$-TPA)$_2$](OTf)$_2$ complex, which contains a comparable hydroperoxo species as a reaction mechanism intermediate shown in Fig. 19.9b (Shan and Que 2005). The interesting aspect about this system is that it facilitated an estimation of the maximal concentration of intermediates that could be formed. For instance it was calculated that if the core rearrangement was the rate-limiting

step, the maximal observed concentration of the mixed valent species would be approximately 7 %. Interestingly this is identical to that detected within AOX by EPR (Berthold et al. 2002). Given the highly clustered nature of the active site and the necessitation of carboxylate movement to open ligation sites, it is likely that the AOX di-iron core would indeed be the rate-limiting step in this part of the mechanism.

An [Fe$_2$(μ-O)(μ O-O)] species has been shown (Kodera et al. 2012) to undergo spontaneous rearrangement between an Fe(III)/Fe(III) and Fe(IV)/Fe(IV) species via a reversible scission of the O-O bond (as shown in Fig. 19.9c). This reaction is dependent upon the surrounding acidity, with evidence that protonation of the bridging oxide increases scission rate (Kodera et al. 2012). We suggest that a potential source for protonation of the active site would be the water channels discussed earlier. The reaction could also be accelerated due to mechanical constraints on the active site; if the bridging species in the di-iron core is a carboxylate ligand, the increased energy provided by a ligand in a now unfavorable position may shift the equilibrium in favor of an Fe(IV)/Fe(IV) species. As with the previous step, the bridging oxygen is likely to be a carboxylate ligand which would affect the oxidation states of the metals, and a simple rearrangement would allow formation of the diamond core favored by other di-iron proteins (Shu et al. 1997).

Figure 19.10 depicts a potential proton-based mechanism for the rearrangement of the scissioned species to the oxo-species

using a proton-based system to liberate a molecule of water. One factor that is difficult to take into account at this time is the extent to which the primary ligation sphere affects the reaction mechanics, with respect to co-ordination saturation and electronic configuration of the metal centers. Computational examination of pKa values using PropKa (Li et al. 2005) reveals that if this rearrangement step is indeed coupled with a carboxylate shift, the acidity of the iron ligating histidine increases significantly, with a pKa decrease from 10.4 to 1.7.

It is also conceivable that this is not the route of electron transport since a shift in the ligation core may well force the reaction in a separate direction. We also have to consider that the picture given by the crystal structure does not truly represent an intermediate in the regular catalytic cycle but is rather a decomposition of the quinol reactive species, which is more stable.

C. Quinol Oxidation Mechanism

TAO has been co-crystallised with colletochlorin B and ascofuranone (Shiba et al. 2013), novel inhibitors of AOXs (Minagawa et al. 1996). The inhibitors are analogs of ubiquinone containing a fully functionalized phenol ring and an isoprene tail. Given the similarities between inhibitor and substrate, these structures add further validity to the idea of the hydrophobic cavity being the quinol-binding site. Both compounds ligate to the protein in an identical fashion, with 2-OH occupying the space between R96, R118 and T219, as depicted in Fig. 19.11.
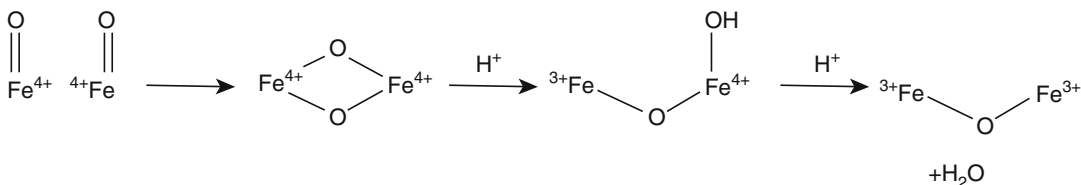

Fig. 19.10. Proposed mechanism for the formation of the quinol reactive species post scission. Formation of the diamond core that is a common intermediate within the di-iron reaction schemes, followed by successive protonation to liberate the first molecule of water. Bridging carboxylate removed for clarity.
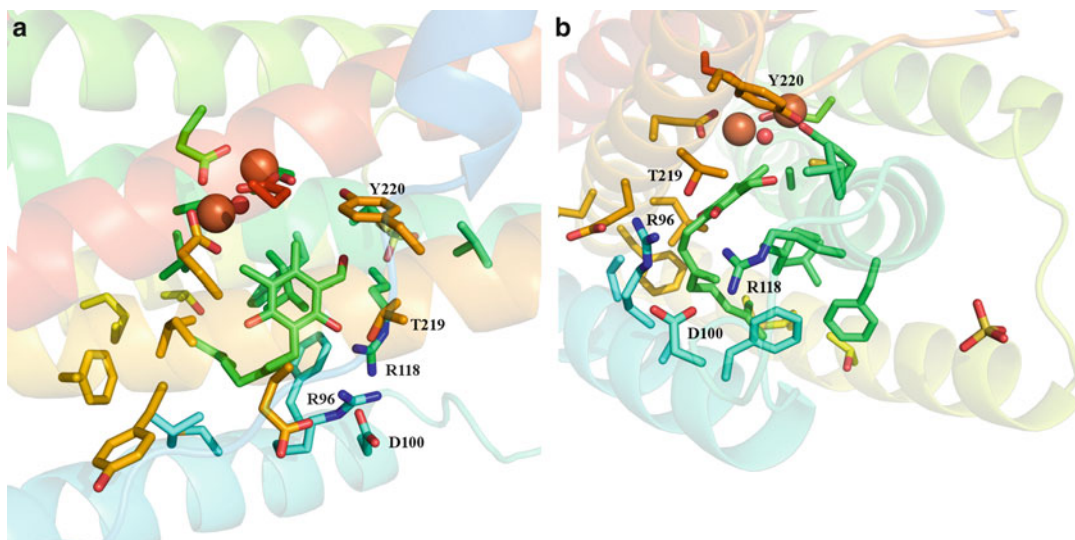
Fig. 19.11. Binding of Colletochlorin B (PDB:3w54), shown primarily in *green*. Amino acids shown are those within 6 Å of the inhibitor, with labelled amino acids indicating potential interaction with the inhibitor. Figure (**a**) depicts the head group interactions with the protein, with 2-OH within hydrogen bond distance to T219, R118 and R96. Figure (**b**) depicts the location of the isoprenoid tail.

Given the polar nature of these residues in an otherwise highly hydrophobic environment, it is highly likely they are involved in the overall reaction mechanism of the quinol-oxidation reaction. Docking studies (Seeliger and de Groot 2010) confirm the validity of the hydrophobic pocket as the substrate binding region since they suggest an identical binding pocket to that seen with the inhibitors in the crystal structure.

The important studies of Maréchal and colleagues (Marechal et al. 2009) demonstrated that the oxygen reactive species was the fully reduced form of the AOX and that reduction of the fully oxidized species occurs in a stepwise fashion of two 2 sequential electron reductions, as depicted in Fig. 19.12. From this, it can be inferred that each reduction step would correspond to a quinol:quinone oxidation step which enters the protein in a stepwise fashion as opposed to simultaneously. While it could theoretically be the case that both quinols bind at once and undergo a single electron reduction each between steps the relative instability of the semiquinone species, combined with the lack of a second substrate
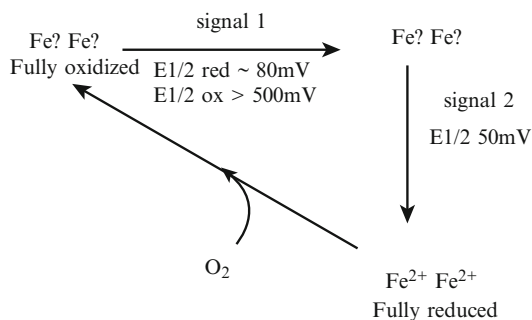
Fig. 19.12. Electrochemical studies adapted from (Marechal et al. 2009). Redox potentials in both oxidative and reductive direction quoted for signal 1, with signal 2 operating under standard reversible redox conditions.

binding site, would suggest that this is probably not the case.

Mutational studies performed on recombinant *Saurumattum guttatum* AOX within the quinol binding pocket revealed that while substitution of the arginine (R96) and aspartic acid (D100) with alanine reduces overall catalytic activity, such mutations do not completely inhibit activity suggesting that their role in the catalytic cycle is non-essential (Young et al. 2014).
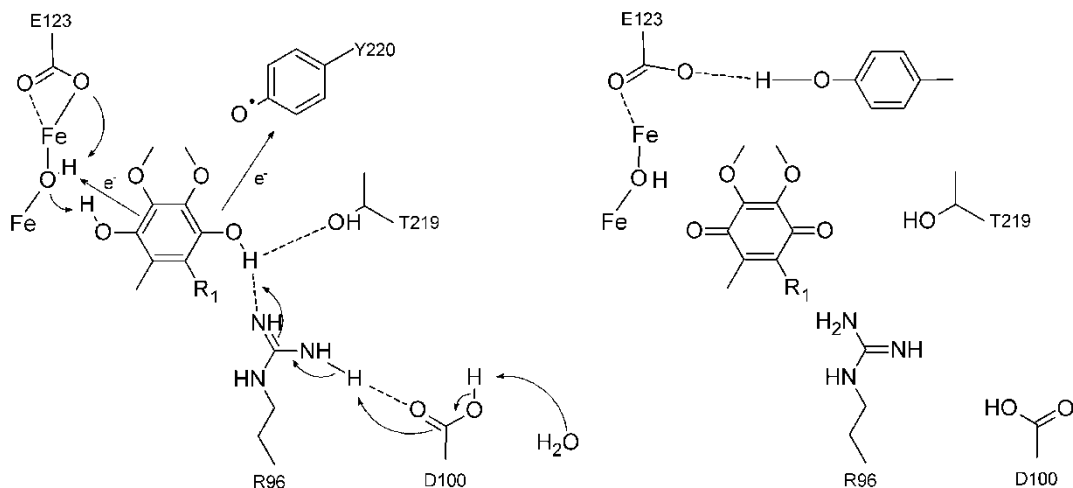
Fig. 19.13. Potential quinol oxidation mechanism based on the docked quinol species. Proton translocation towards the solvent facilitated via R96 and D100, with the second proton entering the di-iron active site, with a potential final resting place between E123 and Y220. Electrons transferred to the di-iron core and tyrosine respectively.

A possible initial reaction with quinol is depicted in Fig. 19.13. As indicated above, docking studies orientate the quinol in a similar manner to that observed for colletochlorin B and ascofuranone in the crystal structures, with one hydroxide between the tyrosine and di-iron core and the second hydroxide ligating to T219 and R96. Given the detection of the tyrosyl radical within the fully oxidized form (A. Maréchal, P.R. Rich and A.L. Moore, unpublished observations), it is likely that the radical reacts with the substrate abstracting a proton and electron from the quinol to form a neutral tyrosine and a semiquinone. While the second electron would transfer to the di-iron core, the fate of the second proton is questionable and probably dependent upon the nature of the water channels. If they do indeed transfer protons into the active site early within the reaction cycle it is feasible that the second proton is transferred out of the active site and into solvent via the arginine-aspartic acid pathway depicted in Fig. 19.13.

IV. Concluding Remarks

A full mechanism for the catalytic cycle of the alternative oxidases has been proposed. While the initial stages for reactivity are fairly well characterized from a number of different sources, the intermediates after the mixed valent species are currently implied from synthetic chemical structures designed to mimic the active site which may not necessarily give a true representation of the catalytic cycle. One of the major problems with attempts to elucidate the mechanism for AOX is its lack of solubility, preventing probing the nature of intermediates to a suitable degree.

On the basis that a peroxo species has been predicted as an intermediate within the cycle, examination of whether the system is able to reduce hydrogen peroxide should confirm whether this is a likely intermediate. Given that rubrerythrin, the closest di-iron structure to AOX, demonstrates a preference for peroxide over oxygen as a substrate (Coulter et al. 2000), it should prove interesting to probe the reactivity of this oxidase towards peroxide.

A fully reduced crystal structure would also go a long way towards elucidation of the full mechanism. While prediction would suggest that the AOX active site adopts a conformation similar to that of the RNR-D84E mutant, there may be structural movements that

we are currently unable to accurately predict. Within the plant species, EPR work on the T179A mutation has revealed a semi-quinone species (P. Heathcote and A.L. Moore, unpublished observations). While this is not necessarily a trapping of an intermediate, a crystal structure of this mutant may well help to clarify what transitional states occur within the di-iron core itself during the oxygen reduction cycle.

Acknowledgments

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Part IV

Superoxide Generation in Cytochrome *bc* Complexes

Mechanisms of Superoxide Generation and Signaling in Cytochrome *bc* Complexes

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Summary

The mechanisms of action of reactive oxygen species (ROS) in plant and animal cells, in chloroplasts, mitochondria, and other sub-cellular compartments such as the endoplasmic reticulum and nucleus, which result in deleterious effects as well as intra-organelle and metabolically significant signaling reactions, are discussed, as well as physiological effects

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induced by the signaling functions of ROS. The role of ROS in chloroplast retrograde signaling and modulation of abiotic stress response and pathogen defense is discussed. Attention is focused on mechanisms of ROS production, and particularly on the intra-organelle reactions mediated through reduction of oxygen to superoxide in the mitochondrial cytochrome bc_1 and the chloroplast b_6f complexes, for which understanding of the mechanistic details is facilitated by the existence of high resolution crystal structures. Attention is directed to the structure-based details that underlie the more than tenfold larger specific rate of superoxide production in the b_6f complex compared to that of the mitochondrial bc_1 complex. Because plastoquinol oxidation by the cytochrome b_6f complex is known to initiate chloroplast state transitions, the effect of superoxide on the intra-membrane chlorophyll protein kinase that is integral to the cytochrome b_6f complex, and which underlies the photosynthetic state transitions that regulate the distribution of light energy between the two photosystems, is considered.

I. Introduction

Superoxide anion radical ($O_2^{\bullet-}$) is a primary product of the univalent pathway of molecular oxygen (O_2) reduction. The complete reduction of O_2 to H_2O requires four electrons. Molecular oxygen, in the ground state, contains paired electron spins. Therefore, a two electron reduction reaction would involve a spin restriction. Instead, the oxygen reduction reaction is favored to proceed through a series of one-electron reduction steps leading to formation of the free radical (Cadenas 1995). The intermediates of the reaction, $O_2^{\bullet-}$, hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\bullet}$), are reactive oxygen species (ROS) that are common by-products of the aerobic metabolism of living cells. Superoxide is a short-lived molecule and, in aqueous solutions, dismutates to O_2 and H_2O_2 spontaneously or via mediation by superoxide dismutase (Miwa et al. 2008).

Mitochondria are a major source of ROS in animal cells (Marchi et al. 2012). Respiratory chain complexes I (NADH:ubiquinone oxidoreductase) and III (ubiquinol:cytochrome c oxidoreductase; cytochrome bc_1 complex) are considered to be the main producers of $O_2^{\bullet-}$ and derived ROS (Boveris et al. 1976; Cadenas et al. 1977; Kushnareva et al. 2002; Turrens and Boveris 1980). Similar ROS production linked to the respiratory electron transfer chain proceeds in plant mitochondria (Suzuki et al. 2012). However, chloroplasts are one of the most significant sources of ROS in plant cells. O_2 reduction is associated with photosynthesis and photorespiration in chloroplasts and peroxisomes, respectively (Foyer and Shigeoka 2011; Mittler et al. 2004). Production of H_2O_2 associated with photosystem I (PSI) was first described (Mehler 1951). Subsequently, the primary reduced product was identified to be superoxide anion, and its disproportionation to H_2O_2 and O_2 was demonstrated (Asada et al. 1974). By analogy to the mitochondrial bc_1 complex, $O_2^{\bullet-}$ production by the b_6f complex (plastoquinol:plastocyanin oxidoreductase) in the oxygenic photosynthetic

Abbreviations: b_p, b_n – b-type hemes on electrochemically positive, negative sides of membrane; cyt – Cytochrome; DBMIB – 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone; E_m – midpoint oxidation-reduction potential; ET – Electron transport; Fe-S – Iron-sulfur center; H_2O_2 – Hydrogen peroxide; HPV – Hypoxic pulmonary vasoconstriction; ISP – Rieske iron-sulfur protein; LHC – Light harvesting complex; NQNO – 2-n-nonyl-4-hydroxyquinoline N-oxide; n-, p-side – Electrochemically negative-, positive side of membrane; $^1O_2$ – Singlet oxygen; $O_2^{\bullet-}$ – Superoxide; PC – Plastocyanin; PEWY – Proline-glutamate-tryptophan-tyrosine; PQ – Plastoquinone; PQH_2 – Plastoquinol; PQH – Plastosemiquinone; $PQ^{\bullet-}$ – Anionic plastosemiquinone; PSI, II – Photosystem I, II; Q_p, Q_n – Quinone binding site on p-, n-side of membrane; ROS – Reactive oxygen species; TDS – Tridecyl-stigmatellin; UQ – Ubiquinone; UQH_2 – Ubiquinol; $UQ_2^{\bullet-}$ – Anionic ubisemiquinone

electron transport chain was proposed (Cape et al. 2006; Cramer et al. 2008), and was subsequently demonstrated quantitatively (Baniulis et al. 2013; Sang et al. 2011).

In addition to respiratory and photosynthetic electron transport systems, ROS production in the endoplasmic reticulum and nucleus has been reported (Ashtamker et al. 2007). Animal and plant cells contain additional $O_2^{\bullet}$ producing enzymes, such as NADPH oxidase, which generates ROS mostly in the extracellular space outside the plasma membrane (Jiang et al. 2011; Marino et al. 2012).

Traditionally, ROS have been regarded as cytotoxic agents that cause oxidative stress and molecular damage. Biochemical pathways including ROS scavenging compounds, such as ascorbate and glutathione, and enzymes including superoxide dismutase, catalase, glutathione reductase that are important for ROS neutralization have been described (Apel and Hirt 2004; Foyer and Shigeoka 2011; Moller et al. 2007). More recently, however, it has been recognized that ROS act not only as toxic compounds but also as signaling molecules associated with responses to abiotic and biotic stress in animals, plants, and microbes (Apel and Hirt 2004; Foyer and Shigeoka 2011; Mittler et al. 2004, 2011; Suzuki et al. 2012). ROS also constitutes a central part of the oxygen sensing mechanism in mitochondria of animal cells, and might be important in a similar capacity in plant cells (Bell et al. 2007; Sasidharan and Mustroph 2011; Schumacker 2014).

A versatile role was disclosed for ROS produced in chloroplasts. Initially, it was regarded as a regulator of activity of the photosynthetic electron transport chain through the balance between different photosynthetic reactions, and a role in excess energy dissipation was investigated. A signaling role for ROS in retrograde signaling from chloroplasts to the nucleus was also demonstrated to be involved in regulation of plant responses to environmental stress, and transcriptional regulation

of photosynthesis (Galvez-Valdivieso and Mullineaux 2010; Nott et al. 2006). ROS produced in chloroplasts has been shown to play a role in triggering a plant response to pathogen infection (Kangasjarvi et al. 2014).

Evidence for $O_2^{\bullet}$ production and its physiological relevance has been presented for *bc* complexes of respiratory and photosynthetic electron transport chains. The two protein complexes catalyze analogous ubiquinol/plastoquinol oxidation reactions linked to electron transport and proton pumping pathways defined in the Q-cycle model proposed by Mitchell (Cramer et al. 2011; Mitchell 1975a, b, 1976; Trumpower 2002).

$O_2^{\bullet}$ production is manifested in *bc* complexes as a bypass reaction of the bifurcated two electron oxidation reaction implicated in Q-cycle electron transfer. Much of the experimental evidence for $O_2^{\bullet}$ production has been presented for the mitochondrial bc_1 complex (Cape et al. 2007; Drose and Brandt 2008, 2012; Forquer et al. 2006; Muller et al. 2002, 2003; Sarewicz et al. 2010; Sun and Trumpower 2003). Similar Q-cycle bypass reactions occur in the b_6f complex (Baniulis et al. 2013; Sang et al. 2011). Despite the fact that general principles of the Q-cycle reactions are considered to be well established [See additional perspective in this volume by Cramer and Hasan], the specific sequence of oxidation-reduction events leading to $O_2^{\bullet}$ production remains a subject of debate (Cape et al. 2006; Rutherford et al. 2012). In addition, many unsolved problems exist for the physiological role of $O_2^{\bullet}$ produced in *bc* complexes. Especially vague is the understanding of the function of $O_2^{\bullet}$ produced in the photosynthetic b_6f complex. Although the general mechanism of Q-cycle bypass reactions may be similar in bc_1 and the b_6f complexes, the surrounding environment and enzymatic reactions, such as oxygen concentration, compartmentalization of reactions, and functional significance of the complexes to other cellular sources of ROS, suggest the possibility of unique functions for the $O_2^{\bullet}$ generated by the b_6f complex.
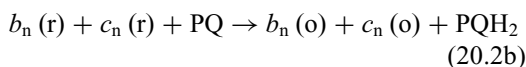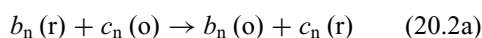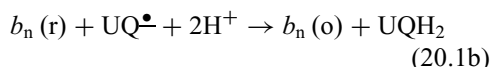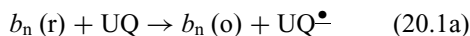
II. Mechanisms of Superoxide Generation in Cytochrome *bc* Complexes

The mitochondrial Q-cycle involves ubiquinol oxidase activity at the Q_p site of the bc_1 complex and ubiquinol reductase activity at the Q_n site (Crofts and Berry 1998). The analogous reaction of plastoquinol oxidation and reduction proceeds at *n*- and *p*-side sites of the b_6f complex (Cramer et al. 2011). It has been proposed that $O_2^{\bullet-}$ is produced as a bypass reaction of the Q-cycle in the bc_1 complex (Cape et al. 2006). While analogous oxido-reductase functions and structural similarity of the b_6f complex suggest a similar mechanism for $O_2^{\bullet-}$ generation, the unique structural properties of the latter complex may have resulted in differences in the mechanism and control of the reaction (Baniulis et al. 2013).

A. Electron Transfer at the Q_n Site

Different electron transfer reactions might function for the mitochondrial and chloroplast *bc* complexes at the negative side of the complex where the ubiqinol/plastoquinol reduction reaction takes place. One electron reactions would involve formation of an intermediate, plastosemiquinone, analogous to the reaction in the bc_1 complex. Two step ubiquinone reduction reactions proposed for the bc_1 complex are described in reactions 20.1a and 20.1b. Ubisemiquinone stabilization at the Q_n site of the bc_1 complex has been demonstrated (Dikanov et al. 2004), and it is believed that this prevents Q-cycle bypass reactions and reduction of O_2. Structural differences at the Q_n site of the b_6f complex involves an additional prosthetic group, heme c_n, which overlaps Q_n in the bc_1 complex (Kurisu et al. 2003). One side of the central heme Fe of heme c_n, 4 Å from the propionate of heme b_n (pdb 1VF5, updated to pdb 4OGO), with which it shares electrons (Zatsman et al. 2006) and ligates the Q_n (Hasan et al. 2013). These properties provide a possibility for different mechanisms

of PQ reduction reaction including the possibility of a two electron exchange with quinone/quinol. Two-electron reduction of PQ could be mediated by the two hemes present at the negative side of the complex (reactions 20.2a and 20.2b). The latter mechanism would prevent $O_2^{\bullet-}$ formation by the b_6f complex in spite of the hyperoxic conditions in oxygenic photosynthetic membranes.

$$b_n\,(r) + UQ \rightarrow b_n\,(o) + UQ^{\bullet-} \qquad (20.1a)$$

$$b_n\,(r) + UQ^{\bullet-} + 2H^+ \rightarrow b_n\,(o) + UQH_2 \qquad (20.1b)$$

$$b_n\,(r) + c_n\,(o) \rightarrow b_n\,(o) + c_n\,(r) \qquad (20.2a)$$

$$b_n\,(r) + c_n\,(r) + PQ \rightarrow b_n\,(o) + c_n\,(o) + PQH_2 \qquad (20.2b)$$

B. Electron Transfer at the Q_p Site and Q-Cycle Bypass Reactions of Oxygen Reduction

At the Q_p site of bc_1 complex, ubiquinol is oxidized in a bifurcated reaction that leads to reduction of the [2Fe-2S] iron-sulfur cluster of the Rieske iron-sulfur protein (ISP) (high potential chain) and heme b_p of cyt *b* in the low potential chain. While the overall principles of the reaction at the Q_p site are well established, several theories on the mechanism of ubiquinol oxidation at the Q_p site of the mitochondrial bc_1 complex have been proposed (Bleier and Drose 2013; Crofts et al. 2013; Xia et al. 2013). Single occupancy models propose simultaneous or sequential electron transfer to the [2Fe-2S] and heme b_p, electron acceptors, while the double-occupancy model involves a cooperation of ubiquinone molecules at the Q_p site during the ubiquinol oxidation reaction.

During the bifurcated sequential electron transfer reaction of ubiquinol oxidation at the Q_p site, a limiting first electron transfer to the FeS center of the Rieske

ISP (reaction 20.3a) generates a neutral ubisemiquinone intermediate that interacts with glutamate residue (Glu87 in cyanobacterial and Glu271 or Glu272 in the mitochondrial bc_1 complex) of the conserved Pro-Glu-Trp-Tyr (PEWY) sequence (Widger et al. 1984) of the cyt *b* subunit (Crofts 2004; Crofts et al. 2013). The interaction is essential for reduction of heme b_p and electron transfer to the low potential chain (reaction 20.3b) as the glutamate residue is proposed to mediate proton delivery to the p-side exit channel by rotational displacement (Crofts et al. 1999b; Yu et al. 2009; Zhang et al. 2001, 2003; Zito et al. 1998) and opens up a niche for the closer access of anionic ubisemiquinone to heme b_p. It has been suggested that the movement of semiquinone to the proximal domain facilitates rapid electron transfer and insulates it from further reaction with the reoxidized Rieske ISP, or with O_2 (Crofts et al. 1999a). Results of kinetic studies using yeast mutants were inconsistent with this sequential semiquinone mechanism and supported the model in which both electrons are transferred simultaneously from UQH_2 located at the same domain of the Q_p site (Covian and Trumpower 2009). However, the formation of $UQ^{\bullet-}$ has been demonstrated by Cape et al. (2007) and the role of the glutamate residue has been supported by later studies using *Rh. sphaeroides* Glu-295 mutants (Cape et al. 2007; Victoria et al. 2013; Zhang et al. 2007).

$$UQH_2 + Fe - S(o) \rightarrow UQ^{\bullet-} + FeS(r) + 2H^+ \tag{20.3a}$$

$$UQ^{\bullet-} + b_p(o) \rightarrow UQ + b_p(r) \tag{20.3b}$$
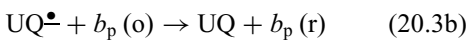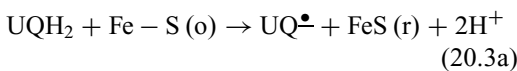
Because ubisemiquinone is a reductant of the low potential heme b_p in the bc_1 complex, it has been inferred that the $UQ^{\bullet-}$ formed at the Q_p site during UQH_2 oxidase activity has a sufficiently reducing potential to form $O_2^{\bullet-}$ in (reaction 20.4) (Trumpower and Gennis 1994). The midpoint redox potential for ubiquinone/quinol is $E_{m7} = +$ 60 mV, (Ohnishi and Trumpower 1980). $O_2^{\bullet-}$ can be formed through a one electron reduction of the oxygen molecule, with a midpoint redox potential $= -0.14$ V (reaction 20.5) (Wood 1988).

$$UQ^{\bullet-} + O_2 \rightarrow UQ(o) + O_2^{\bullet-} \tag{20.4}$$

$$O_2 + e^- \rightarrow O_2^{\bullet-} \tag{20.5}$$

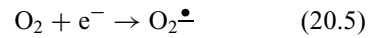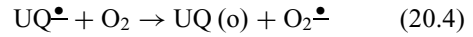A model for formation of $O_2^{\bullet-}$ at the Q_p site involving the semiquinone intermediate was first proposed by (Turrens et al. 1985). Subsequently, $O_2^{\bullet-}$ production by the bc_1 complex was characterized in vitro (Cape et al. 2007; Drose and Brandt 2008, 2012; Forquer et al. 2006; Muller et al. 2002, 2003; Sarewicz et al. 2010; Sun and Trumpower 2003). The latter studies revealed that $O_2^{\bullet-}$ producing activity is effectively controlled under non-inhibited conditions for electron transport. The Q-cycle bypass reaction is activated by *n*-side inhibitors of the bc_1 complex, such as antimycin A, or by an over-reduced state of the electron transfer chain (Fig. 20.1a). When the low-potential electron transport chain is blocked, an unstable semiquinone species can escape from the reaction, or oxygen can diffuse into the reaction site, forming superoxide. It was demonstrated that destruction of protein structural integrity by heat inactivation or proteinase K digestion leads to increased production of $O_2^{\bullet-}$, suggesting that "loosening" of structural integrity may facilitate access of molecular oxygen to the Q_p site or the release of produced $O_2^{\bullet-}$ to the aqueous medium (Yin et al. 2010).

Based on the model of consecutive one electron oxidation of UQH_2, it is inferred that $UQ^{\bullet-}$ is formed as an intermediate of the reaction that follows reduction of Fe-S cluster of ISP. Under inhibited conditions, transfer of electrons to the low-potential chain would be hindered and oxidation of ubiquinone would result in accumulation of the $UQ^{\bullet-}$ at the Q_p site (Crofts et al. 2013). The formation of $UQ^{\bullet-}$ is followed by O_2 reduction. However, recent studies have demonstrated that $O_2^{\bullet-}$ production from the Q_p site is stimulated by
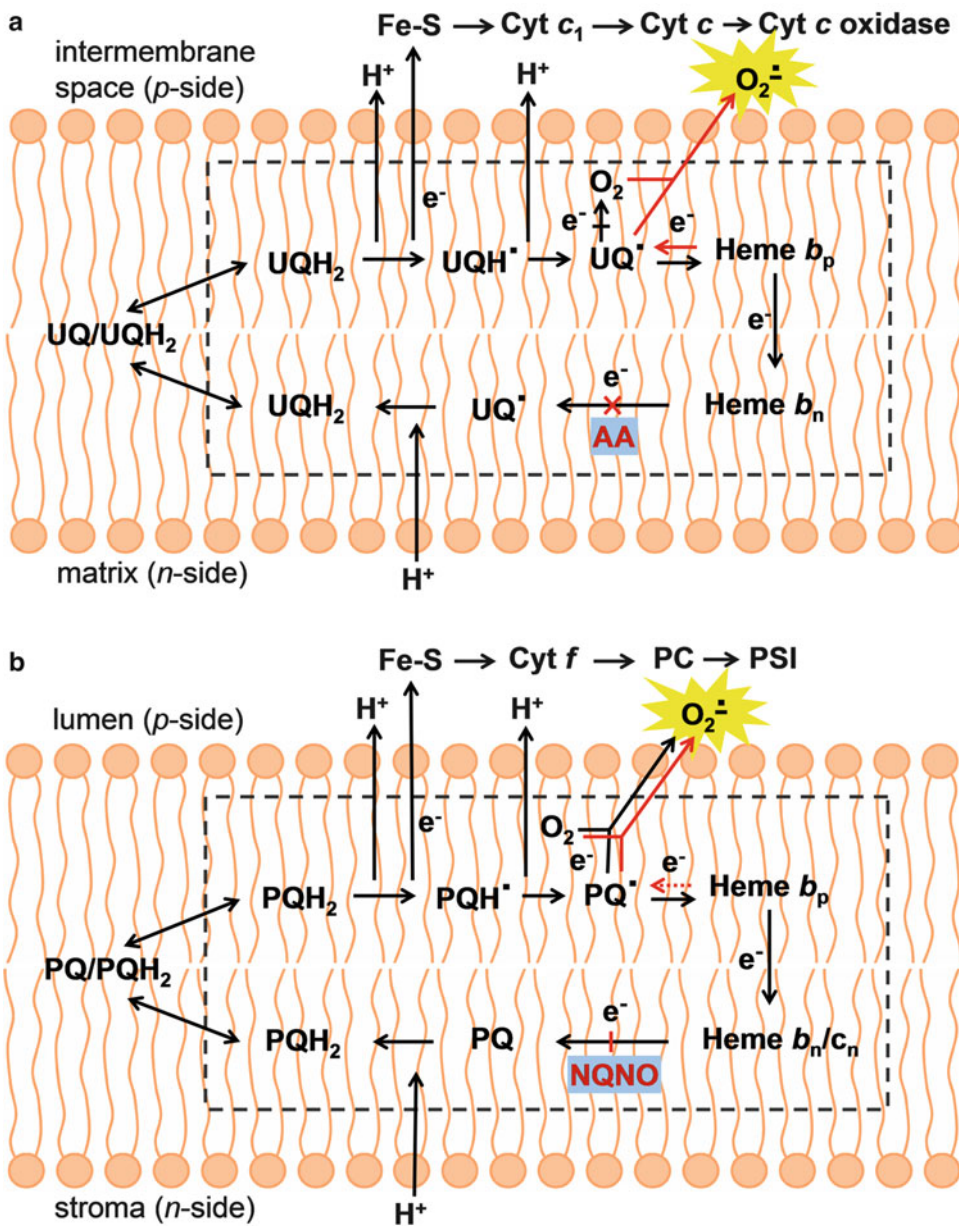
Fig. 20.1. Superoxide producing Q cycle bypass reactions in cytochromes *bc*. *Black and red arrows* indicate Q cycle reactions under uninhibited conditions (reactions 20.1a, 20.1b, 20.2a, 20.2b, 20.3a and 20.3b described in text) and in presence of *n*-side inhibitor, respectively, for cyt bc_1 (**a**) and cyt b_6f (**b**) complexes. Reaction stoichiometry is not represented in the figure and could be obtained from the corresponding reactions presented in text. Oxygen reduction by $UQ^{\bullet}$ intermediate (reaction 20.4) is largerly suppressed under ininhibited conditions for the cyt bc_1 and Q-cycle bypass reaction is activated by *n*-side inhibitor antimycin A (**a**). Accumulation of $UQ^{\bullet}$ due to over-reduced state of the low potential electron transfer chain or through a backward Q-cycle reaction, that involves oxidation of the quinone by heme b_p (reaction 20.6), leads to formation of $O_2^{\bullet}$ (reaction 20.4). The $O_2^{\bullet}$ production equivalent to approx. 2 % of overall ET is sustained in uninhibited cyt b_6f complex (**b**). It is proposed that due to structural differences at the Q_p-site, the life-time of the neutral semiquinone intermediate is expected to be larger and would be important for the enhanced superoxide production (reaction 20.4). Inhibition by *n*-side inhibitor NQNO has small effect on linear ET and has no significant effect on $O_2^{\bullet}$ production, therefore the role of the over-reduced state of the electron transfer chain and "*semireverse*" pathway (shown as *dashed arrow*) in the Q-cycle bypass reaction remains unconfirmed.

the presence of oxidized ubiquinone, suggesting that the dominant reaction for electron transfer to O_2 via ubiquinone might be initiated by reduction of the UQ by heme b_p (reaction 20.6) (Borek et al. 2008; Drose and Brandt 2008; Rutherford et al. 2012; Sarewicz et al. 2010). The reverse Q-cycle reaction leads to formation of $UQ^{\bullet-}$ that is followed by the O_2 reduction reaction (reaction 20.4).

$$b_p\,(r) + UQ \rightarrow b_p\,(o) + UQ^{\bullet-} \qquad (20.6)$$
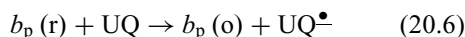
Different mechanisms were also proposed for the control of the Q-cycle bypass reaction leading to O_2 reduction that is largely suppressed in the non-inhibited bc_1 complex (Cape et al. 2006). In agreement with different models of UQH_2 oxidation at Q_p site, the control mechanisms could involve (1) control of stability of the $UQ^{\bullet-}$ anionic semiquinone (Junemann et al. 1998; Link 1997), (2) formation of the reaction intermediates that support the oxidation reaction of heme b_p (Cooley et al. 2004; Crofts et al. 1999a; Rich 2004), (3) a structural barrier that prevents diffusion of O_2 into the reaction site or $UQ^{\bullet-}$ into surrounding media (Yin et al. 2010), (4) a gating mechanism (Mulkidjanian 2005; Osyczka et al. 2005), or (5) overall avoidance of formation of the semiquinone intermediate (Ding et al. 1995).

C. Unique Superoxide Production Mechanism of b₆f Complex

A high rate of $O_2^{\bullet-}$ production by the cyt b_6f complex of oxygenic photosynthesis has been demonstrated experimentally under in vitro conditions using isolated b_6f complex (Baniulis et al. 2013; Sang et al. 2011). Based on structural analogy with the cytochrome bc_1 complex, it is presumed that bifurcated Q-cycle reactions following oxidation of PQH_2, at the Q_p site of the b_6f complex, are analogous to the Q_p site reactions of the bc_1 complex (reactions 20.1a, 20.1b, 20.2a, 20.2b, 20.3a and 20.3b) (Cramer et al. 2011). While this is supported by observations for Q-cycle

reactions involved in photosynthetic electron transfer, considerable differences may be present when Q-cycle bypass reactions that involve reduction of O_2 are considered (Fig. 20.1b). Experiments with isolated yeast mitochondrial cytochrome bc_1 complex and b_6f complex from cyanobacterial thylakoid membranes demonstrated a greater than one order of magnitude higher $O_2^{\bullet-}$ production rate by the b_6f complex compared to the mitochondrial bc_1 complex (Baniulis et al. 2013). Such a high rate of $O_2^{\bullet-}$ production in the b_6f complex could, in principle, be a consequence of a more negative redox potential of heme b_p which, however, is not supported by existing redox titration data, or an increased residence time of the $PQ^{\bullet-}$ in its binding niche near the 2Fe-2S cluster of the Rieske ISP.

Regarding redox potentials, there is appreciable variation in E_m values of the *b* hemes in cyt *bc* complexes (e. g., (Baymann et al. 1999)), which are pH-dependent, dependent on heme exposure to solvent (Kassner 1972), and on the presence of uncoupling agents (Böhme and Cramer 1973). For the isolated yeast bc_1 complex, the E_m of heme b_p is -90 mV (Brandt and Trumpower 1994). There is a large variation in the literature of midpoint redox potentials for heme b_p in the isolated b_6f complex, which may be partly attributed to a difference between titrations done in vitro with isolated b_6f complex compared to those done with thylakoid membranes. Reported E_m values are: -90 mV, pH 7 (Böhme et al. 1971); -172 mV, pH 6.5 (Gwak et al. 1987); -80 mV, pH 6 (Rich and Bendall 1980); -158 mV, pH 8.0 (Hurt and Hauska 1982). Thus, it seems unlikely that a difference in redox potentials is responsible for the difference in efficiency of superoxide formation.

According to the model of a sequential one-electron quinol oxidation reaction at the Q_p site of the bc_1 complex (Crofts et al. 2013), the one-electron oxidation of PQH_2 leads to formation of $PQ^{\bullet-}$, and this would be followed by generation of $O_2^{\bullet-}$ in the Q-cycle bypass reaction (reaction 20.5). The high rate of $O_2^{\bullet-}$ production implies that the

rate constant for the one-electron oxidation of PQH$_2$ leading to O$_2$ reduction by PQ$^{\bullet-}$ is increased relative to that for electron transfer from UQ$^{\bullet-}$ to heme b$_p$ in the bc_1 complex. The source of this difference can be PQ$^{\bullet-}$ generated in the b_6f complex having a longer life-time in the Q$_p$ pocket relative to UQ$^{\bullet-}$ in the bc_1 complex. A suggested structure-based cause of this longer residence time may be the partial occlusion of the Q$_p$-portal for quinone entry/exit by the phytyl chain of the unique chlorophyll *a* molecule embedded in each monomer of the complex (Hasan et al. 2013). However, this occlusion would not affect the overall rate of electron transfer through the b_6f complex and the isolated b_6f complex supports electron transfer rates (approx. 250–300 s^{-1} for spinach and cyanobacterium *M. laminosus* b_6f complexes) comparable to the activity of the bc_1 complex from yeast (approx. 150 s^{-1}) (Baniulis et al. 2013).

As described earlier, the neutral semi-quinone species bound within the Q$_p$ site of cytochrome bc_1 complex undergoes deprotonation via the Glu residue of the conserved PEWY motif (Widger et al. 1984) to form an anionic semiquinone, which can be an electron donor to the low potential chain containing the *b* hemes (Crofts et al. 2013). It has been suggested that Glu78 in subunit IV of the b_6f complex is similarly involved the second electron transfer in the b_6f complex. However motion of the Glu78 side chain is expected to be restricted due to the interaction with Arg87 (Hasan et al. 2013). It has previously been reported that the conserved Arg87 of trans-membrane helix B in the cytochrome b_6 polypeptide (b_6f complex) is replaced by a small uncharged residue, such as Ala84 (or Ala83), in the cytochrome *b* (bc_1 complex) (Widger et al. 1984)*. In the crystal structure of the b_6f complex, the Glu78 side chain is found to be distant from the quinone analogue inhibitor TDS and it is found in a heme b_p-proximal position, in which it interacts with the Arg87 over a short distance of 2.8 Å (Hasan et al. 2013). The TDS-distal orientation of the Glu78 side chain in the cytochrome b_6f complex is in

contrast to the position of the homologous Glu271 in the cytochrome bc_1 complex, where the Glu271 side chain is oriented toward the quinol analog stigmatellin bound within the Q$_p$-site (Esser et al. 2004). It is suggested that this interaction restricts motion of Glu78, thereby making proton translocation a less efficient process. As a consequence, the life-time of the neutral semiquinone would be larger within the Q$_p$-site of the cytochrome b_6f complex. This mechanism would be important for enhanced superoxide production assuming a one-electron oxidation of PQH$_2$ takes place at the Q$_p$ site of the b_6f complex, and that it is involved in superoxide production (reaction 20.4) (Baniulis et al. 2013). Another mechanism for formation of semiquinone through a backward Q-cycle reaction would involve oxidation of the semiquinone by heme b_p as proposed for bc_1 complex, and return of the electron to heme b_p, a "*semireverse*" reaction (reaction 20.6). As shown for the Q$_n$ site of the bc complexes, it cannot be excluded that the Q$_p$ site of the b_6f complex might have unique structural and functional aspects that lead to differences in electron transfer, as well as a Q-cycle bypass reaction mechanism. Therefore, in the absence of experimental evidence, it is suggested that either mechanism of formation of the PQ$^{\bullet-}$ intermediate could be involved in O$_2$ reduction that takes place at the Q$_p$ site of the b_6f complex, and this might be associated with unique functions involved in cell signaling.

In the case of the bc_1 complex, only negligible quantities of O$_2^{\bullet-}$ are produced by the enzyme in the uninhibited state, meanwhile O$_2^{\bullet-}$ producing activity is a result of over-reduction of the low potential chain (that could also be invoked by application of *n*-side inhibitors of the bc_1 complex, such as antimycin A) and would signal the reduced state of the electron transfer chain. For the b_6f complex, O$_2^{\bullet-}$ is produced at rates that constitute a significant part of uninhibited electron transfer rate and accumulation of substantial amounts of ROS in lumen of thy-lakoids would be associated with high photo-

synthetic electron transfer activity. An effect of over-reduced state of the photosynthetic electron transfer chain on $O_2^{\bullet-}$ production could thus far not be confirmed explicitly (Baniulis et al. 2013).

The study of the Q-cycle bypass reactions in the b_6f complex has been impeded due to absence of an *n*-side inhibitor comparable in efficacy to that of antimycin A used in studies with bc_1 complex. A quinone binding site homologous to that utilized by antimycin is altered by the presence of the heme c_n, unique to the b_6f complex, whose presence would hinder interactions with an *n*-side quinone analogue inhibitor such as antimycin (Hasan et al. 2013; Kurisu et al. 2003). Another quinone analogue inhibitor, NQNO, binds specifically to the *n*-side heme c_n (Yamashita et al. 2007), and inhibits the oxidation of heme b_n (Furbacher et al. 1989; Jones and Whitmarsh 1988; Rich et al. 1991). However, unlike the inhibition of the respiratory chain that results from the action of antimycin A, the inhibition of linear ET in the b_6f complex is small (Jones and Whitmarsh 1988) and does not have a significant effect on $O_2^{\bullet-}$ production under in vitro conditions (Baniulis et al. 2013). An increase in ROS production in the presence of NQNO was demonstrated for cyanobacteria in vivo, but the mechanism that leads to this effect and the implications for $O_2^{\bullet-}$ producing activity at the Q_p site are unknown (Brantmier 2013).

III. Signaling Function of ROS Produced by Cytochrome *bc* Complexes

A. Role of Superoxide Produced in the bc$_1$ Complex in Regulation of Mitochondrial Function

Lipid membranes are highly impermeable to the charged superoxide anion (Takahashi and Asada 1983). Therefore $O_2^{\bullet-}$ produced from sources including complex I, pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase and electron transfer proteins may accumulate in the mitochondrial matrix

(Brand 2010). It is notable that alongside glycerol-3-phosphate dehydrogenase, the Q_p site of the bc_1 complex is the major source for $O_2^{\bullet-}$ accumulation in the intermembrane space of mitochondria (Brand 2010). It could also be expected that $O_2^{\bullet-}$ produced in the intermembrane space could also be released to the cytoplasm through voltage-dependent anion channels (Han et al. 2003) or other outer membrane channels such as the translocase of the outer membrane (Perry et al. 2008) and the peptide-sensitive channel (Henry et al. 1996). $O_2^{\bullet-}$ can also be scavenged by cytochrome *c* that is electrostatically bound at the surface of the inner mitochondrial membrane (Lenaz 1988). In addition, reaction with nitric oxide would yield peroxynitrite, another reactive species important in cell signaling (Liaudet et al. 2009). However, most of the $O_2^{\bullet-}$ could be expected to dismutate to H_2O_2 spontaneously or at rates higher by three orders of magnitude through the superoxide dismutase catalyzed reaction in the intermembrane space and cytoplasm (Miwa et al. 2008). Due to the short lifetime of the $O_2^{\bullet-}$ the implications of the separation of $O_2^{\bullet-}$ production and transfer between different mitochondrial compartments and the cytoplasm for cell signaling pathways are unclear. It is possible that superoxide signaling generated by the bc_1 complex is mostly mediated by H_2O_2 or nitric oxide. Under hypoxia conditions, H_2O_2 has been shown to mediate mitochondrial signaling that involves $O_2^{\bullet-}$ production at the Q_p site of the bc_1 complex (Chandel et al. 1998).

Generation of ROS from the bc_1 complex in mammals (including humans) is increased under hypoxia conditions (Chandel et al. 1998). The molecular reactions that follow the increase in ROS generation under low O_2 conditions are not fully understood. However, it has been suggested that ROS produced by the bc_1 complex is involved in stabilization of Hypoxia-Inducible Factor-1α (HIF-1) and the vasoconstriction response to alveolar hypoxia in the lungs (Waypa and Schumacker 2005). In addition, it has been demonstrated that low concentrations of ROS

are involved in protective mechanisms during the ischemic preconditioning that diminishes cardiac damage by ischemia reperfusion injury (Penna et al. 2009).

Production of ROS was shown to be important as an O_2 sensor to trigger a physiological response under hypoxic conditions (Schumacker 2014). Genetic studies using knockdown mutants and application of inhibitors of the Q_p site of the bc_1 complex linked the response to $O_2^{\bullet-}$ producing activity of the bc_1 complex (Bell et al. 2007; Brunelle et al. 2005; Chandel et al. 2000; Mansfield et al. 2005). The molecular basis through which lower O_2 concentrations increased the $O_2^{\bullet-}$ production at the Q_p site is unknown. In addition, other mitochondrial sources of ROS could be involved in the response to hypoxic conditions (Chandel 2010; Paddenberg et al. 2003). A model has been proposed in which hypoxia induces increased generation of ROS within mitochondria resulting in activation of signaling cascades leading to: (1) a decrease in Na^+/K^+ ATPase activity that decreases cellular demand for O_2 and diminishes the risk of cells reaching anoxia; (2) accumulation of HIF-1 protein which leads to transcriptional up-regulation of vascular endothelial growth factor that stimulates angiogenesis and ensures a better supply of O_2.

As part of the mechanism of control of O_2 concentration in body tissues, mitochondrial ROS might be implicated in hypoxic pulmonary vasoconstriction (HPV) (Sylvester et al. 2012). The process is mediated though an increase of intracellular calcium concentration in the pulmonary artery smooth muscle cells and evokes constriction of pulmonary arteries that is important for maintaining ventilation-perfusion ratio and blood oxygenation during hypoxia. Several mechanisms have been proposed for the O_2 sensing required to initiate HPV (Connolly and Aaronson 2010), including the suggestion that reduced O_2 levels cause a fall in ROS production and a shift of cells to a more reduced state that triggers calcium-mediated HVP response (Weir and Archer 1995), However, recent evidence suggests that acute hypoxia leads to an increase in mitochondrial ROS production (Waypa et al. 2006, 2010). In this case, $O_2^{\bullet-}$ released from the bc_1 complex diffuses into the cytosol, triggering a calcium release from sarcoplasmic reticulum and secondary Ca^{2+} entry through sarcolemmal store-operated Ca^{2+} channels into the cytosol (Wang et al. 2012) and the HPV response of smooth muscle cells (Waypa et al. 2013).

$O_2^{\bullet-}$ and H_2O_2 generated by electron transfer complexes of mitochondria might play a role in the triggering of cardioprotection by heart pre-conditioning (Baines et al. 1997; Tritto et al. 1997). Although the mechanism of redox signaling involved in preconditioning is still not completely understood, it was demonstrated that cardioprotection is mediated by mitochondrial ATP-sensitive potassium channels and mitochondrial ROS. Mitochondrial ROS signaling that occurs during the early phase of reperfusion was shown to be necessary for effective protection of the preconditioned heart (Hausenloy et al. 2007; Liu et al. 2008).

Concerning plant mitochondria, a similar signaling function might be expected to be involved in hypoxic stress of plant cells that is induced by water flooding or high metabolic activity. Although the mechanism of oxygen sensing involved in response to such hypoxic conditions is unknown, an increase in ROS accumulation is observed under hypoxic conditions (Ershova et al. 2011), and a role for ROS in oxygen sensing mechanisms has been proposed (Sasidharan and Mustroph 2011).

B. Signaling Role of $O_2^{\bullet-}$ in Chloroplasts

Chloroplasts, on the other hand, are plant cell organelles specialized for light energy transduction. In addition, the components of the electron transport system can function as mediators of environmental signals (Pfannschmidt et al. 2001). High irradiance and photosynthetic activity create hyperoxic conditions in chloroplasts. Therefore, O_2 reduction in chloroplasts is inevitable and leads to the formation of ROS. In

chloroplasts, O_2 photoreduction is estimated to be about 5–27 % of the total electron flow (Cakmak and Marschner 1992) and the organelle is a major source of ROS in plant cells (Foyer and Shigeoka 2011).

The major sources of ROS generated in chloroplasts are PSII ($^1O_2$) and PSI ($O_2^{\bullet-}$) (Asada et al. 1974; Telfer et al. 1994; Hideg et al. 1998). In addition, $O_2^{\bullet-}$ production in vivo by the b_6f complex has been documented (Horn 2004), and the activity has been characterized in vitro (Baniulis et al. 2013; Sang et al. 2011). The accumulated $O_2^{\bullet-}$ is metabolized to a more stable form of ROS H_2O_2, as well as other reactive species such as hydroxyl radical or peroxinitrite, the latter in the presence of iron ions or NO (Apel and Hirt 2004; Huie and Padmaja 1993).

The production of ROS during photosynthetic reactions in chloroplasts is a major cause of oxidative damage of membrane lipids, proteins and DNA (Moller et al. 2007). However, ROS that evolve during photosynthetic ET are important for dissipation of excess excitation energy (Mullineaux and Karpinski 2002), and as the ROS produced in mitochondria, the reactive oxygen species mediate signals from the organelle to the cytosol and nucleus in stress response and pathogen defense (Foyer and Noctor 2003; Galvez-Valdivieso and Mullineaux 2010; Trotta et al. 2014; Zurbriggen et al. 2010).

As discussed above, the signaling function of ROS produced in mitochondria is mostly linked to cell adaptation under hypoxic and anoxic conditions. However, $O_2^{\bullet-}$ production during photosynthesis proceeds under different conditions compared to mitochondrial respiratory electron transfer. High irradiance and photosynthetic activity create hyperoxic conditions in chloroplasts. Therefore, functions other than oxygen sensing would be expected for $O_2^{\bullet-}$ generated by the b_6f complex during photosynthetic electron transfer. In addition, spatial localization of $O_2^{\bullet-}$ production is different in mitochondria and chloroplasts. While $O_2^{\bullet-}$ generated by bc_1 complex is directed to the intermembrane space of mitochondria, the Q_p site of the b_6f complex is oriented towards the lumenal side

of the thylakoid membrane and, therefore, $O_2^{\bullet-}$ produced by the b_6f complex is released to the thylakoid lumen.

C. Interaction Between $O_2^{\bullet-}$ Producing Activities of the Photosynthetic ET Chain

In addition to the linear electron flow from water to $NADP^+$ used for CO_2 assimilation and photorespiration, photosynthetic ET includes a cyclic electron transfer pathway and 'pseudocyclic electron pathway' or a water–water cycle (Asada 1999). For the latter, electrons that are transferred by photosynthetic ET chain from the oxygen evolving complex of PSII are donated to O_2 through photoreduction, leading to formation of $O_2^{\bullet-}$ by the PSI reaction center coupled to the PSI electron acceptors ferredoxin, thioredoxin and ferredoxin:$NADP^+$ oxidoreductase (Mehler reaction) (Foyer et al. 2012; Mehler 1951). The $O_2^{\bullet-}$ subsequently disproportionates to H_2O_2 and is detoxified to water by the ascorbate peroxidase pathway (Asada 1999). The Mehler reaction is important as a bypass or branch used to control over-reduction of the photosynthetic ET chain. In addition, the production of ROS mediates redox signaling that regulates activity of photosynthetic ET chain and gene expression, as discussed above and in Foyer et al. (2012).

While the Mehler reaction proceeds in the chloroplast stroma, $O_2^{\bullet-}$ produced by b_6f complex is released to the thylakoid lumen. The presence of Cu, Zn-superoxide dismutase in the lumen has been demonstrated (Hayakawa et al. 1984). This ensures fast dismutation of $O_2^{\bullet-}$ to H_2O_2 that can diffuse from the lumen through thylakoid and chloroplast membranes. Therefore, $O_2^{\bullet-}$ production by the b_6f complex inside the lumen contributes to signaling or excess energy dissipation mediated by the ROS produced during the Mehler reaction that proceeds in the stroma. It could be presumed that distribution of ROS between the two compartments, thylakoid lumen and stroma, might contribute to control of redox balance between the PQ pool in the thylakoid membrane and PSI acceptors in the stroma. Under favorable light conditions and the presence of a
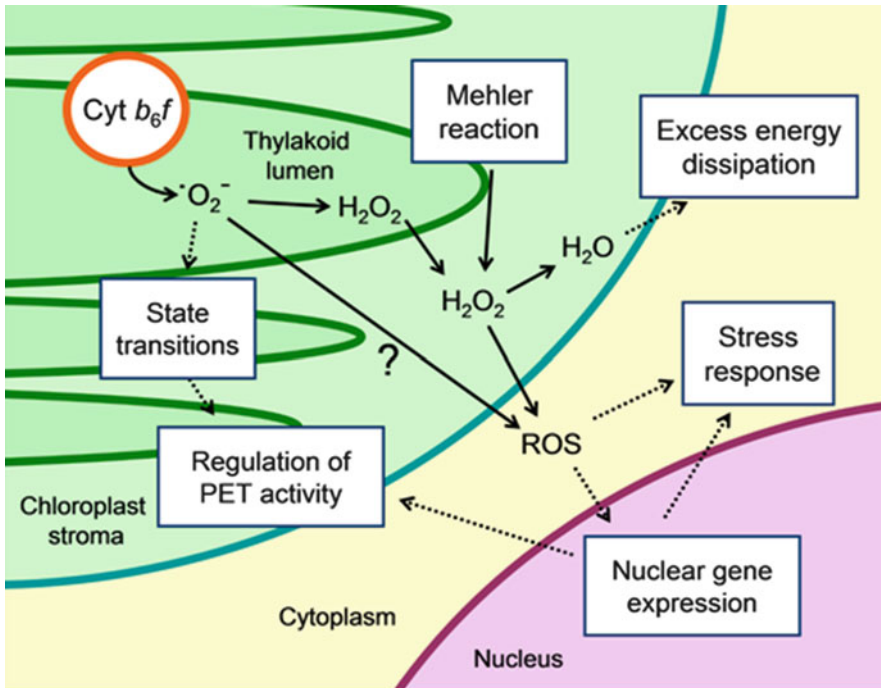
Fig. 20.2. Function of superoxide produced in chloroplasts. Schematic representation shows translocation within the cell of $O_2^{\bullet-}$ produced by photosynthetic cyt b_6f complex and its transformation reactions to other ROS (*solid arrows*). Cellular processes regulated by the ROS in different cellular compartments linked with *dashed arrows*. Mehler reaction is shown in the figure as another major contributor to H_2O_2 production in chloroplast stroma that migh have synergistic effect in regulation of photosynthetic and other cellular processes together with the cyt b_6f complex producing $O_2^{\bullet-}$ in chloroplast lumen.

reduced PQ pool and oxidized stroma, linear ET would cause ROS accumulation in the lumen, due to the $O_2^{\bullet-}$ producing activity of the b_6f complex and the Mehler reaction would proceeds at a slow rate. Over-reduction of both ET chain components, PQ pool and PSI acceptors in the stroma, would cause an increase in the rate of the Mehler reaction, and ROS accumulation would become balanced between the lumenal and stromal sides of the thylakoid membrane. In addition, this balance would be modulated by the activity of PSI, as a low PSI activity would cause: (1) over-reduction of the plastocyanin pool which would slow down linear ET, and $O_2^{\bullet-}$ producing activity of the b_6f complex; (2) a decrease in the rate of the Mehler reaction which would lead to reduction of overall $O_2^{\bullet-}$-producing activity by the ET chain (Fig. 20.2).

D. Regulation of Photosynthetic State Transitions

Photosynthetic state transitions maintain the redox poise of the thylakoid membrane PQ pool (Allen 1992; Wollman 2001). Light harvesting complexes preferentially associate with PSII when the PQ pool and the b_6f complex is oxidized, and with PS I when the PQ pool is reduced. The presence of reduced plastoquinone activates an Stt7/Stn7 protein kinase that phosphorylates light-harvesting complex LHCII and leads to the reversible displacement of the mobile LHCII between the photosystems (Rochaix 2007). The b_6f complex plays a key role in the activation of the kinase (Wollman 2001). It has been demonstrated that the Stt7/Stn7 is bound to the b_6f complex in thylakoid membranes, in a molar ratio of

approximately 1:20 kinase:b_6f complex (Lemeille et al. 2009). In spite of the identification of the kinase and the PQH_2 binding to b_6f as a key step in the activation of the kinase, the mechanism by which the PQH_2 is sensed in the Q_p-portal and the nature of the transmembrane signal remain unknown.

It was proposed that redox signaling that involves b_6f complex might be associated with regulation of state transitions (Kallas 2012), which could be linked to the $O_2^{\bullet-}$-producing activity of the b_6f complex (Fig. 20.2). Although in vivo experiments with application of exogenous ROS did not confirm that ROS signaling is required for regulation of state transitions in cyanobacteria (Brantmier 2013), this does not exclude the possibility that regulation of the Stt7/Stn7 kinase activity could be directly linked to Q-cycle bypass reactions that proceed at the Q_p-site.

Vener et al., proposed a model according to which the Stt7/Stn7 kinase activation is related to occupancy of the Qp site of the cytochrome b_6f complex by plastoquinol (Vener et al. 1997). In addition, it has been suggested that upon binding of PQH_2 at the Q_p-site, a signal is transduced from the ISP soluble domain through the ISP transmembrane helix to the N-terminus of the ISP on the n-side, which may provide the interaction site for the kinase (Hasan and Cramer 2012). As discussed in previous sections, the high rate of $O_2^{\bullet}$ production in the b_6f complex could be associated with an increased residence time of the $PQ^{\bullet}$ in its binding niche near the Fe-S cluster of the Rieske ISP. Therefore, it was proposed that the increased residence time of $PQ^{\bullet}$ generated at the Q_p-site in the b_6f complex may be a consequence of the formation of a long-lived ISP-$PQ^{\bullet}$ interaction that is required to provide enough time for the transmission of the signal through the ISP transmembrane helix to the *n*-side of the complex, where the Stt7/Stn7 kinase is recruited for interaction with the b_6f complex.

E. ROS in Chloroplast Retrograde Signaling and Modulation of Abiotic Stress Response and Pathogen Defense

Chloroplast proteins are encoded by genes located in genomes of both the nucleus and the plastid. Bidirectional signaling pathways ensures coordination of the protein synthesis and transport. Chloroplasts not only receive signals from the nucleus, but can also influence nuclear gene expression via chloroplast-to-nucleus retrograde signaling (Jung and Chory 2010; Nott et al. 2006). The redox state of photosynthetic electron transport components and ROS production comprise one of the retrograde signaling pathways that is crucial in regulation of plant responses to environmental stress (Barajas-Lopez et al. 2013; Fey et al. 2005; Nott et al. 2006). Increased levels of ROS serve as a signal of the functional and developmental state of the chloroplasts that is transferred to the nucleus and leads to change in nuclear gene expression. Chloroplast ROS-initiated signaling is implicated not only in the regulation of expression of nuclear genes associated with chloroplast development but has also been shown to be involved in plant acclimation to a variety of environmental stresses (Li et al. 2013; Pfalz et al. 2012; Suzuki et al. 2012) and pathogen defense signaling (Kangasjarvi et al. 2014; Karpinski et al. 2003, 2013; Trotta et al. 2014; Zurbriggen et al. 2010).

ROS accumulation in different plant cell compartments and routes by which such signal might reach the nucleus has been reviewed recently (Shapiguzov et al. 2012). Several major ROS signals that evolve from the activity of the photosynthetic ET chain, including $^1O_2$, $O_2^{\bullet-}$ and H_2O_2, may act as signal mediators of the retrograde signaling network (Barajas-Lopez et al. 2013; Inaba et al. 2011; Yurina and Odintsova 2011). The function of different ROS molecules is defined by their chemical properties and sites of generation. Singlet oxygen ($^1O_2$) generated in PSII is most likely retained within chloroplasts considering its short lifetime (on the

order of 0.1 μs (Baker and Kanofsky 1992)) and distance traveled. Despite this fact, it was demonstrated that $^1O_2$ accumulates in chloroplasts and affects the expression of several nuclear genes (Kim et al. 2008).

As discussed in the previous section, $O_2^{\bullet-}$ produced by components of the ET chain (such as PSI or b_6f complex) could be converted into H_2O_2 that has a longer half life-time and diffuses across the chloroplast membrane into the cytoplasm (Pfannschmidt 2003). It has been demonstrated that H_2O_2 can interact with downstream mediators of signaling networks, including MAP kinase cascades or *rimb* (redox imbalanced) components (Heiber et al. 2007; Kovtun et al. 2000), which lead to activation of Rcd1 and Rap2.4 transcription factors and expression of stress responsive genes (Shaikhali et al. 2008). The Gun1 (*genomes uncoupled 1*) protein was demonstrated to be a ROS signal mediator, although the mechanism underlying its function remains undefined (Cottage et al. 2008).

To identify ROS-responsive genes and to clarify the function of ROS as signaling molecules, transcript analyses have been performed using ROS-generating agents or mutants lacking antioxidative enzymes (Gadjev et al. 2006; Gechev et al. 2004; Vandenabeele et al. 2004). Increases in H_2O_2 concentrations have been shown to be important for the induction of the ascorbate peroxidase gene, APX2, and for the expression of a number of genes involved in plant development and stress responses (Vanderauwera et al. 2005). Recently, light was shed on H_2O_2 signaling and expression of ROS-responsive genes through the use of plants expressing glycolate oxidase that generate endogenous H_2O_2 in chloroplasts (Balazadeh et al. 2012). The study revealed a rapid and coordinated expression response of ROS-affected genes. These studies demonstrated the existence of multiple pathways implicated in ROS signaling. Interactions between signaling pathways are thought to modulate plant responses to abiotic and biotic stress.

ROS produced in chloroplasts interacts with signals from other intracellular and extracellular ROS sources in the modulation of local and systemic responses of plant immune defense (Kangasjarvi et al. 2014; Karpinski et al. 2013; Zurbriggen et al. 2010) (Fig. 20.2). Conditions that stimulate reduction of the photosynthetic electron transport chain and the overproduction of ROS induce gene expression of pathogen resistance and trigger a pathogen-induced hypersensitive response (Karpinski et al. 1999; Rossel et al. 2007). Studies with the *LSD1* gene mutant *Arabidopsis thaliana* plants demonstrated that induction of the "cell death response" is regulated by ET transfer chain activity and the redox status of the PQ pool (Muhlenbock et al. 2008). Treatment of plant leaves with the quinone analog inhibitor DBMIB decreases the rate of PQH_2 pool oxidation and triggers production of H_2O_2 required for induction of defense response. This observation suggests that the $O_2^{\bullet-}$-producing activity of the b_6f complex does not contribute to the ROS production required for the plant defense signaling process, as $O_2^{\bullet-}$ production at the Q_p site is blocked by the presence of DBMIB (Baniulis et al. 2013).

The research during last decade revealed that the redox state of photosynthetic electron transport components acting through multiple ROS-mediated chloroplast retrograde signaling pathways is crucial in regulation of plant responses to environmental stress. Despite the fact that the role of plant ROS has been the subject of intensive research, the available data about the extent to which the ROS evolving from activity of different photosynthetic ET chain components, and specifically the b6f complex, contribute to plant responses to environmental stress are still fragmented.

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Electron Transfer Reactions at the Q$_o$ Site of the Cytochrome *bc*$_1$ Complex: The Good, the Bad, and the Ugly

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Summary

The cytochrome *bc*$_1$ complex of eukaryotic mitochondrial and prokaryotic energy transducing membranes functions as a protonmotive quinol:cytochrome *c* oxidoreductase. The enzyme utilizes a highly characteristic 'Q-cycle' reaction mechanism in which oxidation of substrate quinol leads to a bifurcated electron transfer pathway. This mechanism generates a highly-reducing, reactive radical semiquinone (SQ$_o$) intermediate at the site of quinol oxidation (Q$_o$) within cytochrome *b* which must be tightly managed to prevent energetically wasteful electron transfer side reactions or reduction of dioxygen to biologically harmful superoxide. In this chapter we discuss mechanistic strategies harnessed by the *bc*$_1$ complex

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to minimize unproductive electron transfer from SQ_o with particular reference to superoxide generation. Recent work suggests that the bc_1 complex achieves this by kinetically trapping the SQ_o anion within a hydrophobic region of the Q_o pocket as a destabilized, low-occupancy intermediate, conserving redox energy to reduce the native electron acceptor (ferriheme b_L) while minimizing the probability of unwanted (and potentially damaging) electron transfer bypass reactions.

I. Introduction

The cytochrome bc_1 complex (cyt bc_1, respiratory complex III) is a homodimeric, multimeric enzyme that functions as a protonmotive (ubi)quinol:cytochrome c oxidoreductase that is central to the respiratory biochemistry of eukaryotes and many prokaryotic organisms (Crofts 2004). The cytochrome b_6f complex, a close cousin of bc_1, is found in the thylakoid membrane of oxygenic photosynthesisers (Baniulis et al. 2008; Cramer et al. 2011). The monomeric unit of cyt bc_1 in eukaryotic organisms has a molecular mass of approximately 240 kDa and is formed from 10 to 11 discrete polypeptides. The hydrophobic cytochrome b subunit is encoded by mitochondrial DNA, with the remaining subunits encoded within the nucleus. During a complete catalytic cycle, cyt bc_1 oxidizes two molecules of ubiquinol, reducing two molecules of cytochrome c and one molecule of ubiquinone. Four protons are translocated into the intermembrane space (IMS) against the electrochemical gradient

Abbreviations: [2Fe-2S] – The iron-sulfur cluster of the 'Rieske' iron-sulfur protein; Cyt – Cytochrome; ENDOR – Electron nuclear double resonance spectroscopy; EPR – Electron paramagnetic resonance spectroscopy; ESEEM – Electron spin echo envelope modulation spectroscopy; HHDBT – 5-n-heptyl- 6-hydroxy-4,7-dioxobenzothiazole; ISP – The 'Rieske' iron-sulfur protein; PEWY – The Pro_{271}-Glu-Trp-Tyr_{274} sequence within the Q_o site of cytochrome b (yeast numbering); QH_2 – Quinol or hydroquinone; Q_i – Quinone reductase site of the cytochrome bc_1 complex; Q_o – Quinol oxidase site of the cytochrome bc_1 complex; SQ – Semiquinone; SQ_o – Semiquinone associated with the quinol oxidase site of the cytochrome bc_1 complex

per two electrons transferred to the soluble acceptor, cytochrome c, with the associated uptake of two protons from the matrix (Berry et al. 2000; Schultz and Chan 2001).

Three subunits form the highly conserved electron transferring catalytic core of the enzyme; cytochromes b (cyt b) and c_1 (cyt c_1), and the 'Rieske' iron-sulfur protein (ISP) (Berry et al. 2000; Hunte et al. 2003). Cytochrome b is located within the inner mitochondrial membrane (IMM) and contains the substrate binding sites for quinol oxidation (Q_o) and quinone reduction (Q_i), located on opposite sides of the energy transducing membrane. Cyt b binds two heme b molecules, of differing potential (approximately -100 and $+50$ mV) forming a low potential electron transfer chain across the membrane. The prosthetic groups of cyt c_1 and the ISP, which are within protein domains located within the intermembrane space, form a high potential electron transfer chain within the cyt bc_1 complex.

Cyt bc_1 operates via the unique and energy-conserving 'Q-cycle' mechanism (Fig. 21.1), proposed by Peter Mitchell (1975) and since modified by others (see, for example Trumpower 1990; Crofts 2005; Zhang et al. 2008; Crofts et al. 2008). This mechanism requires the bifurcation of electron flow between the high- and low-potential electron transfer chains from the site of quinol oxidation at Q_o, reducing a quinone molecule bound at Q_i, recycling one electron back into the quinone pool per quinol molecule oxidized at Q_o. This recycling mechanism doubles the number of protons translocated across the energy transducing membrane per quinol molecule oxidized at Q_o compared to that achieved by
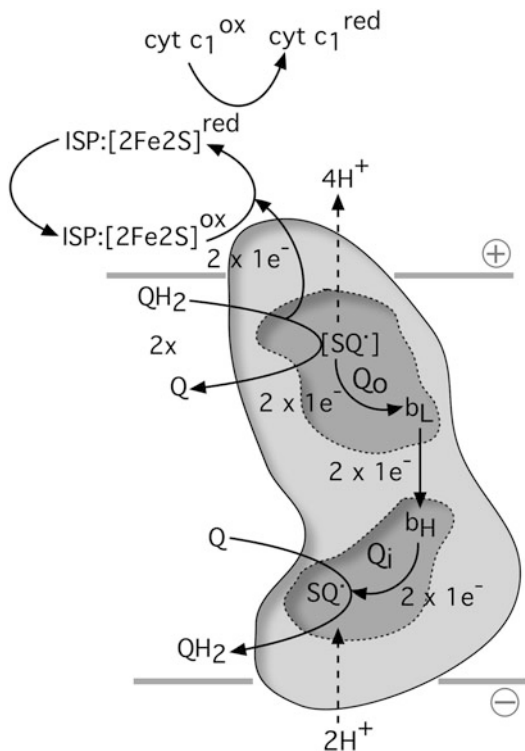
Fig. 21.1. The modified Q-cycle of the cytochrome bc_1 complex. Cytochrome b is represented in cartoon form, with the Q_o and Q_i pockets indicated by *dark shading*. Oxidation of two molecules of (ubi)quinol at Q_o results in the transfer of two electrons through the 'high-potential' chain (the Rieske ISP/cytochrome c_1) with the reduction of one molecule of (ubi)quinone at Q_i mediated by the transmembrane 'low- potential' chain of hemes b_L and b_H. In mitochondria, four protons are translocated into the intermembrane space against the electrochemical gradient with the associated uptake of two protons from the matrix during the complete catalytic cycle of the bc_1 complex.

simple double reduction of the high potential chain. The first electron transfer reaction at Q_o, from quinol to the ISP, generates a highly reducing semiquinone (SQ_o) intermediate, which serves as the reductant for heme b_L. Reduced b_L (-100 mV) is oxidized by heme b_H ($+50$ mV), which then reduces a quinone at the Q_i-site, generating a tightly bound and relatively unreactive semiquinone intermediate (SQ_i). A second quinol molecule is then oxidized at Q_o, and the cycle repeats itself. In contrast to SQ_i, the SQ_o species is kinetically

unstable and capable of transferring its electron to oxygen, generating harmful superoxide (Rutherford et al. 2012; Bleier and Dröse 2013; Lanciano et al. 2013). The bifurcation mechanism at the Q_o-site must thus be controlled to avoid energetically wasteful (and biologically damaging) electron transfer reactions (Osyczka et al. 2005; Cape et al. 2006). Note that Q_p and Q_n are alternative designations for the Q_o and Q_i sites, respectively, referring to their locations near the electrochemically positive (p) and negative (n) sides of the membrane (see Chap. 9 by Cramer and Hasan in this volume for further discussion).

II. Superoxide Production by the bc_1 Complex and the Question of SQ_o Reactivity

Electron leakage to oxygen during normal catalytic turnover of the bc_1 complex is often undetectably low (Muller et al. 2002). In general superoxide production is only observed in the presence of (i) Q_i-site antagonists (such as antimycin) which maintain a reduced low potential chain within the enzyme (Chance et al. 1979; Turrens et al. 1985; Zhang et al. 1998a; Sun and Trumpower 2003), (ii) mutant forms of the cyt bc_1 complex, some of which have been implicated in human mitochondrial disorders (Sun and Trumpower 2003; Borek et al. 2008; Meunier et al. 2013), (iii) under conditions of high membrane potential and/or a highly-reduced UQ pool, impeding electron egress at Q_i (Dröse and Brandt 2008; Rottenberg et al. 2009) or (iv) certain types of Q_o inhibitors, such as myxothiazol and mucidin, which bind proximal to haem b_L (Muller et al. 2002, 2003; Sun and Trumpower 2003). It should be noted that semiquinone is essentially the only progenitor for superoxide at the Q_o-site; autooxidation of ferrocytochrome b_L or the reduced [2Fe-2S] cluster are kinetically insignificant sources of superoxide production (Muller et al. 2002; Forquer et al. 2005; Cape et al. 2009).

Aside from oxygen reduction, there are a number of thermodynamically favorable SQ-mediated bypass reactions that can occur at Q_o. These include (i) double reduction of the high potential chain, lowering the proton-motive efficiency of the bc_1 complex, similarly (ii) reduction of cytochrome c by SQ that has escaped the confines of the Q_o site, and (iii) futile reduction of SQ_o (or Q) by ferroheme b_L. Typically, however, these reactions are not observed during uninhibited turnover of the native enzyme. The bc_1 complex has evolved to minimize the probability of these energetically wasteful (and potentially biologically harmful) reactions from occurring.

A priori, bypass reactions at Q_o, with particular reference to electron transfer from SQ_o to oxygen, may be controlled by thermodynamic or kinetic mechanisms (or combination thereof), or may be avoided completely by the absence of a semiquinone intermediate during the reaction cycle (Cape et al. 2006; Rutherford et al. 2012). This latter phenomenon necessitates a concerted electron transfer mechanism requiring the simultaneous reduction of the ISP and ferricytochrome b_L with proton movements orchestrated accordingly (Snyder et al. 2000; Osyczka et al. 2004). A study of the activation energetics for superoxide production and quinol oxidation at Q_o in the presence and absence of antimycin using wild type and engineered forms of the yeast bc_1 complex with altered ISP redox potentials revealed identical activation enthalpies for superoxide production and normal catalytic turnover of the Q_o site, strongly suggestive of common transition states (Forquer et al. 2006). As such, a truly concerted electron transfer mechanism at Q_o can be ruled out, and the quinol oxidation reaction can be considered to occur sequentially.

A. Thermodynamic Control of SQ_o Reactivity

Protein-bound SQ species may be strongly stabilized (by five to ten orders of magnitude) by local environmental interactions (such as

hydrogen bonding), as is observed at the Q_i site of the bc_1 complex and in other enzymes such as prokaryotic bo_3 and aa_3 quinol oxidases, and the Q_B site of photosystem II (Ingledew et al. 1995; Hastings et al. 2000; Gunner et al. 2008; Yi et al. 2010). A stabilised SQ may be expected to be relatively unreactive with oxygen as additional energy must be supplied to the system for electron transfer to occur. Such a SQ species is expected to be relatively long-lived (with a lifetime of tens to hundreds of milliseconds) (Gunner et al. 2008), and thus be present at a relatively high concentration (occupancy) under equilibrium conditions. In contrast, the reactivity of a destabilized SQ intermediate with oxygen is expected to be moderated by the very short lifetime of the former species, with a correspondingly low equilibrium concentration.

B. Kinetic Control of SQ_o Reactivity

Superoxide production during the catalytic cycle at Q_o may be moderated by 'kinetic steering' of semiquinone oxidation pathways towards productive Q-cycle chemistry (i.e. reduction of cytochrome b_L) and away from deleterious side reactions. By controlling the orientation and distance between reacting centers, the Q_o site influences the activation entropies for the respective electron transfer pathways (with minimal effect on the overall driving force for the reaction) to lessen the probability of electron leak to oxygen. Such kinetic steering is implicit in many 'gating' mechanism models (discussed in Osyczka et al. 2005; Cape et al. 2006; Crofts et al. 2006 and references therein), which are described further in Sect. III.A.

III. Structure and Mechanism of the Q_o Site: Keeping the Chemistry Under Control

The Q_o site forms a large, bifurcated and predominantly hydrophobic volume within cyt b. It is composed from components encompassing the C-terminal region of trans-

membrane helix C, surface helix cd1 and the stretch encompassing the "PEWY" (Pro$_{271}$-Glu-Trp-Tyr$_{274}$, yeast notation) loop/ef helix to transmembrane helix F1 of cytochrome b (Zhang et al. 1998b; Berry et al. 1999; Hunte et al. 2000; Esser et al. 2004, 2008). Ligands binding within Q_o may be broadly classified as 'b_L proximal' or 'b_L distal' species depending on their positioning within the site. The b_L-proximal inhibitors, such as the natural antibiotics myxothiazol and strobilurin, characteristically have hydrogen bonding interactions with the backbone amide moiety of glutamate residue E272 in the Q_o ef helix, in close proximity to heme b_L. In contrast, b_L distal inhibitors, such as stigmatellin bind in a volume of the Q_o site in close proximity to the ISP, typically forming a strong hydrogen bond to a histidine ligand (H181) of the [2Fe-2S] cluster (Berry et al. 2000; Crofts et al. 1999; Gao et al. 2002).

The b_L-proximal inhibitors stimulate superoxide production at 50 % of the rate observed for the Q_i inhibitor antimycin as they permit the generation of SQ_o by the (forward) one-electron transfer reaction from distally-bound quinol to the oxidized ISP, but inhibit its oxidation by ferricytochrome b_L. Accordingly, superoxide production is not observed in the presence of the b_L-distal inhibitor stigmatellin, as this initial electron transfer reaction to form SQ_o cannot occur (Muller et al. 2002, 2003).

A. Electron and Proton Transfers, and Associated Gating Mechanisms

1. The First Electron Transfer Reaction

If we accept the assertion that substrate quinol is oxidized to quinone in a sequential manner at Q_o, which is the probable case (Forquer et al. 2006), then a generally accepted mechanism for the first electron transfer reaction (to the oxidized [2Fe-2S] cluster of the Rieske protein) is outlined below. For a more comprehensive treatment of electron and proton transfer chemistry within the Q_o site, the reader is referred to (Crofts et al. 2006, 2013, and references therein).

The atomic structure of stigmatellin-inhibited bc_1 provides a useful model for the initial enzyme-substrate complex for Q_o-bound quinol (Zhang et al. 1998b; Crofts et al. 1999; Berry and Huang 2003). Accordingly, it is assumed that the enzyme-substrate complex for the initial electron transfer consists of quinol occupying the distal region of the Q_o site, with a hydrogen bond between the benzoquinone hydroxyl and the histidyl sidechain of the H181 ligand of the [2Fe2S] cluster. The donor-to-acceptor distance for electron transfer is approximately 7 Å. A weaker hydrogen bond may be present between the carboxylate group of E272 (yeast notation, equivalent to E271 in the bovine structure and located within the highly conserved 'PEWY' Q_o motif of cyt b) and the benzoquinone hydroxyl *para* to that interacting with H181 (Berry and Huang 2011). It is through the hydrogen bond to H181 (which is considered to confer a bell-shaped pH dependency on the quinol oxidation reaction in the pH 6.0–8.0 range (Brandt and Okun 1997)) that the electron transfer proceeds in a strongly rate-limiting, energetically uphill, proton-coupled electron transfer reaction (Guergova-Kuras et al. 2000). This results in the formation of a semiquinone species within the Q_o distal region, and a reduced [2Fe-2S] cluster with H181 in the protonated form. The headgroup of the ISP then rotates away from the surface of cytochrome b, and reduces cytochrome c_1, releasing the proton carried by H181 to the bulk phase. The diffusion of the extrinsic domain of the ISP between cytochromes b and c_1 is not rate-limiting for catalysis at the Q_o site in the native enzyme (Crofts et al. 2003). The initial (neutral) semiquinone generated at Q_o is likely to have a very transient existence and has not been detected spectroscopically (Cape et al. 2007). Its remaining proton is immediately stripped away to generate a quasi-stable semiquinone anion prior to the second electron transfer reaction (see Sect. III.A.2).

Subtle, Q_o inhibitor-dependent structural and hydrogen-bonding changes within the Q_o site that affect the positioning of the ISP

headgroup and cyt b cd1 helix and loop connecting transmembrane helices E and F, have been analysed by Berry and Huang (2011). These structural perturbations, many of which were recognized in early crystallographic studies (Crofts et al. 1999; Berry et al. 2000), have been interpreted in the context of 'conformational' gating mechanisms for the first electron transfer reaction. Allosteric control of the ISP movement and positioning by occupants at Q_o forms a component of the 'catalytic switch,' electron bifurcation control mechanism proposed by Brandt and colleagues (Brandt and von Jagow 1991; Brandt 1999). Berry and Haung identified a potential hydrogen bonding interaction from the tyrosyl group of the Q_o-site ef helix residue Y279 to the histidyl sidechain of ISP residue H181 in famaxodone-inhibited avian bc_1, offering a possible explanation as to why the ISP headgroup exhibits high occupancy at the surface of cytochrome b in the associated atomic structure even though no hydrogen bonding interaction is predicted between this inhibitor and the ISP. Y279 is proposed to act as a 'surrogate quinol' in this instance (Berry and Huang 2011). A subtly different hydrogen-bonding interaction is observed between Y279 and H181 in the stigmatellin-inhibited complex, with the peptide carbonyl group of H181 acting as the hydrogen bond acceptor (Berry and Huang 2011). Y279 has been proposed as the gating 'trapdoor tyrosine' controlling access to the b_L proximal region of Q_o through simple steric hindrance, the positioning of which is governed by displacement of the PEWY loop (Crofts et al. 2013). Mutation of Y279 is frequently deleterious to the catalytic activity of the bc_1 complex (Fisher et al. 2004) with a concomitant increase in superoxide production, and has been implicated in human mitochondrial disorders (Meunier et al. 2013). Mutation of the equivalent residue (Y268) in the human malarial parasite (*Plasmodium falciparum*) confers resistance to the anti-malarial drug atovaquone, but at the cost of a fitness penalty to the organism (Fisher et al. 2012).

2. The Second Electron Transfer Reaction

The mechanism of the second electron transfer reaction at Q_o, from the semiquinone to oxidized heme b_L, is a matter of debate. A plausible scenario as proposed by Crofts et al. (2006, 2013, and references therein) is sketched here. The first electron transfer results in the semiquinone in the distal region of the Q_o site, hydrogen bonded to the carboxylate sidechain of E272, which immediately deprotonates, generating the semiquinone anion. The sidechain of E272, considered a mobile actor within the Q_o site (Crofts et al. 1999; Palsdottir et al. 2003), rotates by >130° about its C_α-C_β bond, transferring the proton to water molecules in an internal chain, that forms an effective proton channel to the bulk phase. Rotation of the E272 sidechain opens the b_L-proximal volume of the Q_o site to diffusion of the semiquinone anion into position to reduce heme b_L. In contrast to the first electron transfer reaction, electron transfer from the semiquinone to heme b_L is exergonic and rapid, minimizing the lifetime of the SQ_o intermediate. Movement of SQ_o from the distal to proximal domains of the Q_o site decreases the distance for electron transfer to b_L, exponentially increasing the rate constant for b_L reduction. Oxidation of the semiquinone by b_L generates quinone, which diffuses back to the distal pocket of Q_o, and out of the enzyme.

Changes in the local electric field of the proximal domain by the redox state of heme b_L are proposed as a key component of an elegant 'Coulombic' gating mechanism (Crofts et al. 2013; Victoria et al. 2013). The anionic semiquinone is suggested to be attracted to the partial positive charge of ferriheme b_L, decreasing the rate constant for electron transfer to b_L. Contrastingly, ferroheme b_L, which will persist if the membrane potential is high or heme b_H reduced (due to the absence of an electron acceptor at Q_i), is predicted to present a repulsive field to the anionic semiquinone, lessening the probability of the re-reduction of SQ

by b_L. Additionally, the pK of the carboxylic sidechain of E272 is predicted to be lowered by the presence of ferriheme b_L, facilitating proton transfer through this residue from the semiquinone to the water channel (Crofts et al. 2013; Victoria et al. 2013).

E272 is completely conserved in mitochondrial cyt b, but is replaced by valine or proline in β- and γ-proteobacterial sequences (Kao and Hunte 2014). Whether this residue is essential for catalysis at Q_o has been examined by site-directed mutagenesis studies in yeast, *Paracoccus denitrificans* and *R. capsulatus bc*$_1$ (Osyczka et al. 2006; Wenz et al. 2006; Kleinschroth et al. 2008; Seddiki et al. 2008; Victoria et al. 2013). Typically, replacement of this residue has relatively minor effects on substrate binding at Q_o, although the kinetics of steady-state and single turnover (b_L/b_H reduction) are significantly affected. However, changes in the residue can result in large changes in superoxide production at the Q_o site. Of particular interest is the effect of E272 mutants. It has been suggested that there are considerable structural and mechanistic redundancies within the Q_o site to maintain activity, and that protonatable residues within the site (such as H253 in the yeast bc_1 complex) may partly compensate for the missing E272 in site-directed mutants (Osyczka et al. 2006; Wenz et al. 2006). On the other hand, it is argued that because E272 is essential only for the semiquinone-oxidation step at Q_o, which is not rate limiting; the degree of inhibition due to mutagenesis of E272 may be underestimated in steady-state kinetic assays with the mitochondrial enzyme (Crofts et al. 2013).

3. Double-Gated and 'Kinetic' Mechanisms for Controlling Electron Transfer and Chemistry at Q_o

The important concept of the reversibility of electron- and proton-transfers associated with quinone chemistry in the Q-cycle (Mitchell 1975) has been emphasized by Osyczka et al. (2004), and used as a conceptual framework in the proposal of the 'double-gated' mechanism for control of

the electron transfer reactions at the Q_o site (Osyczka et al. 2005).

The 'double gated' models have one dominant feature: quinol oxidation is only permitted when *both* the ISP [2Fe-2S] and heme b_L are oxidized (or reduced, for the reverse electron transfer reaction to quinone). Quinol oxidation is forbidden if the [2Fe-2S] is reduced, or if heme b_L is reduced. This requires that SQ reactivity be gated in some fashion by the redox state of the complex.

A fully reversible, 'logic-gated' scheme was proposed by Rich (2004) for orchestrating electron transfer at Q_o that depends on the redox-linked protonation status of the hydrogen bond donors and acceptors (H161 and E272 in this instance). Partly in response to the controversy concerning the essential role of E272 for catalysis, Osyczka et al. (2005) independently proposed a 'double-gated' model wherein Q_o occupancy is gated by the hydrogen-bonding of a quinoid molecule to the water molecules in the Q_o site. The orientation of these water molecules was proposed to be influenced by the redox status of heme b_L and the [2Fe-2S] cluster, moderating quinoid reactivity accordingly. These double-gated models have been criticized for lacking experimental tractability (Crofts et al. 2006), although that proposed by Rich may be amenable to Fourier transform infra-red spectroscopic investigation.

Osyczka and colleagues developed a 'kinetic' model of control of Q_o site chemistry (Borek et al. 2008; Sarewicz et al. 2010) in which superoxide generation is a function of (i) the occupancy of the [2Fe-2S] cluster at Q_o and (ii) the midpoint potential of cytochrome c_1. Relative to wild type, superoxide production by *R. capsulatus bc*$_1$ in the presence of antimycin decreased when the mobility of the ISP was reduced by insertion of alanine residues in its flexible linker region, increasing the occupancy of the 2Fe-2S cluster at Q_o. Contrastingly, when the midpoint potential of heme c_1 was markedly lowered by mutagenesis of the cyt c_1 residue M183, impeding 2Fe-2S oxidation, superoxide production increased. These results were

interpreted as a simple kinetic competition for electrons between the semiquinone occupant, heme and [2Fe-2S] cofactors at Q_o, and oxygen (Borek et al. 2008; Sarewicz et al. 2010). Steric hindrance, which could limit access of oxygen to SQ_o when the ISP headgroup is docked at cytochrome b, was downplayed as a factor regulating superoxide production in the discussion of Osyczka's data, although it seems likely that this is a contributory factor.

A kinetic model for the control of Q_o-site chemistry was recently proposed by Quinlan and colleagues (Quinlan et al. 2011). The basic premise is that the reduction status of *both* hemes b_L and b_H, as influenced by the membrane potential or redox state of the Q pool, affects the kinetics of quinol oxidation and the occupancy of SQ at Q_o, which accordingly moderates superoxide generation. A similar model linking Q-pool redox status with bc_1-mediated ROS production has been described by Dröse and Brandt (Dröse and Brandt 2008). Using succinate (in the presence of malonate) as the electron donor in antimycin-inhibited rat mitochondrial preparations, Quinlan observed that maximal rates of hydrogen peroxide production showed a bell-shaped response, peaking when heme b_L was 70–80 % reduced. Imposition of a membrane potential by addition of ATP altered the redox equilibration between hemes b_L and b_H, disfavoring electron accumulation on b_H, and limiting peak hydrogen peroxide production. Mathematical modelling suggested that quinol oxidation (i.e. SQ_o generation) was slowed when b_H was reduced, and slowed further by reduction of b_L, perhaps by redox-induced conformational change within the enzyme (Quinlan et al. 2011). Coulombic effects on the redox potential of b_L due to reduction of b_H are also expected (Crofts et al. 2013).

It is notable that the transfer of the *first* pair of electrons from quinol at Q_o in the fully oxidized enzyme is unaffected by antimycin (Crofts et al. 1983), while some proteolytic and EPR spectroscopic data suggest that antimycin can act as an effector of the ISP and perturb its interaction with cytochrome b

(Valkova-Valchanova et al. 2000; Cooley et al. 2005; Cooley 2010). Intra- and intermonomeric allosteric regulation of Q_o chemistry was investigated by Trumpower and co-workers, and used to develop the 'half-of-sites' model for anti-cooperative regulation of bc_1 activity (Covian and Trumpower 2008a, b). They concluded that only one Q_o site per bc_1 dimer could be occupied by quinol during turnover, and that occupancy of the opposing Q_o site was excluded by conformational changes transmitted cross-dimer. Antimycin was also proposed to have a similar cross-dimer, anti-cooperative effect on Q_o turnover. Intermonomer electron transfer, mediated by the b_L hemes (which are separated by an edge-to-edge distance of 12 Å) was suggested to prevent accumulation of SQ_o (and subsequent electron leak to oxygen) in the half-of-sites model. The thermodynamic and kinetic basis of this model has been criticized by Crofts et al. (2008), although it has since been demonstrated that cross-dimer electron transfer can occur at physiologically relevant rates in Q_o/Q_i knockout mutants of *R. capsulatus* bc_1 (Swierczek et al. 2010). This latter study, however, did not support the half-of-sites hypothesis for anti-cooperative control of bc_1 activity, neither do quinone binding studies that indicate complete occupancy of both monomers (Bartoschek et al. 2001).

The data of Dröse and Brandt (2008) and Sarewicz et al. (2010) have been interpreted as supporting the 'semireverse' model for electron transfer at Q_o in which the predominant pathway for superoxide generation is by reverse electron transfer from ferrocytochrome b_L to oxygen via a semiquinone intermediary (Fig. 21.2). In contrast, the observation by Forquer and colleagues (2006) that the rate-limiting step for quinol oxidation and superoxide generation at Q_o was essentially identical strongly supports the 'semiforward' model, with superoxide generation occurring prior to electron transfer to b_L (Fig. 21.2). The 'semiforward' mechanism is also favored by the kinetic models of Q_o chemistry by Quinlan (Quinlan et al. 2011). Strong support for the 'semiforward' model is also
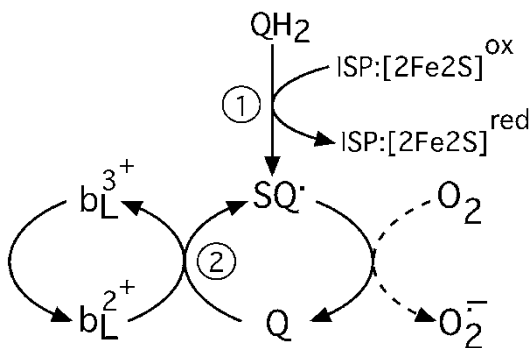
Fig. 21.2. Semiquinone (SQ) formation by the 'semi-forward' (1) and 'semi-reverse' (2) electron transfer pathways at Q_o. In the 'semi-forward' reaction the predominant pathway for SQ formation is oxidation of quinol by the ISP. Contrastingly, SQ is formed by reduction of Q_o-bound quinone by ferroheme b_L in the 'semi-reverse' reaction. Superoxide generation is mediated by SQ in both schemes.

provided by the observation of enhanced superoxide production in the presence of Q_o-proximal domain inhibitors (which prevent b_L reduction) and similarly in mutants of *Rhodobacter sphaeroides* bc_1 lacking heme b_L/b_H (Muller et al. 2002; Yang et al. 2008).

B. Spectroscopic Investigation of SQ_o and Implications for Mechanism

The transient nature of SQ_o has made it a difficult species for spectroscopic investigation. Additionally, it has been questioned whether antiferromagnetic coupling between SQ_o and the reduced ISP would render it invisible to EPR investigation (Link 1997; Jünemann et al. 1998; Sarewicz et al. 2013). Initial EPR experiments detected a radical species in bovine submitochondrial particles under conditions of oxidant-induced reduction of bc_1, which was ascribed to SQ_o due to its abolition by treatment with British Anti-Lewisite (a covalent modifier of the ISP's [2Fe-2S] center) and aerobic sensitivity (De Vries et al. 1981). However, this identification was questioned in later studies where the radical signal was shown to be stigmatellin-insensitive (Jünemann et al. 1998). It is likely that the species reported by de Vries was

located externally from Q_o (or indeed the bc_1 complex in general).

Rapid freeze quench studies under anaerobic conditions have been used to trap SQ_o in *Rhodobacter* chromatophore and bc_1 preparations, prior to EPR analysis (Cape et al. 2007; Zhang et al. 2007). Antimycin was present in these investigations to block semiquinone formation at Q_i and allow electron accumulation on the low potential chain in cytochrome b. Zhang observed a stigmatellin-sensitive SQ_o radical in chromatophores of *R. capsulatus* mutants (lacking heme b_H) after flash excitation of the photosynthetic reaction centers to oxidize the bc_1 complex followed by freezing. Cape et al rapidly mixed *Rhodobacter* bc_1 with decylubiquinol prior to freezing in liquid propane after the reaction had been allowed to proceed for two to three enzymatic turnovers (approximately 10 ms), trapping SQ_o in a small population of centers. SQ_o occupancy was estimated to be approximately 0.01–0.1 SQ per bc_1 monomer. EPR investigation of the frozen samples revealed that accumulation of the radical species (identified as an anionic semiquinone) was sensitive to stigmatellin, as expected, and furthermore was eliminated in a mutant bc_1 complex lacking the ISP [2Fe-2S] cluster. Electron spin echo envelope modulation (ESEEM) analysis, a pulsed EPR technique, indicated an absence of hydrogen bonds between the trapped SQ_o and nitrogen atoms or backbone amide residues, suggesting that the anionic semiquinone was not interacting with H181 of the ISP, and was located in the vicinity of the b_L-proximal niche in the Q_o-site.

The existence of an SQ with occupancy greater than a few percent is not supported by ultra-fast freeze quench experiments on uninhibited bc_1 (Zhu et al. 2007). Oxidation of quinol reduced the [2Fe-2S] center and b_L at the same rates. Even 100 μs after mixing, equal amounts of these two reduced species were produced indicating that either the rate for movement of SQ to the proximal niche and reduction of b_L is much faster than the initial electron transfer to [2Fe-2S], resulting in very low SQ_o concentrations.

A more recent freeze-quench study by the Osyczka group (Sarewicz et al. 2013) examined a broader timescale of 0.012–300 s. SQ was not detected until after about 1 s, peaking near 10 s with antimycin bound. A new EPR line was detected at $g = 1.94$ whose intensity paralleled that of the SQ. These observations were interpreted as indicating accumulation of large occupancy of SQ stabilized in the Q_o-site. The new EPR signal was considered to be from the triplet state of a spin-coupled pair of SQ and reduced [2Fe-2S] center in Q_o. The new signal, seen only when SQ and reduced [2Fe-2S] were both present, was taken to be a state of very-reduced bc_1. These measurements were made in air-equilibrated buffers with oxygen present, and it was argued that a hydrogen bond between SQ and the reduced [2Fe2S] would prevent the SQ from generating superoxide. However, a considerable fraction of the SQ was not in the form of this new EPR signal and is very efficient at generating superoxide (Muller et al. 2003). The measurements, per the Materials and Methods section (Sarewicz et al. 2013), were made with as many as 26 electrons from decylubiquinol per bc_1, of which only eight could be accepted by cyt c. Up to 18 superoxide molecules (some of which can be free radicals with lifetimes on the order of minutes) would be generated for each bc_1 complex, leading to extensive oxidative damage of the enzyme. Unfortunately, it appears that SOD was never added to the reaction mixture as a control to prevent oxidative damage show that the SQ and triplet signals were not products of superoxide.

The new EPR signal at $g = 1.94$ was assigned to a triplet state formed by an interacting SQ and [2Fe-2S] largely on the basis of simulating the new EPR spectrum. There are two major problems with this assignment. First, only one of the three potential EPR peaks from the triplet were clearly observed, the other two are either too broad to detect or obscured by overlap with the EPR spectra of the SQ and reduced [2Fe-2S]. A fit of one EPR line is not very conclusive given the number of parameters that potentially could be involved. Second, the spin coupling model is not very appropriate for the supposed triplet. The [2Fe-2S] does not have a single unpaired spin as assumed in the spin Hamiltonian used by Sarewicz et al. in their supporting information to simulate the $g = 1.94$ line. Rather, the [2Fe-2S] is itself a pair of interacting Fe(II) and Fe(III) ions and their $S = 2$ and $S = 5/2$ spin couple to produce the observed [2Fe-2S] EPR spectrum (Bertrand and Gayda 1979; Bertrand et al. 1985; Gurbiel et al. 1996; Shubin and Dikanov 2006). Rather, a simulation of the EPR spectrum for the triplet proposed by Sarewicz et al. needs to include spin couplings of the SQ, Fe(II) and Fe(III) to provide strong support for a triplet state. The proposal that a triplet composed of SQ and [2Fe-2S] has finally been found is intriguing, but would appear to contradict a large amount of past experimental evidence that SQ_o concentrations are low (see above). In our opinion, this proposal requires more substantial evidence that the signal does not arise from oxidative damage products from superoxide generation and that it is a triplet state composed of SQ and reduced [2Fe-2S] by simulation of its entire EPR spectrum by a realistic spin Hamiltonian.

Recently, the freeze quench studies of Cape et al. (2007) have been developed further using yeast bc_1 preparations, which serves as a more appropriate model for the mammalian enzyme (Vennam et al. 2013). As in the *Rhodobacter* SQ_o study (Cape et al. 2007), ESEEM studies revealed no hydrogen bonding interactions between the yeast SQ_o and the surrounding protein structure. This result was confirmed by electron-nuclear double resonance (ENDOR) spectroscopy indicating that the SQ anion was located within at unusual trapping site within the Q_o-site when compared to other protein-bound SQ species (Vennam et al. 2013).

The temperature dependency of the paramagnetic relaxation enhancement (PRE) between paramagnetic centers in proteins provides useful information concerning the distance between these centers (Bertini and Turano 1995). As such, the PRE effect between the trapped SQ anion and hemes c_1 and b_L/b_H
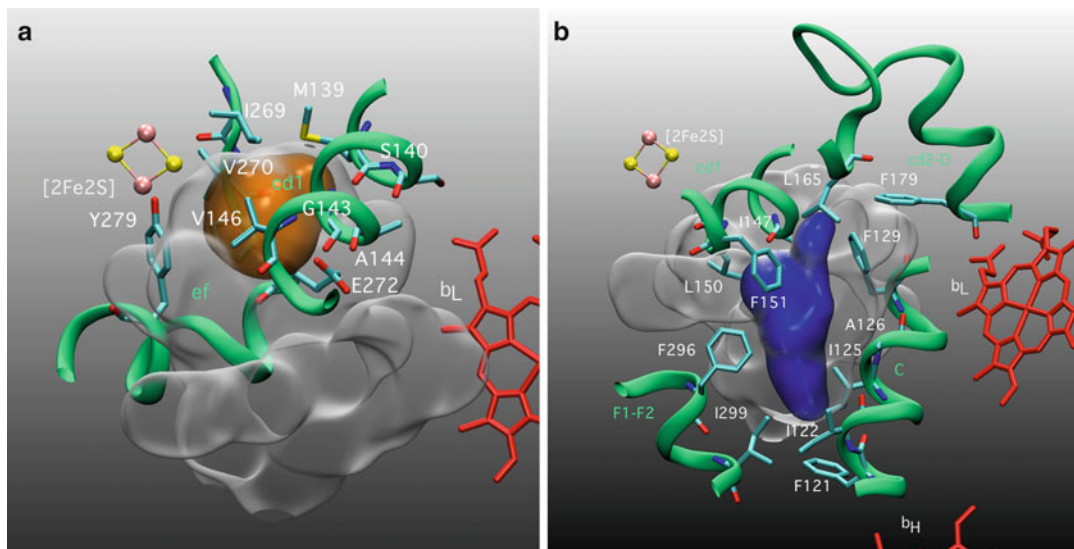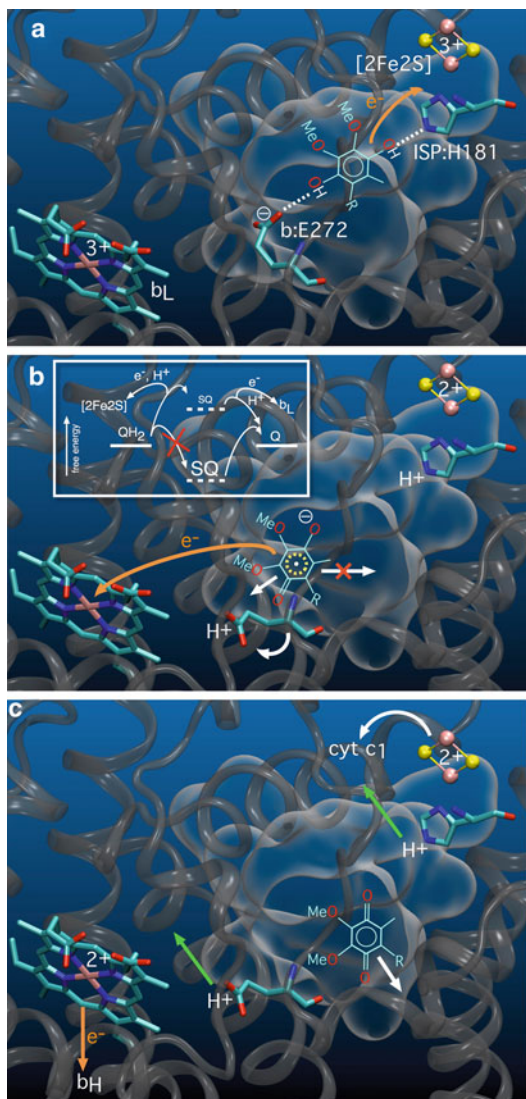
Fig. 21.3. Volumetric representation of the SQ binding sites within yeast cyt bc_1 (constructed using the PDB coordinates 3CX5 (Solmaz and Hunte 2008)). The Q_o void volume is represented by a glass surface. The Q_o site void volume overlapping the intersection of the spherical regions defined by the b_L/c_1(intramonomer) paramagnetic relaxation enhancement distance constraints (site I) is represented in orange (panel **a**). Heme c_1 itself is not shown in the figure. The b_L(intramonomer)/b_L(cross-dimer) distance constraints (site II) are shown in *blue* (panel **b**). Also shown are hemes b_L and b_H (*red*) of cyt b and the [2Fe-2S] cluster of the ISP (*yellow*/*pink*). Cyt b residues in close contact (<4 Å) with site I and site II are labelled and shown in stick form. The cyt b polypeptide backbone is represented in *green*, with helices identified as shown.

of the freeze-quenched yeast bc_1 samples was used to calculate a set of distance constraints for identification of the location of the binding niche for SQ within Q_o (Vennam et al. 2013). It should be noted that the spin relaxation arising from the reduced [2Fe-2S] centre was too slow to contribute to the PRE effect in this study. As it was not possible to distinguish between the PRE effect of hemes c_1 and b_L on the trapped SQ species, two possible localities for SQ_o that satisfied the PRE distance constraints (1.65–1.95 nm from the iron atom of one heme, and 2.55–3.00 nm from the iron of another) were identified within Q_o (Fig. 21.3a, b).

Site I is within the vicinity of the b_L-distal (stigmatellin-binding) niche, however no hydrogen-bonding interactions between SQ_o and the headgroup of the ISP or E272 of cyt b were observed. In comparison, Site II is localized in a hydrophobic niche deep within Q_o, located more proximally to heme b_L, in concordance with the mobile SQ_o models

of Crofts et al. (Crofts 2004; Crofts et al. 2013). The enthalpic cost of accommodating the anionic SQ_o species within the relatively aprotic environment of this hydrophobic region within Q_o is likely met by entropic terms; i.e. the trapped SQ_o can adopt multiple conformations within this volume. These data support the model of a destabilized, low occupancy and mobile SQ anion that is stripped of both hydroxyl protons during the first electron transfer reaction to the ISP (Fig. 21.4) (Kramer et al. 2005). The anionic SQ becomes kinetically (electrostatically) trapped within the hydrophobic confines of Q_o, conserving redox energy for reduction of heme b_L and minimizing the possibility of diffusion into the lipid bilayer. The lack of hydrogen bonding interactions to the surrounding protein structure will result in a highly destabilized, strongly reducing SQ_o (as evidenced by the observed low occupancy (Cape et al. 2007; Zhang et al. 2007)). Thus the redox properties of SQ_o resemble those

of the photosystem I phylloquinone, which is considered to be destabilized with respect to the solution (Shinkarev et al. 2000). The destabilized SQ_o is consistent with the minimization of Q-cycle bypass reactions as its reduction by ferroheme b_L is thermodynamically unfavorable, and the probability of electron transfer to oxygen greatly reduced due to its very low occupancy. Contrastingly, a stabilized SQ_o, while present at high occupancy and likely to be a poor reductant for oxygen, would also be expected to lack sufficient free energy for the reduction of ferriheme b_L. Thus the Q_o site has evolved to strike a balance between maintaining the kinetic efficiency of the Q-cycle while minimizing the probability of bypass reactions through substrate destabilization (Fig. 21.4, panel B) – in contravention of the Pauling paradigm for enzymatic catalysis (Pauling 1948), using the simple device of hydrophobic containment. For further perspectives on reaction mechanisms and superoxide formation in bc_1-b_6f complexes, see the Chaps. 14 and 20 by Sarewicz et al., and Baniulis et al., in this volume.

Acknowledgments

Fig. 21.4. The 'proton-stripping' model for catalysis at Q_o. Electron and proton movements are indicated by *orange* and *green arrows*, respectively. (**a**) A quinol molecule binds in the b_L-distal region at Q_o, forming hydrogen bonds between the benzoquinol hydroxyls, the imidazole ring of ISP residue H181 and an acceptor species within cytochrome *b*, here shown as the sidechain carboxylate of E272. The Q_o site is shown as a glass volume, as in Fig. 21.3. (**b**) Proton-coupled ET between the bound quinol and the ISP [2Fe-2S] cluster occurs through the QH2:H181 H-bond, reducing the [2Fe-2S] cluster and converting the sidechain of H181 to the imidazolium form. A second proton transfer occurs between the neutral semiquinone and an acceptor at Q_o (the carboxylate of E272 in this model), resulting in a semiquinone anion, which diffuses to the b_L-proximal niche within Q_o after this volume is opened up by the rotational flipping

Fig. 21.4. (continued) of the protonated sidechain of E272. The semiquinone anion is enthalpically trapped within Q_o as it cannot be solvated by the hydrophobic residues surrounding the entrance to the site. It should be noted that oxidation of reduced b_L by anionic SQ is thermodynamically unfavorable, considerably lessening the probability of this unproductive bypass reaction. The *inset* represents the energetics for the overall reaction at Q_o; a low occupancy, energetically destabilized SQ_o intermediate is the preferred model. (**c**) Oxidized b_L is reduced by the SQ anion, with the resulting neutral quinone molecule diffusing away into the membrane. The timing of proton transfer as represented in this panel is indicative only, although deprotonation is assumed to occur more quickly than the competing bypass reactions. Coordinates from yeast bc_1 structure 3CX5.PDB (Solmaz and Hunte 2008) were used to generate this figure.

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Part V

Cytochrome Complexes, Signaling, and Regulation

Chapter 22

The Cytochrome b_6f Complex: A Regulatory Hub Controlling Electron Flow and the Dynamics of Photosynthesis?

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Summary

Photosynthetic organisms must respond to several external stimuli to adjust their photosynthetic performances in order to keep a high capacity of carbon assimilation and avoid photodamage. They do so integrating processes occurring at different time scales starting from very fast light harvesting, down to slow changes of electron flow and protein composition of the photosynthetic apparatus. Central to these regulatory mechanisms is the cytochrome b_6f complex, which regulates intersystem electron flow via photosynthetic control and modulates light harvesting capacity by acting as a redox sensor to trigger an acclimation process named state transitions. In this chapter we describe how the

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cytochrome b_6f complex performs to regulate these different functions in both plants and microalgae, proposing new mechanisms of regulation, and critically discussing how these processes are compatible with the existing literature on the structure and function of this complex.

I. Introduction

Photosynthesis is an old invention of nature, which, since its onset, has drastically shaped life on earth (Gould et al. 2008). O_2 evolution by early oxygenic photosynthetic organisms opened up the possibility of land colonization, while imposing new constraints on photosynthesis, mostly related to the interactions between molecular oxygen produced by water splitting in photosystem (PS) II and excited states of the pigment. This interaction triggers the generation of reactive oxygen species (ROS), which are harmful for the photosynthetic apparatus and biological systems in general. ROS production becomes particularly high when photochemical light conversion (charge separation) is impaired by a decrease in electron flow capacity, leading to a reduction of the electron acceptors for the two photosystems. Thus, the photosynthetic electron transfer chain must be regulated to avoid ROS production. Key regulatory processes are linked to the generation

of a proton motive force (a pH gradient, $\triangle pH$) across the thylakoid membrane. Acidification of the thylakoid lumen results in down regulation of light harvesting capacity of PSII via the onset of Non Photochemical Quenching (NPQ), a process which dissipates excess light energy as heat. This mainly occurs in the pigment-containing proteins of PSII, thanks to the $\triangle pH$ induced synthesis of specific xanthophylls (e.g. zeaxathin in plants and green algae, diatoxanthin in other microalgae) and the activation of protein sensors (PsbS in plants and LHCSR3 in eukaryotic algae) (Li et al. 2009).

Regulation of the pH gradient across the thylakoid membrane is central to the regulation of photosynthesis. To understand how the $\triangle pH$ is controlled, however, we need to consider the (literally) central role of the cytochrome b_6f complex (Cyt b_6f). This complex is both crucial in generating $\triangle pH$ but is also itself directly affected by that gradient. Lumen acidification modulates the rate of electron flow through the Cyt b_6f complex (termed "*photosynthetic control*", see Eberhard et al. 2008 and Fig. 22.1).

In what is usually seen as its primary role, in the linear electron flow (LEF) pathway, Cyt b_6f catalyzes the transfer of electrons originating from PSII, and transferred through the membrane via the plastoquinone (PQ) pool, to a hydrophilic one-electron acceptor protein (plastocyanin (PC) or a c-type cytochrome (Cyt c_6)). The electron transfer activity is coupled to proton translocation across the membrane. Reduced PC or Cyt c_6 (depending on species and growth conditions) is released from Cyt b_6f and binds to PSI, where it reduces the primary electron

Abbreviations: CBB – Calvin Benson Bassham cycle; CEF – Cyclic electron flow; Cyt – b_6f the cytochrome b_6f complex; Cyt – Cytochrome; Fd – Ferredoxin; FNR – Ferredoxin-NADP$^+$ oxidoreductase; FQR – Ferredoxin quinone reductase; GAPDH – Glyceraldehyde-3-phosphate dehydrogenase; LEF – Linear electron flow; NDH – NAD(P)H dehydrogenase; NPQ – Non Photochemical Quenching; P_{700} – Primary electron donor to PSI; PC – Plastocyanin; PQ – Plastoquinone; PQH$_2$ – Plastoquinol; PS – Photosystem; ROS – Reactive oxygen species
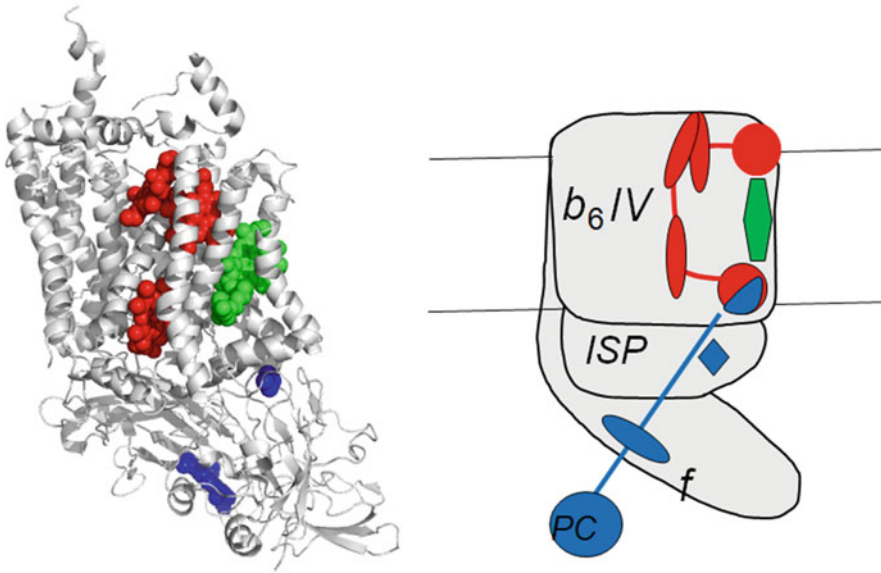
Fig. 22.1. Structure of the cytochrome $b_6 f$ complex (Stroebel et al. 2003). *Left*: atomic structure of the complex from *Chlamydomonas reinhardtii*. *Right*: Schematic representation of the complex. The low potential and high potential paths are represented in *red* and *blue*, respectively. The chlorophyll is shown in *green*. Catalytic activity of the high potential path is linked to H^+ release in the lumen, and consequently regulated by the establishment of a $\triangle pH$ across the membranes. The main subunits of the complex are represented: IV, subunit IV; b_6 and f, cytochrome b_6 and f, respectively; *ISP* Rieske protein containing and Fe-S cluster (*square*). *Closed circles*, *ellipses* and *polygons* indicate the quinone binding sites, hemes and chlorophyll, respectively. Note that only one monomer of the cytochrome $b_6 f$ dimer is shown.

donor of this complex, P_{700}. Electrons arriving at the donor side of PSI are transferred to its acceptor side via charge separation and there, either continue their journey towards the Calvin Benson Bassham cycle (CBB) via ferredoxin (Fd), ferredoxin NADP reductase (FNR) and then $NADP^+$, or are employed directly to provide reducing power for other cellular metabolic processes (such as nitrogen and sulfur metabolism, lipid, amino acid, pigment biosynthesis) via Fd oxidation (Eberhard et al. 2008). In addition to this linear pathway, the electrons from PSI can also be transferred back to Cyt $b_6 f$ via cyclic electron flow (CEF) around PSI (Shikanai 2007; Johnson 2011). This process, the significance of which was debated for many years, is now thought to occur in all oxygenic photosynthetic systems. Cyt $b_6 f$ is critical in controlling the relative efficiency of linear and cyclic flow, either indirectly (by setting the rate of plastoquinol (PQH_2) oxidation) or directly, e.g. via the formation of a biochemically stable PSI-Cyt $b_6 f$ complex (CEF supercomplex), which is seen in the green alga *Chlamydomonas reinhardtii* (see Chap. 23 by Minagawa). Finally, Cyt $b_6 f$ plays a specific role, which is not seen in its respiratory counterparts, the cytochrome bc_1 complexes. Being the site of PQH_2 oxidation, this complex senses the redox state of the photosynthetic electron transfer chain, and triggers the induction of state transitions, a process regulating the relative light absorption capacity of the two photosystems via the reversible migration of (a part of) the light harvesting complexes between the two photosystems (see Chap. 24 by Rochaix).

II. H^+/e^- Coupling in the Cytochrome b_6f Complex and Its Role in Photosynthetic Control

Photosynthetic electron flow is kinetically controlled at the level of PQH_2 oxidation at the lumenal side of the Cyt b_6f complex. Regulation can occur in response to acidification of the thylakoid lumen. This kinetic effect, known as *"photosynthetic control,"* is the consequence of the tight coupling between electron and proton transfer during oxidation of PQH_2 (West and Wiskich 1968). During this reaction, electrons originating from PQH_2 are divided between the Rieske Fe-S protein, the first step in the so-called high-potential chain (Fig. 22.1) and a cyclic route that comprises the b hemes (Q cycle; reviewed in Cramer et al. 2011), and possibly the c' (or c_n) heme located on the stromal side of the complex (see Chap. 15 by Zito and Alric). In isolated Cyt b_6f isolated from *Chlamydomonas* (Nitschke et al. 1992; Pierre et al. 1995) the Rieske protein has an E_m of $+290$ mV. Cyt f, which oxidizes the Rieske Fe-S cluster, has an E_m of $+330$ mV, and the two Cyt b_6 hemes have E_m values of -84 and -158 mV, respectively.

Due to the less positive E_m of the cytochrome comprising the b-heme pathway (as compared with Cyt f and the Rieske Fe-S protein), this route is referred to as the low-potential chain. According to the Q cycle mechanism proposed by Mitchell (1975) and modified by Crofts et al. (1983), quinones are oxidized and reduced at two distinct sites in the protein, the Q_o and Q_i (or Q_p and Q_n) sites, respectively, which are located on opposite sides of the membrane (Cramer et al. 2011). PQH_2 oxidation on the lumenal side is associated with the reduction of both Cyt f and heme b_L (Crofts et al. 1983) and the release of protons into the lumen. Oxidation of the b_6 hemes occurs through a two-step reduction of a PQ molecule at the Q_i site, possibly involving the recently discovered c' heme (Cramer et al. 2011; and Chap. 15 by Zito and Alric). The electron transfer sequence is (1) the reduction of Cyt b_L by PQH_2, a process that is coupled to proton release to the lumen, (2) electron transfer to b_H, and then (3) most likely a double electron transfer from these hemes to a PQ molecule bound at the Q_i site, a process that is coupled to proton uptake from the stroma. Overall, the Q cycle results in additional proton pumping into the lumen, and therefore increases the H^+/e^- ratio of photosynthetic electron transfer.

Previous work has suggested that this cycle is constitutively active in physiological conditions (Sacksteder et al. 2000), and can be bypassed only upon drastic modifications of the redox features of the low potential chain (Malnoe et al. 2011). On average, each electron passes twice through the Cyt b_6f complex, once through the low- and once through the high-potential pathways and transports 2 protons from the stroma to the lumen. The reaction $PQH_2 + PC^+ \leftrightarrow PQ + PC + 2H^+$ is expected to be sensitive to the lumenal H^+ concentration and this is simply as a result of a mass transfer effect. In particular, the pH effect would reflect the pH modulation of the formation of hydrogen bonds between the PQH_2 and protonatable residues in the Q_o site. One of these protonatable residues is His126 of the Rieske protein of *C. reinhardtii*. The role of His126 in the modulation of the pH effect on the quinol oxidation rate has been first demonstrated in the case of cytochrome bc_1 complexes (reviewed in Berry et al. 2000). Its involvement in the control of Cyt b_6f activity was confirmed by the pH profile of this complex in *Chlamydomonas*, where a pK of 6.25–6.5 has been detected (Finazzi 2002). This value is very close to the one previously attributed to His126 (Berry et al. 2000) of the Rieske protein in bacterial systems. The second residue involved in proton release in the lumen during PQH_2 oxidation is Glu78 of the PEWY sequence, which is extensively conserved in respiratory and photosynthetic cytochromes (Berry et al. 2000). This residue is located in a key position in the lumenal binding pocket

(see e.g. Hasan et al. 2013) and its removal significantly altered the pH dependence of PQH_2 oxidation in *Chlamydomonas* cells (Zito et al. 1998).

Although plausible, a careful examination of the features of PQH_2 oxidation in the Cyt b_6f complex suggests that the simple mass effect explanation invoked previously to explain the photosynthetic control is probably a too simplistic view. Indeed the E_m differences between the initial electron donor ($PQH_2 \sim 0$ mV) and the terminal acceptor (PC) is ~ 300 mV. This means that a very large pH gradient would be needed to counteract the driving force for electron transfer under steady state coupled conditions. However, it has been proposed that no such large proton gradient can be built in the thylakoid membranes under physiological conditions (Kramer et al. 2004). Measurements of the kinetics of P_{700} reduction following a light-dark transition, an indicator of the flux through the Cyt b_6f complex, indicate that no inhibition of PQH_2 oxidation normally occurs in unstressed plants across the range of light intensities experienced in nature (Harbinson and Hedley 1989; Ott et al. 1999). This also implies that the in vivo lumen pH is not acidic enough to inhibit Cyt b_6f activity, consistent with previous in vivo measurements of the size of the H^+ gradient in microalgae (pH gradient of about 1.5 units; Finazzi and Rappaport 1998).

Interestingly, a mutant of *Arabidopsis*, *pgr1*, has been shown to have a mutation in the Rieske protein that results in an altered pH sensitivity, with a pK of 6.5–7 (Jahns et al. 2002). This shift results in plants that are deficient in qE (the $\triangle$pH-mediated 'high-energy' quenching of PSII), and the electron flux through Cyt b_6f is inhibited at a pH that is too high to induce significant quenching. This is in principle consistent with the notion that the lumenal pH does not affect the turnover of the Cyt b_6f complex in vivo, unless its pH sensitivity is modified. On the other hand, Joliot and Johnson (2011) demonstrated that partial uncoupling of the thylakoid membrane, by infiltrating leaves with nigericin, resulted in an inhibition of NPQ, as expected if the $\triangle$pH is inhibited, but also in a net reduction of the electron transport chain. Overall, these observations can be rationalized assuming that the lumen pH is involved in regulating the activity of the Cyt b_6f complex, although possibly via a different mechanism than a simple H^+-mediated mass effect.

As an alternative explanation for the photosynthetic control, one can assume the existence of a pH dependent equilibrium between two kinetic states of the Cyt b_6f complex (characterized by a fast or a slow electron transfer rate). Under the hypothesis that the equilibration rate between the two states is faster than the electron transfer reaction, the pH dependence of Cyt b_6f activity can be mimicked in the frame of this very simple model, where only two kinetic states and a pK regulating their equilibration is conceived (Rappaport and Finazzi, unpublished data). The resolution of the structure of the Cyt b_6f and of its respiratory counterpart (cytochrome bc_1) has provided a structural basis supporting this model. It has been shown that diversion of electrons into the high and low potential branches occurs via a conformational change at the level of the Rieske iron sulfur protein. In the related mitochondrial bc_1 cytochrome complexes, the rotational swing of the Rieske protein extra-membrane domain leads to a cluster movement over 20 Å, which prevents a double electron injection into the high potential chain (for a review see Berry et al. 2000). Evidence for this movement was provided by three-dimensional Cyt bc_1 structures (Xia et al. 1997; Iwata et al. 1998; Zhang et al. 1998), electron paramagnetic resonance spectroscopy (Brugna et al. 2000), and mutagenesis studies (for review, see Darrouzet et al. 2001). Several lines of evidence support the existence of a similar movement in the b_6f complex. These are two-dimensional crystals (Breyton 2000a), viscosity studies (Heimann et al. 2000), electron transfer from the Rieske ISP to Cyt f in vitro (Soriano et al. 2002), inhibitor binding (Schoepp et al. 1999), mutagenesis studies (de Vitry et al. 2004), and three-

dimensional Cyt b_6f structures (Kurisu et al. 2003; Stroebel et al. 2003). Thus, one may speculate that the fast kinetics would be the one where the ISP and the PQH$_2$ lie close together in the lumenal site, while the slow one would correspond to the ISP protein being close to the Cyt f moiety, thereby preventing proper interaction of PQH$_2$ with its binding site. The pK involved in this regulation would mainly be the one of His126, which is involved in making H–bonds with the quinone.

As mentioned above, the main consequence of the Q cycle is the increase in the H$^+$/e$^-$ stoichiometry. A corollary of this model is that, although increased, the stoichiometry of protons pumped into the lumen per electron is fixed, being set by the conformational changes in the lumenal site. This could be a limitation for photosynthesis where the H$^+$ gradient is required not only for photoprotection, but obviously also for ATP synthesis. In oxygenic photosynthesis, carbon assimilation is mainly driven by LEF, which produces NADPH as well as ATP, although probably in a ratio not sufficient to support the formation of glyceraldehyde-3-phosphate, the exported product of the CBB cycle (see Allen 2003 for a discussion). On the other hand, other electron fluxes, e.g. to reduce nitrogen, have different ATP:NADPH requirements. Thus every means available to modulate the H$^+$/e$^-$ ratio in a flexible way, depending on demand, would allow plants to better regulate carbon assimilation, other metabolism and photoprotective responses. This possibility was investigated in *Chlamydomonas* using a genetic approach.

Mutants with an engineered Glu78 residue in subunit IV of Cyt b_6f were generated and their responses to pH changes were tested (Zito et al. 1998; Finazzi 2002). Spectroscopic analysis revealed that this residue not only controls PQH$_2$ oxidation, as previously reported (Finazzi 2002), but also modulates the number of charges that are transferred through the Cyt b_6f complex. This suggests that a transfer of protons from the stroma to the lumen during catalysis takes place in the complex itself, and that this is linked to PQH$_2$ oxidation at the lumenal pocket; suggesting the existence of a H$^+$ pumping activity in the complex (Finazzi 2002). This is in agreement with the finding of a relevant isotopic effect on the initial rate of charge transfer within the complex (Deniau and Rappaport 2000).

To account for this observation a model was proposed (Fig. 22.2), which reconciles the Q cycle with experimental observations showing that dicyclohexylcarbodiimide (DCCD), a chemical capable of modifying protonatable residues, induces a significant proton slip during catalysis (Brandt and Trumpower 1994). The model assumes that a channel exists between the lumenal and stromal sites for PQ redox reactions, providing an internal shuttle for protons (Finazzi 2002). This channel would be operational either in the stroma to lumen direction (proton pumping) or in the opposite way (proton slip) depending on the pH of the two compartments and on the redox state of the electron flow cofactors within Cyt b_6f (i.e. the high and low potential b_6 hemes). The transition from proton slip to proton pumping, observed in the pH 5–6 region, i.e. in the range compatible with the pK of this residue was compatible with the observation that the number of charges transferred through the cytochrome complex during turnover becomes constant in mutants where the Glu78 residue has been changed to a neutral residue, suggesting that this glutamic acid residue would act as a gate, modulating the opening and closing of this channel depending on lumenal pH. Overall the activity of the redox-linked proton could represent a useful mean to help maintain pH homeostasis in the chloroplast lumen by modulating the H$^+$/e$^-$ stoichiometry in vivo.

Although appealing, this hypothesis has not yet found a solid structural support. Indeed, no clear proton wire has been detected between the stromal and lumenal sides in the structure of Cyt b_6f complexes, at variance with the H$^+$ chain that is observed at the level of Cyt f, to channel proton release into the lumen (Cramer et al. 2011).
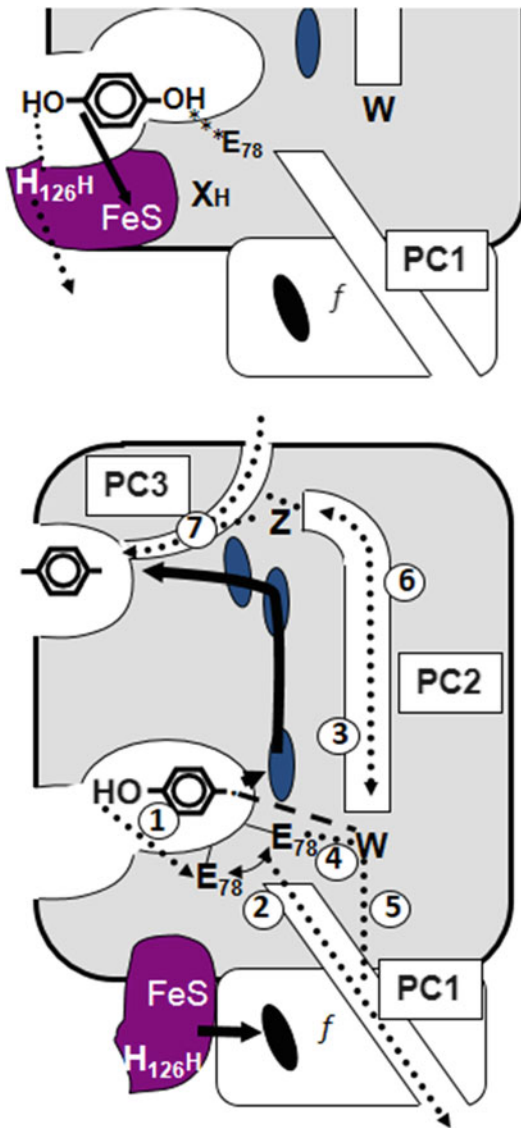
Fig. 22.2. Proposed mechanism for the redox controlled H^+ pump in the Cyt b_6f complex. PQH_2 binding at the lumenal sites occurs via H^+ interaction with the H_{126} and E_{78} residues. This results in H^+ and electron transfer to the Rieske protein (*top*). The Rieske protein is then oxidized by Cyt f (*thick arrow*) thanks to conformational changes of its head. In parallel, a movement of the protonated semiquinone allows the transfer of an electron to the b_L heme. Oxidation of neutral semiquinone is proposed to take place via the E_{78} residue (1), which is connected to a proton channel PC1 (2). E_{78} would modulate the activity of a transmembrane H^+ channel (PC2, (2)) gated by two protonatable residues (*W* and *Z*). Their protonation would be regulated by electrostatic interactions with the semiquinone (*dashed line*) and the b_6 hemes.
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III. A Redox Kinetic Control of Cytochrome b_6f?

As discussed above (Sect. II), the effective rate limiting step in the electron transport chain is generally thought to be the oxidation of PQH_2 by Cyt b_6f. However, the factor that limits overall photosynthesis will often be downstream of this, either the supply of CO_2 for ribulose-5-phosphate reduction or the recycling of inorganic phosphate. For example, if a leaf is deprived of CO_2, either naturally, due to drought induced stomatal closure, or artificially, by lowering the external CO_2 supply, demand for both NADPH and ATP declines. In simple terms this should result in a reduction of the whole electron transport chain, through a simple blockage of electron flow. This is not, however, what is seen. If CO_2 is abruptly removed, PSII acceptors, including the PQH_2 pool, become reduced, but Cyt f and the high potential chain become more oxidized, the opposite of what might be expected (Golding and Johnson 2003; Golding et al. 2005). This observation clearly shows that flux through the high potential chain is regulated at the level of the Cyt b_6f complex.

As already discussed, a simple effect of pH on the oxidation of PQH_2 might explain this observation; however various observations suggest that more is occurring. Importantly, another major pH-dependent process, high-energy state quenching, differs both in terms of kinetics of induction and steady state levels from the down-regulation of electron transport. For example, a wide range of NPQ values can be observed with no change in the flux through Cyt b_6f and, conversely, Cyt b_6f can be downregulated without a corresponding increase in NPQ (Ott et al. 1999).

Fig. 22.2. (continued) The activity of this channel would affect the H^+/e^- stoichiometries of PQH_2 oxidation, because of oscillation between proton pumping ((3) + (4) + (5)) and proton slip (6). (7) Protonation of the quinone at the stromal side. *Dotted lines*: proton transfer reactions. Solid lines electron transfer reactions (Modified from Finazzi 2002).

This led Johnson (2003) to put forward a model for feedback regulation by the redox poise of the PSI acceptor pool. Initially it was suggested that this may be via thioredoxin; however, later evidence pointed to NADPH redox poise being the crucial factor (Hald et al. 2008). Mutants in which photosynthesis was inhibited by antisense repression of glyceraldehyde-3-phosphate dehydrogenase, which consumes NADPH, showed a strong downregulation of Cyt b_6f activity and an oxidation of the high potential chain. Meanwhile, mutants repressed in FNR had a reduced electron transport chain. Both showed high levels of pH-dependent fluorescence quenching (a form of NPQ), indicating that the difference could not be explained by lumen pH alone.

Experiments such as these speak against a simple role for lumen pH controlling the flux of electrons through the Cyt b_6f complex. A notable example would be the *Arabidopsis pgr5* mutant, where the control of electron flux through Cyt b_6f fails to operate (Munekage et al. 2002b; Nandha et al. 2007; Joliot and Johnson 2011). This mutant was first identified as a result of a screen for plants deficient in NPQ (Munekage et al. 2002a); however, in contrast to the *pgr1* mutant (Jahns et al. 2002), identified in the same screen, *pgr5* has a reduced electron transport chain across all conditions, implying that the PQH$_2$ oxidation is not being down regulated. At low CO$_2$ it does, however, generate NPQ, implying the formation of a large △pH (Nandha et al. 2007). This alone is not sufficient to cause an inhibition of PQH$_2$ oxidation. In another study by Joliot and Johnson (2011) it was shown that infiltration of leaves with the ionophore nigericin, which dissipates △pH without affecting the potential gradient, did not inhibit PSII electron transport but did prevent both NPQ formation and the downregulation of Cyt b_6f activity. Results such as these speak in favor of a role of pH in regulating electron flow, but not through a simple photosynthetic control mechanism. Rather, the pH may modulate the redox sensitivity, shifting the E_m of redox sensitive components involved in regulation (Fig. 22.3). Johnson (2003) showed that inhibition of the Cyt b_6f complex by dithiothreitol is pH
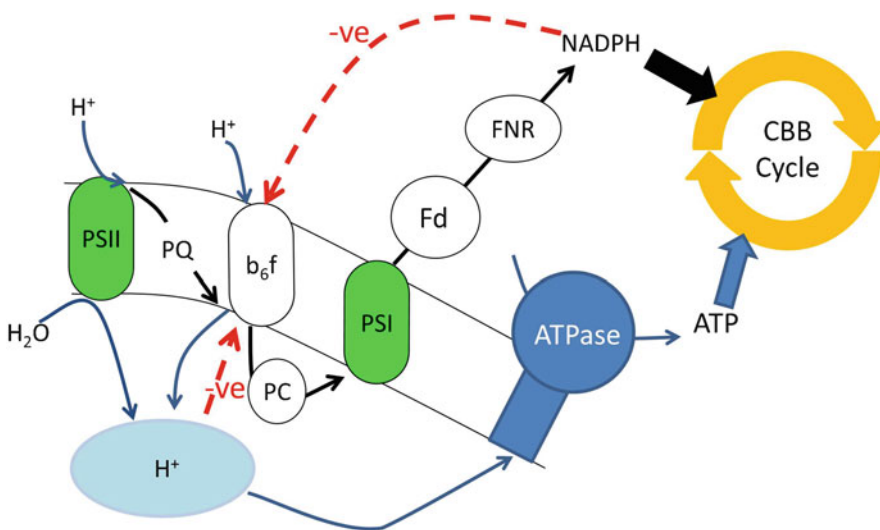
Fig. 22.3. Feedback control of Cyt b$_6$f. Linear electron transport generates NADPH (*black arrows*). At the same time, there is a net movement of protons into the thylakoid lumen, generating a △pH, which is used to drive ATP synthesis (*blue arrows*). Under optimal conditions, NADPH and ATP are used in the Calvin-Benson-Basham (CBB) cycle. If the CBB cycle is inhibited, e.g. due to drought, NADPH feeds back to down-regulate the Cyt b$_6$f complex, while the △pH, which is no longer being dissipated in ATP synthesis, inhibits electron flow either by directly inhibiting PQH$_2$ oxidation or indirectly by modulating the sensitivity to NADPH. See text for further explanation.

sensitive, with a shift in E_m of -90 mV/pH. Hence, changes in the pH in the thylakoid lumen may increase the sensitivity of the Cyt b_6f complex to reducing agents, promoting at the same time redox regulation of electron flow, most likely exerted by NADPH.

IV. Linear Versus Cyclic Electron Flow

Among electron transport processes alternative to LEF, cyclic electron flow (CEF), which only involves PSI, has a major role in allowing plants and algae to control their ATP production via the regulation of the proton gradient, $\triangle$pH. CEF in flowering plants has been the subject of a number of reviews in recent years (e.g. Shikanai 2007; Johnson 2011) and we do not intend here to review in detail the evidence for its occurrence. Rather the focus will be on the regulation of this pathway and the involvement of the Cyt b_6f complex. The functioning of CEF in plants follows the same mechanism: electrons produced at the PSI acceptor side are oxidized in a way that results in the reduction of PQ, feeding electrons back into the electron transport chain between the two photosystems. That reaction may be catalyzed by either an NAD(P)H dehydrogenase (NDH) complex homologous to Complex I in mitochondrial electron transport (Rumeau et al. 2007; Shikanai 2007), or via an alternative reaction possibly involving Cyt b_6f. The relative contribution of the two paths is still under debate. Plants deficient in the NDH complex appear to perform well across a wide range of conditions, though there is evidence that they have increased sensitivity to drought stress (Rumeau et al. 2007; Lehti-maki et al. 2010). It seems, however, that NDH is not essential for plant growth. On the other hand, a recently identified mutant with enhanced CEF activity in *Arabidopsis* shows higher accumulation levels of this complex (Livingston et al. 2010), and it was recently proposed that this complex could mediate an H_2O_2 driven activation of CEF (Strand et al. 2015).

In addition to the NDH pathway, electron transfer can take place via a pathway often referred to as the FQR (ferredoxin quinone reductase) pathway (Bendall and Manasse 1995). In early studies, the FQR pathway was proposed to involve the Cyt b_6f complex (Tagawa et al. 1963). However, later studies hypothesized a distinct enzyme for FQR that bypasses Cyt b_6f and directly interfaces with PQ pool reduction by Fd (Bendall and Manasse 1995). No one, however, was able to identify or purify the FQR specific enzyme until recently where evidence was presented for FQR being a complex of PGR5-PGRL1 (Hertle et al. 2013).

The physical association of the Cyt b_6f complex with PSI and FNR has also been suggested as a platform for the electron transfer in the FQR pathway (Carrillo and Vallejos 1983; Arnon 1995). Mathematical modeling of the electron transfer (Laisk 1993) and in vivo observation of its high efficiency (Joliot and Joliot 2002) suggest that FQR activity might operate in a complex involving Cyt b_6f and PSI. Indeed, Cramer and his coworkers reported that the Cyt b_6f complex co-purified with FNR and was reduced by Fd (Zhang et al. 2001). Physical interactions between PGRL1 and PsaD (subunit of PSI), PetB (Cyt b_6), FNR, and PGR5 were shown by a yeast two-hybrid assay using the corresponding genes from *Arabidopsis* (DalCorso et al. 2008). This suggests that the formation of a CEF competent supercomplex is likely required to stabilize this pathway in a system where it could otherwise be outcompeted by linear flow.

While no such supercomplex was found in spinach leaves (Breyton et al. 2006) and indeed it has been argued that no such complexes are required (Johnson 2011; Joliot and Johnson 2011), biochemical analysis of thylakoid membranes from *Chlamydomonas* allowed Minagawa and colleagues (Iwai et al. 2010) to isolate such a complex from State 2-locked cells, where CEF is the prominent electron flow pathway. Using a sucrose density gradient, they purified a super-supercomplex composed of the PSI-LHCI supercomplex

with LHCIIs, Cyt b_6f, FNR, and PGRL1 in a fraction heavier than that of the PSI-LHCI supercomplex alone. Spectroscopic analyses of this super-supercomplex indicated that, upon illumination, reducing equivalents downstream of PSI were transferred to Cyt b_6f, while the oxidized PSI was re-reduced by reducing equivalents from Cyt b_6f (Iwai et al. 2010; see Chap. 23 by Minagawa for a further description of this CEF supercomplex).

What is the role of this supercomplex, and why is it observed only in *Chlamydomonas*? To answer these questions one has to consider that a key point in our understanding of CEF is the question of how the relative fluxes through LEF and CEF are regulated. It is acknowledged that photosynthetic complexes are not evenly distributed in the thylakoid membrane (Albertsson 2001). Functional PSII is localized in the membrane stacks. PSI is distributed between the grana margins ($\sim$70 %) and the stromal lamellae. Cyt b_6f is evenly distributed throughout the membrane. It has been shown that the diffusion of PQ/PQH_2 in the thylakoid membrane is highly restricted, probably due to the very high protein concentrations that characterize this membrane (Tremmel et al. 2003). This means that PQ localized in the granal stacks will primarily act in mediating electron transport between PSII and those Cyt b_6f complexes localized in the stacked regions. For reasons of access, it is unlikely that electrons will be transferred from Fd to Cyt b_6f complexes localized in the granal regions. Rather, a separate pool of Cyt b_6f, with associated PQ will be found in the stromal membranes. The physical distance between the different domains and the limitations on PQ diffusion mean that these different pools could be quite separate, thus promoting independent functioning of the photosynthetic complexes present in the grana and grana margins (LEF) and the stromal lamellae (CEF). On the other hand, thylakoid stacking is less defined in *Chlamydomonas* and algae in general, and therefore the functional segregation of LEF and CEF based on the physical segregation of the photosynthetic complexes in two distinct domains hardly applies to these algae. It is therefore possible that the super-supercomplex, which localize the mobile electron carriers (PQ, Fd, and PC) within a physically restricted space would ensure the compartmentation of the two pathways, thereby preventing CEF from being outcompeted by LEF activity. By doing this, super-supercomplex formation would play a similar role to that suggested for the segregation of PSI and PSII in the granal and stromal lamellae in plants.

V. Role of Cytochrome b_6f in State Transitions

In oxygenic photosynthesis, the two photosystems working in series have distinct pigment containing antenna complexes with distinct light absorbance properties. PS I has an enriched absorption capacity in the far-red region, whereas PS II lacks the far-red absorption capacity, but has a higher efficiency in the spectral regions where chlorophyll *b* absorbs (i.e. around 475 and 650 nm). Thus, in natural environments, where light quality and quantity fluctuate with time (Allen 1992; Bellafiore et al. 2005) unbalanced absorption can take place. This effect can be minimized by a phenomenon known as state transitions. Discovered independently by Murata and Sugahara (1969) and Bonaventura and Myers (1969), this process relies on a redox-triggered phosphorylation of the PSII antenna complexes (LHCII), which leads to a physiological displacement of LHCII from PSII to PSI under conditions where absorption of the former is enhanced (i.e. by enhanced light absorption by chlorophyll *b*). This state is called State 2. Conversely, over-excitation of PSI (e.g. by far red light) leads to the reassociation of LHCII with PSII (State 1; Allen 1992).

Pharmacological and genetic work has shown that state transitions are triggered by the PQ pool, which acts as a redox regulator of the phosphorylation of LHCII (Wollman 2001), via the activation of an LHCII kinase. However, reports exist claiming that LHCII

kinase(s) is/are not only activated by the reduced PQ pool, but also deactivated by the reduced thioredoxin pool that is downstream of PS I in the stroma of the chloroplasts in pumpkin (Rintamaki et al. 2000) and pea (Hou et al. 2002), and in *C. reinhardtii* (Vink et al. 2004). Why is the PQ pool responsible for such a redox signaling role? Being functionally located between PSII and PSI this redox active molecule can "sense" the relative light harvesting capacity of the two photosystems, being largely reduced when PSII activity overcomes that of PSI, and becoming oxidized when PSI prevails.

The nature of the kinase responsible for LHCII phosphorylation has been first identified in *Chlamydomonas* thanks to a genetic screening: it is a Ser/Thr kinase (Stt7) present in the chloroplast thylakoid membranes (Depege et al. 2003). Later, it was shown that an ortholog exist in plants (STN7; Bellafiore et al. 2005). The reversible phosphorylation of LHCII observed with thylakoids during state transitions implies that a phosphatase (TAP38/PPH1) is also active, being responsible for the dephosphorylationn of LHCII (Pribil et al. 2010; Shapiguzov et al. 2010).

Signal transduction from PQH_2 to the Stt7/STN7 kinase is performed by the Cyt b_6f complex, as shown by several independent pieces of evidence. Wollman and Lemaire (1988) found that *C. reinhardtii* mutants lacking Cyt b_6f were unable to undergo a State 2 transition, indicating an essential role of this complex in the kinase activation. Later, spectroscopic (Vener et al. 1997) and genetic approaches (Zito et al. 1999) revealed that the committed step in kinase activation is the binding of PQH_2 to the lumenal quinone-binding pocket (Q_o site) of Cyt b_6f. While a consensus exists on the nature of the partners involved in the signaling from light to the kinase, the mechanism leading to the activation of Stt7/STN7 by reduced PQ is still under debate.

Based on a pharmacological analysis of kinase activation (Finazzi et al. 2001) a model has been built for this activation (Finazzi 2005), which involves several steps (Fig. 22.4). The signal from PQH_2 would be transduced by an "active" Cyt b_6f complex, capable of switching between a docking conformation, which activates the kinase, and a releasing one, where the active kinase becomes capable of interacting with LHCII. Docking would involve conformational changes at the level of the Rieske protein and results in the phosphorylation of the Cyt b_6f-associated subunit PetO (Hamel et al. 2000; Finazzi et al. 2001; Wollman 2001). This step would precede phosphorylaytion of LHCII, which likely requires the release of the Stt7 kinase by a subsequent conformational change. This is suggested by the observation that while the PQ analog tridecylstigmatellin blocks Cyt b_6f catalysis and LHCII phosphorylation by preventing the movement of the Rieske protein head (Zhang et al. 1998; Breyton 2000b; Finazzi et al. 2001), this chemical does not affect the phosphorylation of PetO (Wollman 2001).

How are the conformational changes occurring in the lumenal region of the complex transduced to the stromal site where the active moiety of the kinase is located? Analysis of the Stt7 kinase has suggested that this protein contains a putative transmembrane helix. This helix might, therefore, be directly involved in sensing PQH_2 binding to the Q_o site, as already suggested (Vener et al. 1997). However, the existence of a direct signaling within the cytochrome complex is also possible, and supported by phenotypic analysis of Cyt b_6f mutants of *Chlamydomonas*. In particular, a mutant was generated (Zito et al. 2002) to fuse a small Cyt b_6f subunit (PetL) with subunit IV to "transform" the b_6f complex, which contains seven helices in its core subunits (Cyt b_6 and subunit IV), into a bc_1-type complex (which contains eight helices in its unique core complex subunit, Cyt b). At variance with expectations, this mutation did not lead to an altered electron flow, or PQH_2 binding to the lumenal Q_o site of the modified b_6f complex, but led to a complete abolition of state transitions.

Based on these results, it was proposed that docking of the kinase, which follows the structural changes induced by the PQH_2-
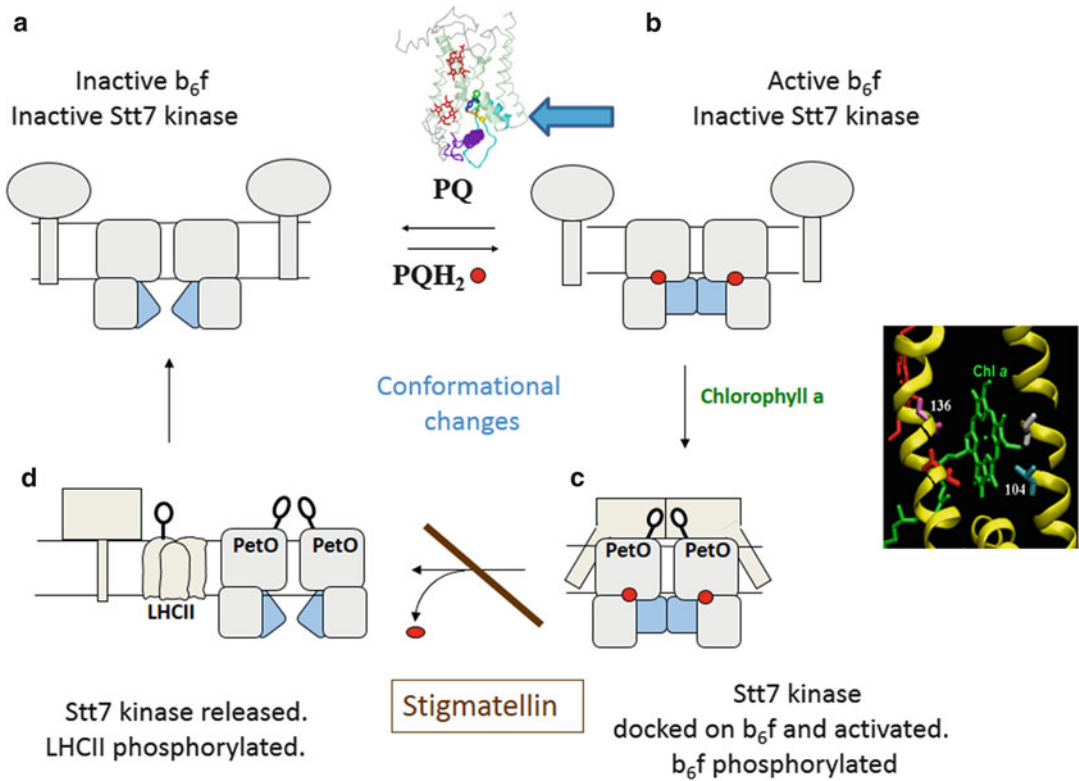
Fig. 22.4. Hypothetical mechanism of Stt7 activation by the cytochrome b_6f complex. The model conceives that upon binding of the PQH_2 molecule (*red dot*) to the lumenal Q_o site of Cyt b_6f, conformational changes in the lumenal part of the Rieske protein (*blue*) would take place (transition form "**a**" to "**b**"). This would allow docking of the kinase (*spheres* and *sticks*) on the "lipid exposed" part of the complex, in close vicinity to the chlorophyll ring (**c**), leading to kinase activation (represented as *boxes* and *sticks*) and phosphorylation of the Cyt b_6f associated subunit PetO (*black loops*). Release of the active kinase after dissociation of the quinone from its binding pocket (**d**) would trigger LHCII trimer-kinase interactions, as required for antenna complex phosphorylation. The site of PQH_2 and kinase docking on Cyt b_6f, as well as the site of inhibition by stigmatellin are shown.

Cyt b_6f interaction, might take place on the cytochrome portion located in the proximity of the C terminus of subunit IV (where the PetL subunit was fused). This being the case, the mutant phenotype could be explained assuming that because of the presence of an additional helix close to its docking site, the formation of the kinase-Cyt b_6f complex was prevented, even if the cytochrome complex was in its "active" state. Interestingly, the 3D structure of Cyt b_6f from *Chlamydomonas*, (Stroebel et al. 2003), has revealed the presence of a chlorophyll molecule, which is exposed to the lipid phase through its tetrapyrrole ring, being sandwiched between helices F and G of subunit IV (see also Chap. 9

by Cramer and Hasan). Its phytol chain goes deeply into the complex structure ending up in the lumenal, PQH_2 binding pocket. Thus it is conceivable that the chlorophyll could "sense" the binding of PQH_2 to the Cyt b_6f complex via the phytol chain and transduce this information to the kinase docking site, via the chlorophyll ring (Finazzi 2005).

The possible involvement of the chlorophyll ring in kinase activation was later confirmed by the analysis of mutants affecting the environment of the chlorophyll ring in the complex, again in *Chlamydomonas* (de Lavalette et al. 2008). This study showed that in one mutant strain where the chlorine ring was likely displaced from its original site

because of steric hindrance, state transitions were largely compromised, although PQH_2 binding to the Q_o site was not significantly affected. Based on these results, a scenario can be proposed to account for the mechanism of Stt7 activation by the Cyt b_6f complex (Fig. 22.4). However, further work will be needed to further confirm this model at the experimental level.

VI. Conclusions

The role of the cytochrome b_6f complex in electron flow is a topic that has been investigated for many years combining functional and genetic approaches in both plants and microalgae. The resolution of the structure of this complex has led to major advances in this field, bridging the functional mechanism to the precise structural changes within the complex. Biochemical analyses have since then been instrumental in defining a further level of regulation, which involves a different interaction of the cytochrome with other photosynthetic complexes, as exemplified by the isolation of the PSI-Cyt b_6f supercomplex from *Chlamydomonas*. However, despite these major achievements, big questions are still open concerning both the intimate electron transfer mechanisms (the proton electron stoichiometry during catalysis, the obligatory occurrence of the Q cycle during turnover, proton versus redox regulation of the activity, and the possible existence of a signal transduction pathway within the complex allowing the activation of the kinase responsible for state transitions by reduced plastoquinone molecules). Major multidisciplinary efforts will be needed in the future to cast light on these fundamental aspects of bioenergetics.

Acknowledgments

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Chapter 23

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A Supercomplex of Cytochrome *bf* and Photosystem I for Cyclic Electron Flow

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Summary

In oxygen-evolving photosynthesis, photochemical energy conversion occurs via electron transport in the thylakoid membranes, resulting in the reduction of $NADP^+$ in the stroma and the concomitant pumping of protons into the thylakoid lumen. Those electrons flow into two different pathways in the thylakoid membranes: the linear electron flow pathway from water to $NADP^+$ via photosystem II and photosystem I (PSI) in series and the cyclic electron flow pathway around PSI. While the operational site(s) for the cyclic electron flow has been elusive, recent studies are unraveling its molecular details. After providing an overview of the general understanding of the cyclic electron flow, this review focuses on a recent report on the super-supercomplex that is composed of the cytochrome b_6f complex, photosystem I with its own light-harvesting complex, the light-harvesting complex for photosystem II, and the ferredoxin-NADPH oxidoreductase, which exhibits cyclic electron flow in the green unicellular alga *Chlamydomonas reinhardtii*.

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I. Introduction

Because plants and algae typically do not have means to escape from adverse environments, their ability to acclimate to the environments is essential if they are to survive in their niche. In natural environments where the quality and quantity of light fluctuates, photoacclimation of the photosynthetic machinery is particularly important for photosynthetic organisms to optimize their photosynthetic efficiency and to protect their machineries from photooxidative damage. Photosynthesis is a process of photochemical energy conversion that occurs via electron transport in the thylakoid membranes of chloroplasts and results in the reduction of $NADP^+$ in the stroma and the concomitant pumping of protons into the thylakoid lumen. The NADPH that is generated by electron flow and the ATP that is synthesized by using the proton motive force across the thylakoid membrane are required to assimilate CO_2 in the Calvin-Benson cycle. Photosystem I (PSI) and II (PSII) are multi-subunit pigment-protein complexes where the charge separations elicited by light energy take place to drive electron flow. Electrons that are generated in the photosystems flow into two different pathways in the thylakoid membranes: the linear electron flow (LEF) pathway from water to $NADP^+$ via PSII and PSI in series and the cyclic electron flow (CEF) pathway around PSI. While

the operational site(s) for CEF has been elusive, recent studies are unraveling its molecular details. In this chapter, I introduce a recent study on a super-supercomplexes that is composed of the cytochrome b_6f complex (Cyt bf), PSI with its own light-harvesting complex (LHCI), the PSII light-harvesting complex (LHCII), the ferredoxin (Fd)-NADPH oxidoreductase (FNR), as well as several other membrane proteins showing CEF activity in the green unicellular alga *Chlamydomonas reinhardtii* (Iwai et al. 2010).

II. Cyclic Electron Flow Is Essential

It is crucial to achieve an ATP:NADPH ratio of 3:2 in the stroma in order to assimilate CO_2 in the Calvin-Benson cycle; however, this balance cannot be achieved by LEF alone (Allen 2003). As illustrated in Fig. 23.1, 4 protons are released into the lumen and 2 reduced plastoquinone (PQ) molecules are released into the intersystem pool after 4 turnovers of PSII upon capturing 5 photons assuming the quantum yield of the photo-chemistry is 0.8 (Björkman and Demmig 1987). At the Q_o (or Q_p) site of Cyt bf, 2 molecules of PQH_2 release 4 protons into the lumen and transfer 2 electrons to Pc. The yield of protons and electrons is doubled here (Sacksteder et al. 2000) by the so-called 'Q cycle mechanism' (Mitchell 1976). In total, 8 protons are released from Cyt bf into the lumen, and 4 Pc and then 2 $NADP^+$ are reduced. After all, 12 protons that are released into the lumen via LEF are used to rotate the CF_o subcomplex of ATP synthase, where 3 ADP molecules are phosphorylated per single rotation. Because the number of the proton-binding c-subunits in the CF_o sub-complex is 14 (Seelert et al. 2000), the 12 protons generated by LEF can only rotate the CF_o subcomplex 0.86 rounds, which is coupled to the synthesis of 2.6 molecules of ATP. To synthesize 3 molecules of ATP, plants and algae therefore need to re-route 1 electron from PSI to Cyt bf in order to

Abbreviations: AA – Antimycin A; CEF – Cyclic electron flow; Cyt bf – cytochrome b_6f complex; DBMIB – 2,5-dibromo-3-methyl-6-isopropylbenzoquinone; DCMU – 3-(3,4-Dichlorophenyl)-1,1-dimethylurea; DMBQ – 2,6-dimethyl-p-benzoquinone; FCCP – Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; Fd – Ferredoxin; FNR – Ferredoxin-NADPH oxidoreductase; FQR – Ferredoxin-plastoquinone oxidoreductase; LEF – Linear electron flow; LHCI and LHCII – Light-harvesting complex protein I and II; NDH – NAD(P)H dehydrogenase; NPQ – Non-photochemical quenching; Pc – Plastocyanin; PQ – Plastoquinone; PSI and PSII – Photosystem I and II
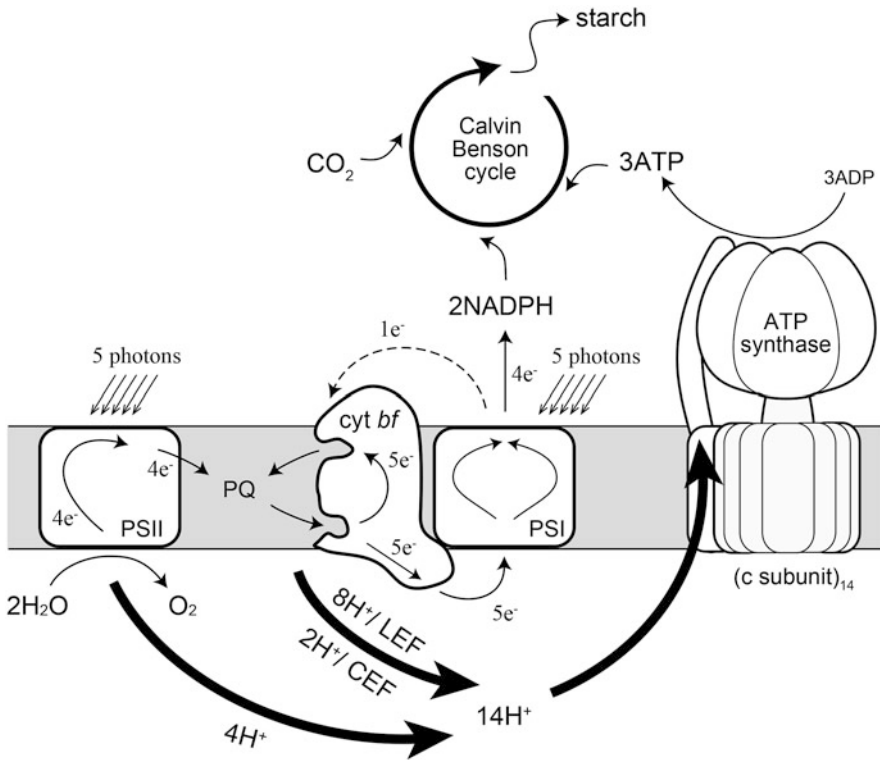
Fig. 23.1. Schematic representation of the movement of protons and electrons by the photosynthetic electron transport chain in chloroplasts. The pathways and stoichiometry of light-driven electron transport, proton translocation, and ATP synthesis in the thylakoid membranes are shown. *Thick arrows* represent the pathways of protons; *thin solid* and *thin dashed arrows* represent LEF and CEF, respectively.

pump 2 extra protons into the lumen. It is CEF that enables such electron re-routing from PSI to Cyt *bf*. CEF is thus essential for CO_2 assimilation (Munekage et al. 2004). The scheme described here indicates that the quantum yield of PSII, in which 5 photons are required for 4 turnovers, is lower than that of PSI, where 5 photons are required for 5 turnovers (Fig. 23.1). To put it another way, the quantum yield in PSII is 20 % lower than in PSI because 20 % of the electrons in PSI are re-circulated by the CEF pathway. In natural systems, ATP and NADPH could be consumed in various other cellular reactions and the demand for each fluctuates from time to time. Therefore, photosynthetic organisms need to constantly adjust the relative ratio of the two modes of electron flow. Another role of CEF in chloroplasts is to establish a high ΔpH across the thylakoid membrane to ac-

tivate non-photochemical quenching (NPQ). This is especially important under adverse environmental conditions where the capacity for CO_2 fixation cannot catch up with the amount of absorbed light. CEF is therefore crucial for achieving the correct balance of NADPH and ATP in the thylakoid stroma for CO_2 assimilation and for protecting its photosynthetic machineries from photodamage. Two possible electron transfer pathways have been proposed for CEF: an Fd-dependent pathway and an NAD(P)H dehydrogenase (NDH)-dependent pathway (Shikanai 2007). In the NDH-dependent pathway, NDH mediates NADPH oxidation and PQ pool reduction in much the same way as the complex I does in mitochondria (Ogawa 1991). However, how electrons on Fd are transferred to the PQ pool in Fd-dependent CEF (Fd-CEF) has, until recently, been unclear.

III. Cyclic Electron Flow and the Redox Status of the Chloroplast

Each of the two charge separation devices in the thylakoid membranes, PSI and PSII, has a distinct pigment system with unique absorption characteristics. Thus, an imbalance in the distribution of energy between the two photosystems tends to occur in natural environments, where light quality and quantity fluctuate with time (Allen 1992; Bellafiore et al. 2005; Minagawa 2011). Because the two photosystems are connected in series under normal conditions, plants and algae need to constantly balance their excitation levels to ensure an optimal efficiency of electron flow. State transitions occur under such conditions to balance the light-harvesting capacities of the two photosystems and to minimize the unequal distribution of light energy. This short-term adaptation involves thylakoid-bound protein kinase(s) that are responsible for the phosphorylation of LHCII is a process called state transitions. When PSII is preferentially excited, LHCIIs are phosphorylated, and the light-harvesting capacity of PSII decreases (State 2). Conversely, when PSI is preferentially excited, LHCIIs are de-phosphorylated, and the light-harvesting capacity for PSII increases (State 1). At room temperature, State 1 can be characterized by a high yield of chlorophyll fluorescence, arising primarily from PSII, and State 2 can be characterized by a low yield of chlorophyll fluorescence. In *C. reinhardtii*, it was suggested that the modulation of CEF occurs in parallel with state transitions (Finazzi et al. 2002). When the light-induced reduction of Cyt *bf* was probed in State 1- and State 2-adapted cells, a differential sensitivity to the addition of the Q_B site inhibitor of PSII 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) was observed. DCMU blocked the reduction of Cyt *bf* in State 1 but not in State 2. An identical sensitivity to an inhibitor of Cyt *bf*, 2,5-dibromo-3-methyl-6-isopropylbenzoquinone

(DBMIB), was observed in both State 1 and State 2, suggesting that PSII-independent CEF only occurs when the cells are in State 2 (Finazzi et al. 1999).

Other reports provided more information about CEF in *C. reinhardtii*. Under anaerobic conditions, the independent knock-down of three thylakoid membrane proteins, PGRL1, CAS, and ANR1, resulted in a decreased CEF activity (Terashima et al. 2012). The ability to undergo state transitions, however, was unaffected in these mutants, suggesting state transitions do not switch between LEF and CEF but rather that state transitions and LEF/CEF switching are coincidental. This was further supported by examining CEF activity in the State 2-locked mutant of *C. reinhardtii* (Takahashi et al. 2013). Although the lateral migration of mobile LHCIIs occurred in the *ptox2* mutant, which has a constantly reduced PQ pool due to the lack of plastid terminal oxidase 2 (Houille-Vernes et al. 2011), CEF was negligible much as in wild type cells in State 1. Thus, the CEF/LEF switching and state transitions can be hypothesized as follows: The chloroplast stroma and PQ pool are oxidized when PSI is preferentially excited. Under these conditions, LHCIIs are bound to PSII (State 1). Upon reduction of the stroma, Cyt *bf* and FNR are associated with PSI to form a super-supercomplex (CEF supercomplex) as described below; and on the other hand, the PQ pool, and the Q_o-site of Cyt *bf,* gets reduced to bring the cells into State 2, where migration of the mobile LHCIIs occurs to the PsaH side of PSI, and a large scale reorganization of the thylakoids occurs to make the remained LHCII attached to PSII energy-dissipative (Nagy et al. 2014) (Fig. 23.2).

IV. A Supercomplex in Charge of Cyclic Electron Flow in *Chlamydomonas*

The machinery for Fd-CEF was identified in *C. reinhardtii* cells treated with carbonyl cyanide 4-(trifluoromethoxy)phenylhydra-
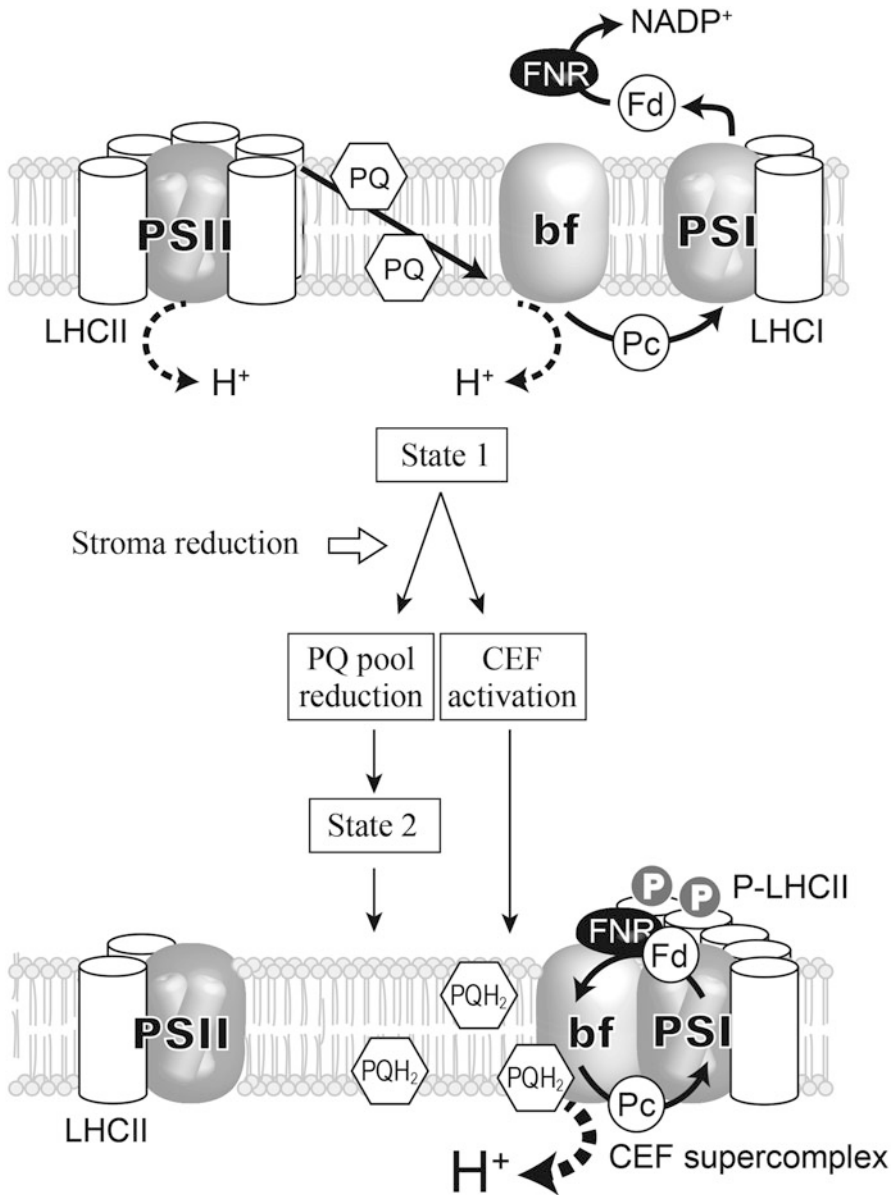
Fig. 23.2. Schematic representation of the regulation of state transitions and electron flow in *C. reinhardtii.* *Upper*, when PSI is preferentially excited, the chloroplast stroma and the PQ pool are oxidized. Under these conditions, LHCIIs are bound to PSII (State 1). The photosynthetic electron flow proceeds in LEF mode, generating NADPH as well as a proton gradient across the thylakoid membrane that is used for ATP production. *Middle*, when the stroma is reduced, on one hand, migration of the mobile LHCIIs from PSII to PSI occurs to establish State 2; on the other hand, CEF becomes activated by association of Cyt *bf* and FNR with PSI to form a super-supercomplex (CEF supercomplex). *Lower*, the cells are in State 2 and the photosynthetic electron flow proceeds in the CEF mode. PQ pool-reduced. Abbreviations: *bf* Cyt *bf*, *Fd* ferredoxin, *Pc* plastocyanin.

zone (FCCP) (Iwai et al. 2010). When FCCP depletes intracellular ATP pools by uncoupling the energized membranes and stimulating glycolysis according to the Pasteur effect, the stroma of the chloroplast is reduced, and probably because of this,

CEF gets activated and the PQ pool in the thylakoid membrane becomes reduced to lead the cells to State 2 (Bulté et al. 1990). We solubilized these thylakoid membranes and loaded them onto a sucrose density gradient, which yielded four discreet green bands including the heaviest fraction (the A4 fraction). The A4 fraction contained PSI with its own antenna system (the PSI-LHCI supercomplex), major LHCIIs, minor LHCIIs, Cyt bf (Cyt b_6, Cyt f, PetC, PetD, PetO, FNR, and PGRL1 with equimolar amount of Cyt bf and PSI. Four different methods were employed to confirm the proteins in the A4 fraction formed a single supercomplex: (1) A single peak with a molecular mass corresponding to 1400–1600 kDa was observed on a gel-filtration chromatogram. (2) The constituents of the A4 band were disassembled by salt treatment into smaller molecular mass complexes, indicating they originally formed a super-supercomplex. (3) The A4 fraction was not present either in the mutant of PSI or the Cyt bf complex. (4) The affinity-purified supercomplex by using a His-tagged PsaA subunit of PSI contained the same protein composition as the original A4 fraction.

To address the question as to whether the supercomplex in the A4 fraction is a functional unit for CEF, we examined the electron transfer activities of the supercomplex on its stromal and lumenal sides by a series of spectroscopic analyses. The stromal side electron transfer was first probed by measuring the reduction of Cyt b in the supercomplex. When Cyt b in the supercomplex was reduced after incubation with NADPH together with Fd, the resulting signal was approximately half as large as that produced upon the reduction of both b-type hemes with dithionite. This suggested that only one of the b-type hemes in Cyt bf, most likely the high-potential heme b_H, was reduced (Fig. 23.3). When we illuminated the supercomplex in the presence of Fd, Cyt b was reduced, manifesting the photoreduction of heme b_H by the electrons from P700. We then evaluated the lumenal side electron transfer by studying the flash-induced kinetics of $P700^+$ re-reduction and Cyt f oxidation within the supercomplex. After oxidized by a single-turnover actinic flash, most of the P700 in the supercomplex accepted electrons from Pc. Cyt f in the supercomplex was rapidly oxidized upon illumination in the presence of Pc (Fig. 23.3).

The physical association of PSI, Cyt bf, and FNR has long been suggested as a platform for Fd-CEF (Joliot and Joliot 2002). Mathematical modelling of electron transfer (Laisk 1993) and the observation of
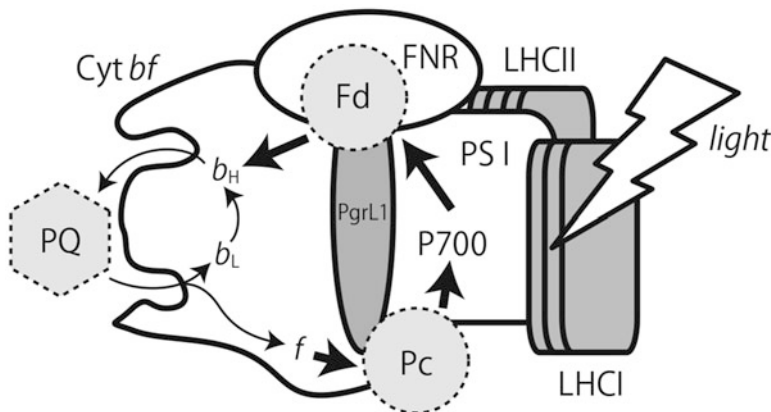

Fig. 23.3. Fd-CEF model in the CEF supercomplex. Major components of the CEF supercomplex (PSI, LHCI, LHCII, FNR, Cyt bf, and PGRL1) and possible electron transfer pathways are schematically drawn along with the mobile electron carriers (Fd, PQ, and Pc). Abbreviations: *P700* primary electron donor in PSI, b_H and b_L high and low potential b hemes in Cyt bf, f heme f in Cyt bf.

its high efficiency in vivo (Joliot and Joliot 2002) suggested that Fd-CEF operates in a supercomplex. Cramer and co-workers reported that Cyt *bf* was co-purified with FNR and was reduced by Fd (Zhang et al. 2001). More recently, physical interactions between PGRL1, PsaD (a subunit of PSI), PetB (Cyt b_6), FNR, and PGR5 were proposed based on a yeast two-hybrid assay using the corresponding genes from *A. thaliana* (DalCorso et al. 2008). However, the enigmatic all-star "super-supercomplex" comprising PSI, Cyt *bf*, and FNR has never been detected or isolated (Breyton et al. 2006; DalCorso et al. 2008). The study in *C. reinhardtii*, however, demonstrated that the CEF supercomplex indeed exists. Moreover, the isolated supercomplex contained PGRL1 as well as a loosely bound Cyt *bf* subunit, PetO (Hamel et al. 2000). Notably, electron transfer activity was detected on both stromal and lumenal sides of the complex (Fig. 23.3). The exact electron transfer pathway within Cyt *bf* is still unclear, but we speculate that the reducing equivalent on heme b_H is transported to b_L via PQ molecules much as proposed for the Q-cycle (Mitchell 1976) (Fig. 23.3). Because CEF and LEF share many redox carriers including PQ, Cyt *bf*, Pc, PSI, Fd, and FNR, the two pathways are potentially in competition with one another. Furthermore, the redox poise of the CEF components could be disturbed if reduced LEF components coexist. However, the formation of a super-supercomplex specific for Fd-CEF (a PSI-LHCI-LHCII-FNR-Cyt *bf*-PGRL1 super-supercomplex or CEF supercomplex) seemingly compartmentalizes CEF by localizing the mobile electron carriers, forming a functional pool of CEF components to sustain the increased CEF activity.

As mentioned above, mutants carrying a mutation in one of the three thylakoid membrane proteins, PgrL1, CAS, and ANR1, have decreased CEF activities under anaerobic conditions (Terashima et al. 2012). Because these three proteins were included in the CEF supercomplex, they are likely additional components for the super-supercomplex (Terashima et al. 2012).

Furthermore, Rappaport and his colleagues showed that the CEF supercomplex is formed under anaerobic, but not aerobic conditions in wild type *Chlamydomonas*. It is also formed in *ptox2* (State 2-locked) and *stt7-9* (State 1-locked) mutant cells, leading them to propose that a reduced stromal environment (which was experimentally substantialized under anaerobic conditions) was responsible for the LEF/CEF switching. The mechanism of how such an increase in reducing power in the stroma promotes the formation of the CEF supercomplex still remains to be unraveled (Takahashi et al. 2013).

Last but not least, I will discuss the possible CEF inhibitor antimycin (AA) and the intriguing CEF components PGR5 and PGRL1. Among NPQ mutant lines, Shikanai and his colleagues isolated several *pgr* mutants that have restricted accumulation of proton motive force across the thylakoid membranes (Munekage et al. 2002). Because the reduction of the PQ pool by way of CEF was earlier described as AA-sensitive (Tagawa et al. 1963), an elusive AA-binding enzyme, Fd-PQ oxidoreductase (FQR), had been proposed to bypass Cyt *bf*, which is not sensitive to AA in contrast to the sister enzyme Cyt bc_1 complex in mitochondria (Huang et al. 2005), when it reduces the PQ pool (Moss and Bendall 1984). Because *pgr5* affected the AA-sensitive fluorescence rise, which is generally ascribed to Fd-mediated PQ pool reduction, PGR5 was postulated to be a component of the FQR enzyme (Shikanai 2007). PGR5 is only an 8 kDa polypeptide, therefore more likely forming a complex with other membrane spanning subunit(s) to constitute any FQR. A *pgrL1* mutant was subsequently reported to display the same phenotype as *pgr5* in *A. thaliana* (DalCorso et al. 2008). A recombinant PGRL1 was shown to be reduced by Fd when it formed a heterocomplex with PGR5, although such a heterocomplex was not detected in vivo (Hertle et al. 2013). The reduced and monomerized PGRL1 in turn reduced the PQ analog 2,6-dimethyl-p-benzoquinone (DMBQ) in an AA-dependent manner in vitro (Hertle et al. 2013).

While genes for both PGR5 and PGRL1 are present in the genome of *C. reinhardtii* (Merchant et al. 2007), and the transcription of both of these genes is at least elicited under specific conditions (e.g., iron deficient conditions) (Petroutsos et al. 2009), only PGRL1 was detected in the CEF supercomplex from *C. reinhardtii* (Iwai et al. 2010). A recent report indicated an impaired electrochromic shift upon switching from light to dark in the presence of DCMU in the *pgr5* mutant and an absence of PGR5 in the *pgrL1* mutant in *C. reinhardtii* (Johnson et al. 2014). These results suggest that Fd-CEF might also be dependent upon both PGRL1 and PGR5 in *C. reinhardtii* as in *A. thaliana*. PGR5 might have been present in the CEF supercomplex, but escaped our scrutiny since it is expressed at a level that is seven times lower than its binding partner PGRL1 (Hertle et al. 2013). Alternatively, it is probable that PGR5 is indeed absent in the isolated CEF supercomplex and so the complex did not exert its maximal CEF activity. The latter scenario is more likely because a significant amount of the reduced form of PGRL1 was present even in the *pgr5* background despite the amount of the reduced form and the reduction rate was somewhat lower than the control (Hertle et al. 2013). These scenarios, however, cannot account for the result in *C. reinhardtii* cells that the re-reduction of $P700^+$ was not sensitive to AA in the presence of DCMU, namely the condition where only CEF can reduce $P700^+$ (Iwai et al. 2010). A recent study on AA sensitivity and the sequence variations of PGR5 in two plant species might provide a possible clue for this problem (Sugimoto et al. 2013). They found CEF was sensitive to AA in *A. thaliana*, but not in pine. PGR5 in the former has Val and in the latter has Lys in the third residue. Because a common wild type strain of *C. reinhardtii* 137c, which was used in our isolation of the CEF supercomplex, harbors Lys at this position in its PGR5, any PGR5-dependent CEF would be AA resistant. We should, however, bear in mind that "AA has multiple effects on photosynthesis, and thus ascribing functional significance to any one process may be misleading" (Fisher and Kramer 2014).

V. Perspectives

In this chapter, I provided an overview of the PSI-Cyt*bf*-PgrL1 supercomplex that conducts Fd-CEF in *C. reinhardtii* (Iwai et al. 2010). Recently, an alternative PGR5-PGRL1 heterocomplex model, which was originally reported for *A. thaliana* (Hertle et al. 2013), was also proposed for *C. reinhardtii* (Johnson et al. 2014). The induction of *Pgr5* transcription was only reported under Fe-limiting conditions in *C. reinhardtii* (Petroutsos et al. 2009), another study reported the PGR5-dependent CEF pathway is constitutively active (Johnson et al. 2014). While PGR5 was not detected in the CEF supercomplex (Iwai et al. 2010), a *pgr5* mutant exhibits decreased CEF activity (Johnson et al. 2014). Although there are currently several differences and discrepancies among these studies and others, such recent studies set the framework toward unraveling the long lasting CEF story at the molecular level. One critical point to push these efforts even further would be sorting out the techniques for measuring CEF activity. These discrepancies may have in part arisen from differences in the methodologies employed for the measurements of CEF activity, such as a rise of PSII fluorescence as a marker of the PQ pool reduction in osmotically ruptured chloroplasts (Munekage et al. 2002; DalCorso et al. 2008; Hertle et al. 2013), the rapid re-reduction of $P700^+$ in the absence of PSII activity in the cells (Munekage et al. 2002; DalCorso et al. 2008; Petroutsos et al. 2009), the dark-interval relaxation of the electrochromic shift in the absence of PSII activity in the cells (Johnson et al. 2014), the reduction of a PQ analog DMBQ by a recombinant polypeptide (Hertle et al. 2013), the Cyt b_H photoreduction in the isolated complex (Iwai et al. 2010), and so on. Future efforts are expected to sort

out these methodological differences. A recent attempt by Fisher and Kramer indeed provided an important step toward clarifying the problem (Fisher and Kramer 2014). They reevaluated the induction kinetics and inhibitor sensitivity of PSII fluorescence from raptured spinach chloroplasts and pointed out that the fluorescence did not rapidly equilibrate with the redox state of the PQ pool. They concluded the fluorescence rise therefore may not reflect the CEF-dependent reduction of the PQ pool, but the reduction of the low potential variants of the primary acceptor (Q_A) in PSII. Further efforts, especially those using key mutants of *A. thaliana* and *C. reinhardtii,* will be much awaited.

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State Transition Kinases and Redox Signal Transduction in Chloroplasts

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Summary

Both linear (LEF) and cyclic electron flow (CEF) occur in the photosynthetic electron transfer chain and are essential to meet the cellular demands in energy and reducing power. Because photosynthetic organisms are constantly subjected to changes in light and nutrient availability, they need to continuously adapt the relative levels of LEF and CEF. A key parameter in this response is the redox state of the photosynthetic electron transport chain. While the molecular mechanisms underlying the regulation of the CEF/LEF ratio by the chloroplast redox state are still poorly understood, considerable progress has been achieved in elucidating how the redox state of the plastoquinone pool is sensed by the Stt7/STN7 protein kinase associated with the cytochrome b_6f complex. This kinase induces both a short term and a long term response, both of which tend to restore the redox poise of the plastoquinone pool and thus to optimize the photosynthetic efficiency. The short term response, called "state transitions," involves the phosphorylation of the light harvesting system of PSII and its reversible migration from photosystem II to photosystem I. The long term response involves a signaling information chain from the chloroplast to the nucleus which ultimately adjusts the stoichiometry between the two photosystems for re-equilibrating their redox state and that of the intervening cytochrome b_6f complex. The Stt7/STN7 kinase together with the Stl1/STN8 kinase and their associated phosphatases form a central quartet which is part of an extended chloroplast signaling network that is important for the dynamic response of the photosynthetic apparatus, especially under adverse environmental conditions.

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I. Introduction

The primary reactions of oxygenic photosynthesis occur on the thylakoid membranes within chloroplasts of land plants and algae. They are mediated by the three major protein-pigment complexes photosystem II (PSII), photosystem I (PSI) with their corresponding light-harvesting systems LHCII and LHCI, and the cytochrome b_6f complex (Cytb_6f). Light energy captured by the two antenna systems is transferred to the reaction centers of PSII and PSI where it induces a charge separation across the thylakoid membrane with ensuing electron flow along the photosynthetic electron transport chain from water to PSII, the plastoquinone pool (PQ pool), Cytb_6f, PSI and NADP$^+$, the final electron acceptor (Fig. 24.1). This electron flow is coupled to proton pumping into the thylakoid lumen and the resulting pH gradient across the membrane is utilized by a fourth major complex, the ATP synthase, to produce ATP from ADP and inorganic phosphate. Besides linear electron flow (LEF) there is a cyclic electron pathway (CEF) in which PSI transfers electrons back to the PQ pool and the Cytb_6f complex through the Ndh (Burrows et al. 1998; Shikanai et al. 1998) and Pgr (Munekage et al. 2002) pathways, respectively (Fig. 24.1). This cyclic electron pathway generates a proton gradient and ultimately ATP, but does not produce reducing power. In addition, a third electron transfer route exists, called the chlororespiratory chain, which feeds reducing power from the stroma to the PQ pool through the Ndh complex with plastid terminal oxidase (Ptox) as final electron

acceptor (Fig. 24.1). Ultimately, the products of the primary reactions of photosynthesis, NADPH and ATP are used to fuel the reactions of the Calvin-Benson cycle for carbon assimilation. To meet the demands of this metabolic cycle ATP and NADPH have to be delivered at a ratio of 3:2, a value that exceeds the maximum capacity of LEF which is 2.6:2 (Allen 2003). Moreover other specific cellular processes, e.g. translation and the carbon concentrating mechanism in algae require an even higher ratio of ATP/NADPH. Therefore fine-tuning of LEF and CEF is essential for maintaining cellular growth and metabolism.

The thylakoid membranes consist of two distinct domains, the grana regions and the stromal lamellae. Grana regions usually consist of cylindrical stacks of 5–20 layers of thylakoid membrane linked together by stroma lamellae. Each granum is surrounded by two stroma-exposed membranes and the highly curved margins that connect the adjacent grana membranes (Shimoni et al. 2005). Importantly, the photosynthetic complexes are not uniformly distributed between these two domains giving rise to lateral heterogeneity (Andersson and Andersson 1980). Because of their bulky extrusion into the stromal phase, PSI and the ATP synthase are confined to the stromal lamellae and the end membranes of the grana but excluded from the interior of the grana where most of PSII resides while Cyt b_6f partitions equally between both domains.

Because PSII and PSI are connected in series through the electron transport chain, their activities need to be coordinated to maintain a proper redox poise of the PQ pool. Over-excitation of PSII relative to PSI would lead to an over-reduction of the PQ pool whereas the reverse case would result in its over-oxidation. In both of these extreme cases this would seriously compromise photosynthetic electron flow and lower the photosynthetic yield. Thus, different processes contribute to the maintenance of a balanced redox state of the electron transport chain. Some of these processes proceed through the transfer of reducing power to molecular

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Abbreviations: CEF – Cyclic electron transfer; Cytb_6f – Cytochrome b_6f complex; DCMU – 3-(3,4-Dichlorophenyl)-1,1-dimethylurea; LEF – Linear electron flow; LHC – Light harvesting system; Ndh – NAD dehydrogenase; PQ – Plastoquinone; PQH$_2$ – Plastoquinol; PSI – Photosystem I; PSII – Photosystem II; Ptox – Plastid terminal oxidase; Q$_o$ Q$_p$ – The quinone binding site on the electrochemically positive side of the membrane
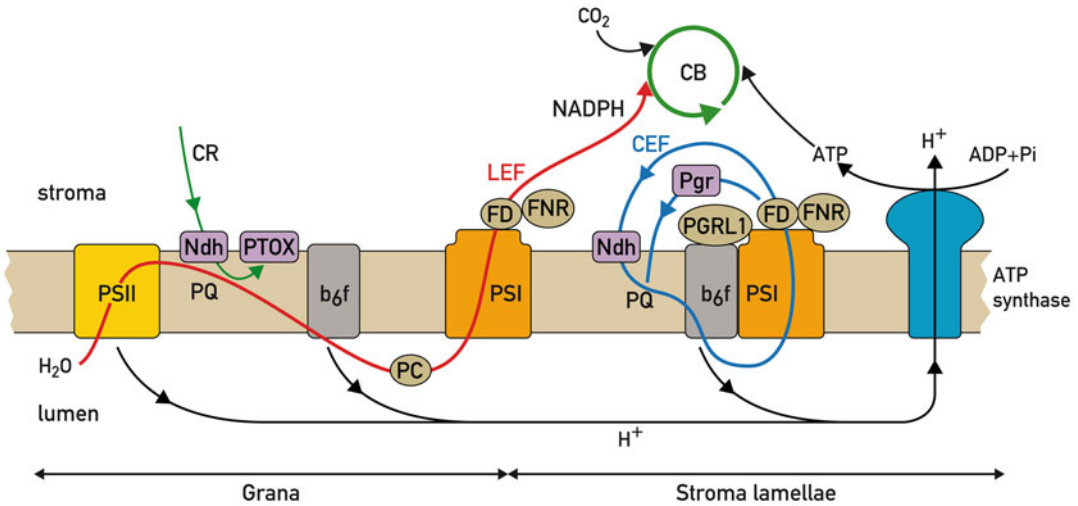
Fig. 24.1. Linear and cyclic electron transfer pathways. Linear electron flow (LEF) and cyclic electron flow (CEF) are shown in *red* and *blue*, respectively with *arrows* indicating the direction of electron flow. The LEF pathway is driven by the two photochemical reactions of PSII and PSI: electrons are extracted by PSII from water and transferred subsequently to the PQ pool, Cytb_6f, plastocyanin (PC), PSI and ferredoxin (FD). Ferredoxin-NADPH reductase (FNR) catalyzes the formation of NADPH at the expense of reduced FD. The CEF pathway with the two Ndh and Pgr branches is driven by PSI in the stroma lamellae. In *C. reinhardtii* PSI forms a supercomplex with Cytb_6f, FNR, PGRL1 and additional factors (Iwai et al. 2010a). Upon reduction of FD, electrons are returned to the PQ pool either through the NADH complex (Ndh) or via PGRL1 (Pgr) which acts as a FD-PQ oxidoreductase. Both LEF and CEF are associated with proton pumping into the lumen. The resulting proton gradient is used by ATP synthase to produce ATP, which together with NADPH drives CO_2 assimilation by the Calvin-Benson cycle (CB). CR, chlororespiratory chain (*green*) which feeds stromal reducing power into the PQ pool with plastid terminal oxidase (PTOX) as terminal electron acceptor. *G* grana, *SL* stroma lamellae (Adapted from Rochaix (2014)) with permission.

oxygen. This can occur on the acceptor side of PSI through the Mehler reaction in which electrons react with oxygen to form H_2O_2. Alternatively, Ptox, the plastid terminal oxidase is able to reoxidize the PQ pool on the stromal side of the thylakoid membrane. These reactions lead to the formation of a proton-motive force and hence to ATP synthesis. Excess reducing power can also be dissipated through the malate shuttle to the mitochondrial respiratory chain also resulting in oxygen consumption and ATP formation (Nunes-Nesi et al. 2008). Another process for avoiding over-reduction of the PQ pool involves state transitions, originally recognized as a mechanism that allows for the balancing in the short term of the light excitation energy of the LHCII and LHCI antenna systems of PSII and PSI, respectively. In the long term the maintenance of this balanced redox state is achieved through

compensatory changes in the expression of the LHC genes and involves a signaling chain from the chloroplast to the nucleus. While state transitions were discovered 45 years ago (Bonaventura and Myers 1969; Murata 1969), it is only recently that the underlying molecular mechanisms have been partially unraveled and they continue to be intensively investigated.

A key feature of state transitions is that the redox state of the PQ pool is perceived through a protein kinase which is associated with the thylakoid membranes. Because this kinase phosphorylates the LHCII proteins, it was named LHCII kinase. Although the activity of this kinase was noticed early (Bennett 1977), the kinase protein was identified only recently and named Stt7 in *Chlamydomonas* and STN7 in *Arabidopsis* (Depège et al. 2003; Bellafiore et al. 2005). Whenever PSII is overexcited relative to PSI, reduction

of the PQ pool promotes the occupancy of the Qo site of Cytb_6f by plastoquinol (PQH_2) and leads to the activation of the kinase and phosphorylation of LHCII (Vener et al. 1997; Zito et al. 1999). This causes the dissociation of LHCII from PSII and its association with PSI and hence an increase in size of the antenna of PSI at the expense of that of PSII and consequently restores the redox poise of the PQ pool. Dephosphorylation of the LHCII proteins is achieved by a phosphatase TAP38/PPH1 which counteracts the action of the Stt7/STN7 kinase (Pribil et al. 2010; Shapiguzov et al. 2010). Besides its involvement in state transitions, the Stt7/STN7 kinase is also implicated in a signaling chain which transmits signals from the chloroplast to the nucleus, a process also called retrograde signaling. This chapter reviews recent advances on the role of the Stt7/STN7 kinase and the TAP38/PPH1 phosphatase in state transitions and retrograde signaling. Earlier work on this topic has been covered in several recent reviews (Eberhard et al. 2008; Lemeille and Rochaix 2010; Rochaix 2013).

II. The Role of the Stt7/STN7 Protein Kinase in State Transitions

The classic model of state transitions is shown in Fig. 24.2. Over-excitation of PSII relative to PSI leads to a reduced redox state of the PQ pool which in turn favors docking of PQH_2 to the Qo site of the Cytb_6f complex. This process activates the Stt7/STN7 kinase (Vener et al. 1997; Zito et al. 1999) and leads to the phosphorylation of LHCII (Depège et al. 2003; Bellafiore et al. 2005). Subsequently, a portion of LHCII, called mobile LHCII, dissociates from PSII and associates with PSI thereby equilibrating the excitation energy between PSII and PSI. This state is called state 2. Conversely, over-excitation of PSI relative to PSII oxidizes the PQ pool which leads to the inactivation of the Stt7/STN7 kinase and to the dephosphorylation of LHCII mediated by the PPH1/TAP38 phosphatase (Pribil et al. 2010; Shapiguzov et al. 2010). The

dephosphorylated mobile LHCII dissociates from PSI and moves back to PSII. This state is called state 1. As seen below this view of state transitions may be too simplistic although its major features are still valid.

Although a direct phosphorylation of LHCII by the Stt7/STN7 kinase could not be demonstrated in vivo, it is very likely that this kinase qualifies as the LHCII kinase because it can be co-immunoprecipitated with LHCII and the phosphorylation of several LHCII proteins depends on Stt7/STN7 (Lemeille et al. 2010). Moreover, this kinase is always associated with large molecular weight complexes and in particular with the Cyt b_6f complex in *Chlamydomonas* (Lemeille et al. 2009). The kinase is phosphorylated under state 2 conditions most likely through autophosphorylation (Lemeille and Rochaix 2010) because, in vitro, recombinant Stt7 kinase autophosphorylates readily (G. Fucile and J.D. Rochaix, unpublished results). In *Chlamydomonas* six phosphorylation sites within Stt7 sites have been identified (Lemeille et al. 2010; Wang et al. 2014) whereas four were found in STN7 of *Arabidopsis* (Reiland et al. 2009). The phosphorylation of the Stt7/STN7 kinase does not appear to play any major role in state transitions as changes of all four sites through site-directed mutagenesis in *Arabidopsis* did not impair LHCII phosphorylation and state transitions (Willig et al. 2011). However replacement of the four phosphorylated amino acids by phosphormimetic amino acids prevented the decline of STN7 which normally occurs upon prolonged state 1 conditions (Willig et al. 2011).

The Stt7/STN7 kinase is conserved in land plants and algae. It contains a single transmembrane region bounded by a conserved kinase domain on the stromal side and a short conserved N-terminal domain with two conserved Cys residues on the lumen side. These two residues play a crucial role because changes of either of these Cys abolishes the activity of the kinase and state transitions although they are outside of the catalytic domain (Lemeille et al. 2009).
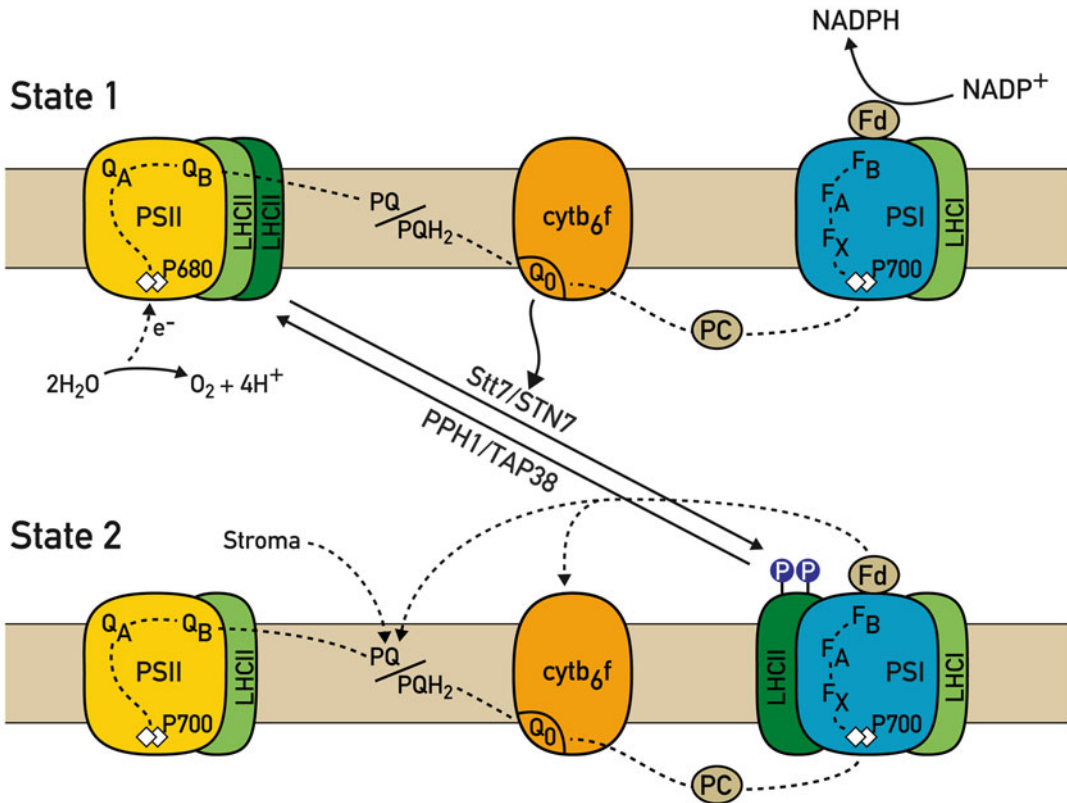
Fig. 24.2. State transitions. Transition from state 1 to state 2 occurs when the redox state of the plastoquinone pool (PQ) is reduced following preferential excitation of PSII relative to PSI. Docking of plastoquinol (PQH$_2$) to the Qo site of Cytb_6f leads to the activation of the protein kinase Stt7/STN7 which phosphorylates LHCII either directly or indirectly. Phosphorylated LHCII dissociates from PSII and binds to PSI (state 2). Upon preferential excitation of PSI relative to PSII, the kinase is inactivated and the PPHI/TAP38 phosphatase dephosphorylates LHCII which moves back to PSII (state 1). Under reducing conditions cyclic electron flow is induced independently of state transitions (Takahashi et al. 2013) (Adapted from Rochaix (2007)) with permission).

However during state transitions induced by changes in light quality or quantity, the redox state of these Cys does not change. It is only under prolonged anaerobic conditions that these Cys are fully reduced (A. Shapiguzov and J.D. Rochaix, unpublished results). Although state transitions can also be induced by anaerobiosis in *Chlamydomonas*, this process occurs significantly faster than the reduction of the Cys residues. One possibility is that these two Cys are involved in intersubunit disulfide bridges and thus responsible for the dimerization of the kinase. In this view only the kinase dimer would be active. The crystal structure of the dimeric Cytb_6f complex of Chlamydomonas reveals

a cavity on the lumen side which could accommodate the interacting N-terminal domains of the kinase dimer (Stroebel et al. 2003). This cavity is presumably also conserved in the cyanobacterial Cytb_6f complex (Hasan et al. 2013a).

There is significant sequence identity between the Stt7/STN7 and Stl1/STN8 kinases. The latter is also associated with the thylakoid membrane and appears to be mainly responsible for the phosphorylation of the PSII core proteins (Bonardi et al. 2005; Vainonen et al. 2005). There is some overlap between the substrates of these two kinases based first on the fact that in the absence of STN7 some residual LHCII phosphorylation

still occurs and reciprocally in the absence of STN8 residual phosphorylation of the PSII core proteins is detectable. Second, these protein phosphorylations are abolished in a double mutant lacking both kinases.

In land plants and *Chlamydomonas* the LHCII kinase is inactivated under high light (1000 μmol photons m^{-2} s^{-1}) (Schuster et al. 1986; Rintamaki et al. 2000). In plants, this process appears to be mediated by ferredoxin and thioredoxin. The question thus arises whether these two proteins localized in the stromal phase could modulate the redox state of the lumenal Cys of Stt7/STN7. This is in principle possible because CCDA and HCF164, two thylakoid proteins required for heme attachment on the lumen side and for the biosynthesis of the Cytb_6f complex, are able to transfer thiol-reducing equivalents across the thylakoid membrane and could therefore mediate the redox control of Stt7/STN7 (Lennartz et al. 2001; Page et al. 2004). However, high light treatment did not change the redox state of the conserved lumenal Cys in *Chlaymdomonas* indicating that the inactivation of the kinase occurs through a different mechanism (A. Shapiguzov and J.D. Rochaix, unpublished results). Besides the conserved luminal Cys, a TRX-like CxxxC motif within the kinase domain of STN7 exposed to the stroma exists in land plants. This motif has been proposed as a possible target for TRX (Puthiyaveetil 2011). However, site-directed mutagenesis of the Cys of this motif did not affect state transitions and LHCII phosphorylation in Arabidopsis (A. Shapiguzov and J.D. Rochaix, unpublished results). Furthermore, while this motif is conserved in land plants and several algae, it is absent from *Chlamydomonas*. Also, the decreased accumulation of Stt7/STN7 under extended state 1 conditions is unlikely to play a role in the control of the activity of the kinase because this decrease is significantly slower than that of its activity under high light (Schuster et al. 1986; Lemeille et al. 2009; Willig et al. 2011). It is therefore not yet clear how the LHCII kinase is inactivated under high light.

Besides its role in state transitions the Stt7/STN7 kinase is also required for optimal adaptation to fluctuations in light intensity. Reversible LHCII protein phosphorylation occurs when *Arabidopsis* plants are subjected to alternative periods of low light and high light with LHCII phosphorylation and dephosphorylation, respectively (Tikkanen et al. 2007). However surprisingly, the distribution of light excitation energy between the two photosystems does not change significantly during these changes in phosphorylation. The STN7 kinase appears to promote a balanced distribution of excitation to both photosystems under these conditions because in its absence the excitation of PSII rises during the low light phases and growth of the plants is strongly diminished (Tikkanen et al. 2007).

Although one cannot exclude that the activity of the PPH1/TAP38 phosphatase is also subjected to redox control, there is no evidence supporting this possibility. It is more likely that this phosphatase is constantly active and that the Stt7/STN7 is the major regulatory factor of the phosphorylation of the LHCII proteins.

III. Mobility and Location of LHCII

Recent results have led to a reconsideration of the classical state transition model which raise new questions concerning the different locations of the mobile LHCII during state transitions. In land plants a PSII supercomplex with four LHCII trimers was identified (Wientjes et al. 2013). It contains up to two additional loosely associated trimers in state 1 which are displaced to PSI during a transition to state 2 so that in the end the PSII supercomplex remains intact (Wientjes et al. 2013). Earlier studies had indicated that stroma lamellae enriched in PSI contain more phosphorylated LHCII than the grana fraction which is enriched in PSII (Kyle et al. 1983; Bassi et al. 1988). The studies of Wientjes et al. however indicate that phosphorylation is not limited to the mobile LHCII but also includes LHCII

within the PSII supercomplex indicating that LHCII phosphorylation is not sufficient for the dissociation of certain LHCII trimers from PSII. In contrast to the earlier view which assumed that a small part of LHCII migrates to PSI in response to low light irradiance, the new results show that a large fraction of the PSI complexes binds one LHCII trimer. This system can transfer excitation energy efficiently to PSI after acclimation to different light conditions, indicating that LHCII is associated with the PSI antenna under many different growth conditions (Wientjes et al. 2013). Thus in land plants the mobile LHCII functions as a highly efficient antenna system for PSI under different light conditions. Mobile LHCII moves back to PSII only under specific conditions, e.g., upon a sudden increase in light intensity or when PSI is over-excited by far red light.

Whereas, evidence for the migration of LHCII between PSII and PSI comes mainly from in vitro studies with thylakoid membranes (Kargul et al. 2005; Takahashi et al. 2006), fluorescence lifetime imaging microscopy has provided new insights into this process in vivo. This technique allows one to differentiate chlorophyll auto-fluorescence based on its lifetime. It revealed a 250 ps lifetime fluorescence component during a state 1 to state 2 transition in live wild-type but not in *stt7* mutant cells of *Chlamydomonas* (Iwai et al. 2010b). This component diffused through the cell and formed large spotted areas. The fact that it could be detected in a mutant lacking PSII and PSI but containing LHCII and that its appearance correlated with LHCII phosphorylation suggest that it originates from phosphorylated LHCII dissociated from PSII in vivo. The existence of a free LHCII pool raises many questions. In particular, does it only form transiently and how and where does LHCII bind to PSI?

State transitions have been thought to represent a process which is mainly involved in acclimation under low light conditions. While this appears to be true for land plants, recent work with *Chlamydomonas* indicates

that besides qE defined as energy-dependent quenching through thermal dissipation of absorbed excess light, state transitions also have a photoprotective role during high light acclimation (Allorent et al. 2013). Comparative analysis of the *Chlamydomonas* mutants *stt7*, *npq4*, lacking the Lhcsr3 protein and the double mutant *stt7-npq4* showed that both qE and state transitions are induced during acclimation to high light and that the double mutant is significantly more affected than either of the single mutants. The *Chlamydomonas* PsbS protein, a major component of the qE response in land plants is not expressed under normal light and does not appear to play a significant role in this response (Bonente et al. 2008). Instead, induction of Lhsr3, an essential factor for qE, requires several hours upon exposure to high light stress (Peers et al. 2009). It was proposed that state transitions could play an important photoprotective role during this induction phase by decreasing the excitation photo-pressure on PSII through the displacement of a large part of the LHCII antenna to PSI, and thereby would diminish the production of reactive oxygen species before qE becomes operational (Allorent et al. 2013).

IV. How Is the Stt7/STN7 Kinase Activated?

While it is well established that the Stt7/STN7 kinase is activated as a result of a more reduced state of the PQ pool and more specifically through the docking of plastoquinol to the Qo site of the Cytb_6f complex, the underlying molecular mechanisms are still unknown. As indicated above, the two conserved lumenal Cys residues are essential for the activity of the kinase and need to be maintained in an oxidized state. They may be involved in two disulfide bridges responsible for dimer formation. Presumably the binding of PQH$_2$ induces a conformational change in the N-terminal luminal domain of the kinase which is transduced through the transmembrane

domain to the catalytic stromal domain of the kinase. Alternatively, PQH_2 binding could provoke a conformational change on the lumenal side of Cyt b_6f which is transmitted to its stromal side where it modulates the kinase activity. Finally, both the kinase and the Cytb_6f complex could be involved in this transmembrane signaling chain. New insights into this problem have come from the comparison of the chloroplast Cytb_6f and the yeast Cytbc_1 complex crystal structures. It is well documented that cytochrome b with its eight transmembrane domains has been replaced by two polypeptides in the Cytb_6f complex, cytochrome b_6 with four and PetD with three trans-membrane helices (Widger et al. 1984). Thus, one transmembrane domain has been lost in the Cytb_6f complex. Interestingly, the crystal structure of this complex reveals the presence of a lipid and chlorophyll a chlorin ring in place of the lost eighth trans-membrane helix in the cytochrome b subunit (Hasan et al. 2011, 2013b). This replacement raises the question whether it occurred as a result of a selective advantage for signaling the redox state of the PQ pool or for coupling the oxidation of PQH_2 in the lumen to the reduction of a factor on the stromal side of the thylakoid membrane. It was proposed that this lipid may mediate this function or could substitute for the transmembrane domain of a signaling protein lost during crystallization of the complex (Hasan et al. 2013b). A prime candidate is the Stt7/STN7 kinase with its single trans-membrane domain.

Clearly, determination of the crystal structure of the Stt7/STN7 kinase is likely to provide key insights into the activation process of the kinase. An important step forward was achieved recently with the determination of the crystal structure of the kinase domain (KD) of the MsStt7d kinase from the marine microalga *Micromonas sp. RCC299,* which is related to that of Stt7/STN7 (Guo et al. 2013). Although MsStt7d does not contain a transmembrane domain and lacks the conserved Cys residues at its N-terminal end, the kinase domain is well conserved. MsStt7d-KD contains two

lobes, a small N-terminal lobe with a sheet of six antiparallel β strands and a single α helix and a larger C-terminal lobe with nine α helices and a pair of short β strands. The protein forms a dimer and is capable of autophosphorylation. A unique characteristic feature of this kinase and of the Stt7/STN7 family is a hairpin structure, essential for the stability of the enzyme, which interacts with the activation loop and thereby activates the kinase. Moreover, MsStt7d is a dual specificity kinase phosphorylating both Thr and Tyr residues that is able to phosphorylate a consensus pentapeptide of the antenna proteins from *Micromonas* (Guo et al. 2013). In this respect, it should be noted that an extensive phosphoproteomic analysis of plastid proteins of *Arabidopsis* did not reveal any Tyr phosphorylation site (Reiland et al. 2009). However this appears to be different in algae as Tyr phosphorylation could be detected in *Chlamydomonas* (Wang et al. 2014). The approach used with the *Micromonas* kinase opens a new framework for a better understanding of the activation process of Stt7/STN7 and raises the question whether the dual specificity of MsStt7d-KD may correspond to the dual role of Stt7/STN7 in both state transitions and in the long term response.

V. Involvement of Stt7/STN7 in the Long Term Response

In addition to its role in short term response, the Stt7/STN7 kinase is also involved in a long term response with alterations in nuclear and chloroplast gene expression which lead to compensatory changes in thylakoid protein accumulation. In this way balancing of the excitation energy occurs between PSII and PSI and the redox poise of the PQ pool is maintained (Brautigam et al. 2009; Pesaresi et al. 2009). In the *stn7* Arabidopsis mutant the chloroplast *psaAB* operon is upregulated at the transcriptional level and the level of the nucleus-encoded PSI antenna protein Lhca1 is increased post-transcriptionally. This long term response

does not involve LHCII phosphorylation and state transitions and diverges from the short term response at, or immediately downstream, of STN7 (Pesaresi et al. 2009). As a consequence the Stt7/STN7 kinase must have other targets that act in the long term response pathway but that are still largely unknown. Comparison of the known LHCII phosphorylation sites of Stt7 in *Chlamydomonas* led to the identification of a consensus motif for these sites. A striking feature of this motif is that the phosphorylated Ser/Thr is flanked on each side by two basic residues (Lemeille et al. 2010) which is similar to that proposed for the specific signature of STN7 phosphorylation sites of *Arabidopsis* (Vainonen et al. 2005). Searching the predicted chloroplast proteins from *Chlamydomonas* for the presence of the Stt7 target phosphorylation motif led to the identification of additional putative substrates of Stt7. Interestingly they include several low-abundance chloroplast- and nucleus-encoded proteins involved in post-transcriptional steps of chloroplast gene expression, such as Tab2 required for translation of the *psaB* mRNA and the chloroplast σ-factor RpoD (Lemeille et al. 2010). These observations raise the possibility that Stt7 may also play a role in chloroplast gene expression. Additional putative target sites of Stt7 were identified amongst proteins required for chlorophyll synthesis (ChlH, Cpx1, PorB, Ppo) and subunits of ATP synthase (AtpA, AtpB).

Further indications that state transitions are not involved in the long term response have emerged from the analysis of several mutants of *Arabidopsis* affected in PSI. Thus *psaI-1* which lacks the PSI subunit PsaI and thereby the docking site of PSI for LHCII (Lunde et al. 2000) is deficient in state transitions but still displays a long term response (Pesaresi et al. 2009). The *psaD1* and *psaE1* mutants which lack one of the isoforms of the PsaD and PsaE proteins, respectively, still accumulate PSI but are impaired in electron flow and deficient in both state transitions and in the long term response. However, in these mutants LHCII is phosphorylated under all light conditions because of the over-reduction of the PQ pool (Pesaresi et al. 2009). The growth defect in these mutants is exacerbated in the double mutants *stn7 psaD1* and *stn7psaE1*. Taken together, these observations indicate that the STN7 kinase does not operate properly when it is permanently activated and that the long term response only occurs if the activity of STN7 can be modulated.

VI. Relationship Between Stt7/STN7 and Cyclic Electron Flow

A salient feature of thylakoid membranes is their lateral heterogeneity with PSII and PSI localized mainly in the grana and stromal lamellae, respectively. In contrast, the Cyt b_6f complex is equally distributed between these two regions but it is partially redistributed to the stroma lamellae during a transition from state 1 to state 2 (Vallon et al. 1991; Fleischmann et al. 1999). Enrichment of the Cytb_6f complex in the stroma lamellae did not lead to an increase in the maximal rate of CEF suggesting that the Cytb_6f complex is not the limiting step in CEF. Rather, it is likely that this limitation arises from the Ndh and Pgr pathways.

The picture that emerges from recent studies is that CEF is involved in the response to a wide range of environmental and metabolic changes to modulate the ATP/NADPH output ratio according to the cellular needs. Moreover, CEF induces a lumenal pH-dependent photoprotection through non-photochemical quenching (Lucker and Kramer 2013). The relationship between CEF and state transitions has been examined under a variety of experimental conditions including both continuous illumination, inhibition of photosynthesis with different inhibitors, darkness, anoxia and nutrient limitation. Studies aimed at determining whether there is a causal relationship between state transitions and CEF have given rise to conflicting results that can be at least partially attributed to the different experimental conditions used. It was initially

reported that CEF is induced in darkness in Chlamydomonas under anaerobic conditions that are also known to induce a transition to state 2 (Finazzi et al. 1999). Moreover, this activation of CEF did not occur in the *stt7* mutant which is blocked in state 1 (Finazzi et al. 2002). Reciprocally, CEF was shown to be inhibited by treatment with DCMU in the light which blocks electron transfer in PSII and leads to the oxidation of the PQ pool, a condition that promotes state 1. These results reveal a correlation between the induction of CEF and a transition from state 1 to state 2 and led to the suggestion that state transitions may act as a switch between LEF and CEF (Finazzi et al. 2002). Along the same line, a large PSI-Cytb_6f supercomplex including FNR and Pgrl1 was identified in *Chlamydomonas* under state 2 but not under state 1 conditions (Iwai et al. 2010a). The fact that this complex could perform light-induced oxidation of cytochrome *f* and reduction of cytochrome *b* of the Cytb_6f complex suggested that it is involved in CEF.

However, other reports appear to contradict the idea that CEF is controlled by state transitions. In one study wild-type levels of CEF were obtained in the *Chlamydomonas stt7* mutant under limiting light conditions in the presence of DCMU (Cardol et al. 2009). Wild-type and *stt7* mutant cells were also examined for CEF under conditions in which inorganic and organic carbon availability changes (Lucker and Kramer 2013). These studies indicated that CEF is activated when the metabolic demand for ATP increases due to the induction of the carbon concentrating mechanism when CO_2 is limiting. However, CEF activation as measured by the decay of the electrochromic shift signal upon a light to dark transfer still occurred in the *stt7* mutant of *Chlamydomonas* as in the wild type, indicating that CEF is not dependent on state transitions in this case (Alric 2014). Moreover, time course experiments revealed that CEF occurred faster than state transitions revealing that there is no causal relationship between state transitions and CEF.

A similar conclusion was reached from a comparative study of wild type and two mutants *stt7* and *ptox2*, locked in state 1 and 2, respectively, independent of the redox conditions (Takahashi et al. 2013). The *ptox2* mutant is deficient in the plastid terminal oxidase which controls the redox state of the PQ pool in the dark (Houille-Vernes et al. 2011). While the accumulation of reducing power and transition to state 2 correlated well with the enhancement of CEF in the wild type, this was not the case for *ptox2*. In this mutant, CEF was not enhanced under aerobic conditions in the dark even though it is locked in state 2 with phosphorylated LHCII. Moreover, CEF enhancement and formation of the PSI-Cytb_6f supercomplex were still observed in the *stt7* mutant when the PQ pool was reduced. Thus both of these processes occurred under reducing conditions with no correlation with state transitions and their associated LHCII reorganization (Takahashi et al. 2013).

Further progress has been achieved in the identification of components involved in CEF. Pgrl1 was shown to transfer electrons from ferredoxin to the plastoquinone pool in vitro and could thus qualify as the ferredoxin-plastoquinone reductase (Bendall and Manasse 1995), a hypothetical enzyme which had escaped biochemical detection (Hertle et al. 2013). The fact that the rate of electron transfer was rather low in these experiments suggests that other components are required for efficient CEF. A prime candidate remains the PSI- Cyt b_6f supercomplex. The latter identified in *Chlamydomonas* under state 2 conditions and proposed to be involved in CEF is also formed in the absence of the Stt7 kinase further weakening the causal link between state transitions and CEF (Takahashi et al. 2013). Furthermore, a similar PSI-Cyt b_6f supercomplex was also identified under anaerobic conditions (Terashima et al. 2012). It contains, in addition, the CAS (Ca^{++} sensing protein) and ANR1 (anaerobic response), a protein that is required for acclimation to anoxia. A decrease of

CAS and ANR1 levels led to a strong inhibition of CEF which could be rescued by addition of external Ca^{++} in the case of CAS but not of ANR1 down regulation. These two proteins interact together, and with PGRL1 in vivo, suggesting that they may be involved in Ca^{++}-dependent regulation of CEF (Terashima et al. 2012). The fact that a decrease in the level of PGRL1, CAS or ANR1 leads to inhibition of CEF but that neither of these proteins is required for state transitions also agrees with the notion that these two processes are independent from each other. Taken together, these studies indicate that CEF is subjected to a rapid redox regulation through several components including metabolic pools (PQ, NADPH, ferredoxin) (Takahashi et al. 2013), reactive oxygen species (Livingston et al. 2010), and ions (Ca^{++}) (Terashima et al. 2012), independent of state transitions.

VII. Conclusions and Perspectives

During the past years an increasing number of protein kinases and phosphatases have been identified that participate in a complex chloroplast signaling network involved in the acclimation response towards changes in environmental conditions (Fig. 24.3). A key parameter in this response is the redox state of the photosynthetic PQ pool which is influenced by intrinsic factors such as the cellular energy balance and by environmental factors including light quality and quantity, CO_2 level, temperature and micronutrient availability. How the redox state is perceived by the chloroplast is still not fully elucidated although some potential candidates involved in this process have been identified. Amongst them a protein quartet consisting of the Stt7/STN7 (Depège et al. 2003; Bellafiore et al. 2005) and STl1/STN8 (Bonardi et al. 2005; Vainonen et al. 2005) kinases and their counterpart PPH1/TAP38 (Pribil et al. 2010; Shapiguzov et al. 2010) and PBCP (Samol et al. 2012) phosphatases plays an important role. The most promising candidate for sensing the redox state of the PQ pool is the Stt7/STN7 kinase. Through its close associa-

tion with the Cyt b_6f complex it is intimately connected to the PQ pool and activated upon binding of PQH_2 to the Q_0 site of the complex. Besides its role in state transitions, a short term response, the Stt7/STN7 kinase is also involved in the long term response which links the redox state of the PQ pool to nuclear gene expression (Pesaresi et al. 2009). However, little is known about the components of this signaling chain except that the two pathways branch off at the level of the kinase.

Another aspect of the chloroplast signaling network is the linkage of chloroplast kinases and phosphatases with each other and, in particular, how they relate to the Stt7/STN7 kinase (Fig. 24.3). The fact that Stl1 is a substrate of the Stt7 kinase suggests the existence of signaling cascade between these two kinases (Lemeille et al. 2010). Although another kinase, CSK, has been proposed to be a redox sensor in the chloroplast based mostly on its relation to bacterial two-component systems (Puthiyaveetil et al. 2008), it remains to be seen whether this kinase acts in an autonomous way or whether it is also linked to Stt7/STN7. CSK is conserved in land plants and some algae, but appears to be absent from *Chlamydomonas*. The casein kinase 2 (PTK) is implicated in the phosphorylation of proteins involved in chloroplast transcription and post-transcriptional regulation (Link 2003; Bollenbach et al. 2004). One phosphorylation site of STN7 was proposed to be a potential substrate for PTK, raising the possibility that this kinase could also be involved in LHCII phosphorylation through STN7 (Reiland et al. 2009). However, the connection of STN7 to the plastid-signaling network is still largely unknown.

Finally, the essential role of chloroplast reducing power for the enhancement of cyclic electron transfer raises the question of the underlying molecular mechanisms, which could involve any of the other kinases mentioned except Stt7/STN7. As pointed out by Takahashi et al. (2013), an interesting aspect of this new conceptual view is that it could apply to the entire green lineage, especially to those photosynthetic organisms in which
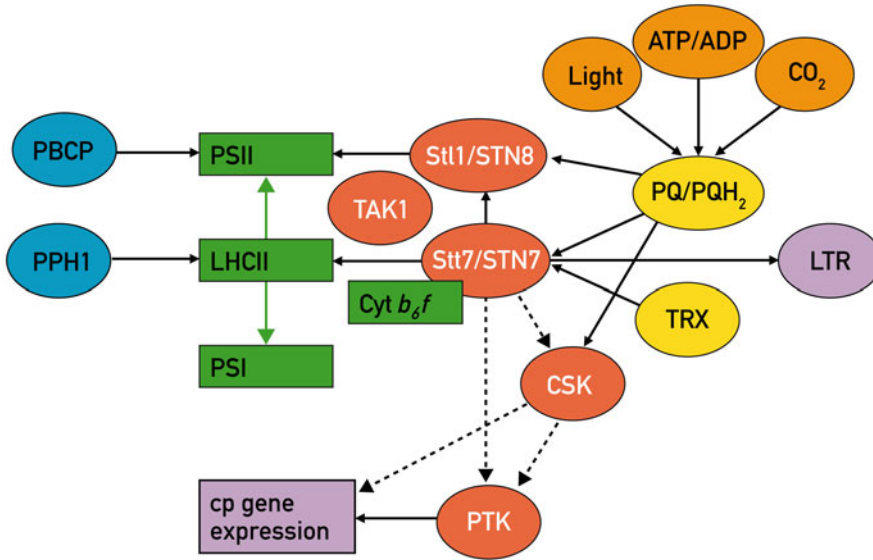
Fig. 24.3. Chloroplast signaling network. The redox state of the plastoquinone pool (PQ/PQH2) is dependent on irradiance, CO_2 level and cellular ATP/ADP ratio. PQH_2, plastoquinol, activates the Stt7/STN7 kinase through the Cytb_6f complex. The major substrates of the Stt7/STN7 and Stl1/STN8 kinases are LHCII and the PSII core proteins. STN7 is also involved in retrograde signaling (LTR). The LHCII kinase has been proposed to be inactivated through reduced thioredoxin (TRX). The PPH1 and the PBCP phosphatases act as the counterparts of the Stt7/STN7 and Stl1/STN8 kinases, respectively. The kinases PTK and CSK act on the chloroplast gene expression system and may be redox-controlled through STN7. The TAK1 kinase has been proposed to be involved in LHCII phosphorylation (Snyders and Kohorn 1999) but its precise role remains to be determined (Adapted from Rochaix (2013)) with permission).

state transitions involve the mobility of only a limited portion of the antenna.

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Regulating Synthesis of Cytochromes

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Summary

Detailed understanding of the regulation of cytochrome synthesis is an area that remains substantially undefined. Evidence to date suggests that the synthesis of cytochromes involves complex coordination of heme biosynthesis with expression of cytochrome apoproteins. Heme biosynthesis in turn is coordinated with the import of iron, as the presence of a coordinated iron in the heme tetrapyrrole is a key feature that allows heme to undertake redox reactions, redox sensing, and to carry diatomic gases such as O_2, CO or NO. Some cytochrome oxidases also contain copper centers that are involved in shuttling electrons to and from heme groups. Since excess of iron and/or copper is toxic to cells, the proper import of these metals is also a significant consideration for the synthesis of some respiratory cytochromes. This review will cover both transcriptional and post-transcriptional control mechanisms that affect the synthesis of heme, the expression of cytochrome apoproteins and homeostasis of iron and copper in photosynthetic organisms.

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I. Introduction

As discussed in other chapters of this volume, cytochromes are essential components in photosynthesis where they function to safely shuttle electrons to and from the photosystems (PS) as well as to a variety of other cellular components. Notable cytochromes include the b_6f complex in cyanobacteria and plants that are essential for shuttling electrons between photosystem II (PSII) and photosystem I (PSI), and the homologous bc_1 complex in anoxygenic photosynthetic bacteria that is essential for photosynthetic cyclic electron transport in these bacteria. Not to be overlooked are various cytochrome oxidases and related reductases that generate membrane potential through the reduction of oxygen or other components such as dimethyl sulfoxide (DMSO).

By definition, all cytochromes contain one or more bound hemes that are either non-covalently or covalently attached to an apoprotein. Heme is composed of a heterocyclic tetrapyrrole ring, termed a porphyrin, that has iron bound to four pyrrolic groups. The synthesis of heme is highly regulated, as unbound free heme is a lipophilic toxic moiety that can catalyze damaging lipid peroxidation, oxidation of proteins and DNA damage (Kumar and Bandyopadhyay 2005; Graça-Souza et al. 2006; Anzaldi and Skaar 2010). All of these deleterious reactions are catalyzed by oxidative and reductive properties of an iron that bound to four pyrrole groups in a porphyrin ring. Free iron is also highly toxic as it can generate damaging oxygen radicals

through a process known as the Fenton reaction (Touati 2000; Chiancone et al. 2004; Cornelis et al. 2011). One can therefore think of cytochromes as a protein moiety that has evolved to safely and effectively control the redox properties of iron bound to heme that additionally is also capable of providing substrate and product specificity. It is because of the toxic nature of free iron and free heme that evolution has selected complex cellular mechanisms of coordinating the transport of iron with the synthesis of the tetrapyrrole ring that binds the iron, which is in turn coordinated with synthesis of cytochrome apoproteins. Coordination of these events ensures that there is minimal free iron or free heme present in most cells. Finally, some cytochrome oxidases also contain two copper centers as prosthetic groups that are also involved in electron transport (Wainio et al. 1959; Tsukihara et al. 1995). As is the case of iron, unbound copper is also highly toxic, necessitating tight control on its transport (Wainio et al. 1959; Tsukihara et al. 1995; Osman and Cavet 2008; Macomber and Imlay 2009; Dupont et al. 2011).

This review is centered on what is known about the control of cytochrome apoprotein synthesis, its relation to iron and copper transport, and the synthesis of the tetrapyrrole component of heme (Fig. 25.1). We will discuss evidence that heme is itself an important feedback regulator of its own synthesis at both the transcriptional and post-transcriptional levels. We also will cover evidence that there is coordination of heme synthesis gene expression with the expression of cytochrome apoproteins. Most of the studies on the control of cytochrome biosynthesis in photosynthetic organisms have been undertaken with purple photosynthetic bacteria, specifically *Rhodobacter capsulatus* and *Rhodobacter sphaeroides*. This review will therefore be focused on these studies, although we will touch on what is known in other classes of photosynthetic organisms.

Abbreviations: ALA – δ−aminolevulinic acid; BChl – Bacteriochlorophyll; cbb_3-Cox – cbb_3 cytochrome oxidase; Chl – Chlorophyll; Cys – Cysteine; DMSO – Dimethyl sulfoxide; GluTR – Glutamyl-tRNA reductase; PAS – Per-Arnt-Sim; PBG – Porphobilinogen; PPIX – Protoporphyrin IX; PS – Photosystem; *Rb.* – *Rhodobacter*
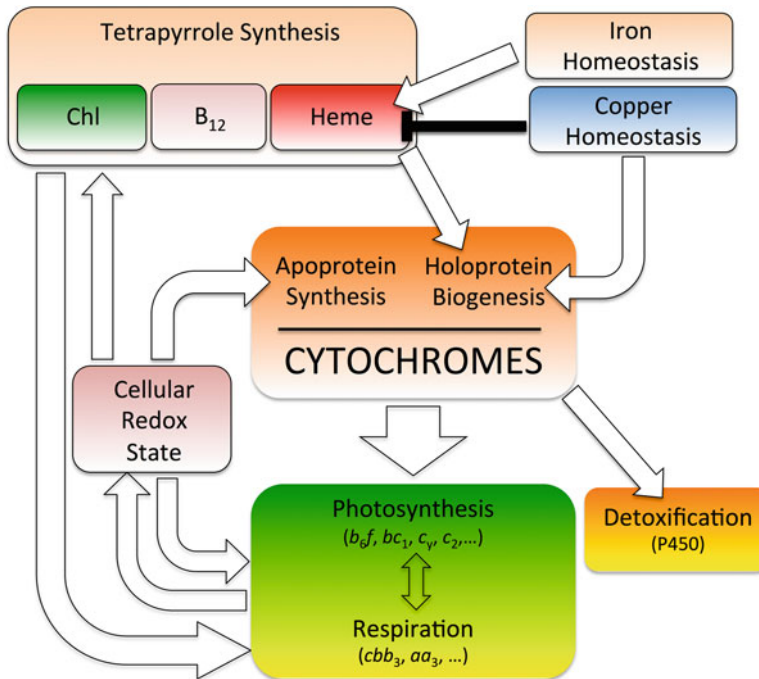
Fig. 25.1. Overview of major cellular signals controlling cytochrome biosynthesis and major cellular functions of cytochromes. Only prokaryotic photosynthetic organisms can synthetize vitamin B_{12}. Only a subset of cytochromes, the terminal oxidases, uses copper as a cofactor. Copper was shown to inhibit heme synthesis (Azzouzi et al. 2013). *B_{12}* cobalamin/vitamin B_{12}, *Chl* chlorophyll, *aa_3* and *cbb_3*, *aa_3* and *cbb_3* cytochrome oxidases, *P450* cytochrome P450.

II. The Bi- and Tri-furcated Tetrapyrrole Pathway

As indicated in Fig. 25.2, the tetrapyrrole biosynthetic pathway is quite complex involving many enzymes and a branched pathway that leads to the synthesis of heme, chlorophyll (Chl) or bacteriochlorophyll (BChl in anoxygenic phototrophs) and cobalamin (vitamin B_{12}). Heme is present in all branches of life (including all photosynthetic organisms) and is thus thought to be the most ancient of these three tetrapyrroles (Yin and Bauer 2013). In contrast, Chl is not present in Archaea. In eukaryotes, Chl is thought to be derived from endosymbiosis of a cyanobacterium that led to the formation of chloroplasts (Xiong et al. 2000; Xiong and Bauer 2002). As a result, in addition to complexities of regulating heme synthesis, photosynthetic cells are also faced with

the challenge of balancing heme synthesis with the synthesis of the second important tetrapyrrole, Chl/BChl, which has bound Mg instead of iron. Heme and Chl biosynthesis branch from each other at protoporphyrin IX (PPIX), which is the point where metal chelation into the tetrapyrrole PPIX occurs (Suzuki et al. 1997; Yin and Bauer 2013). As is the case with heme, free Chl/BChl is toxic, as light excited Chl/BChl is capable of forming a triplet state that can transfer energy to oxygen in the ground state to form highly reactive singlet oxygen. This necessitates that Chl/BChl synthesis also be highly regulated. To add further complexity, bacteria in general and photosynthetic bacteria in particular (anoxygenic and cyanobacteria) also synthesize a third tetrapyrrole cobalamin (vitamin B_{12}) that contains cobalt bound to a tetrapyrrole ring (Yin and Bauer 2013). Cobalamin is not synthesized by algae and
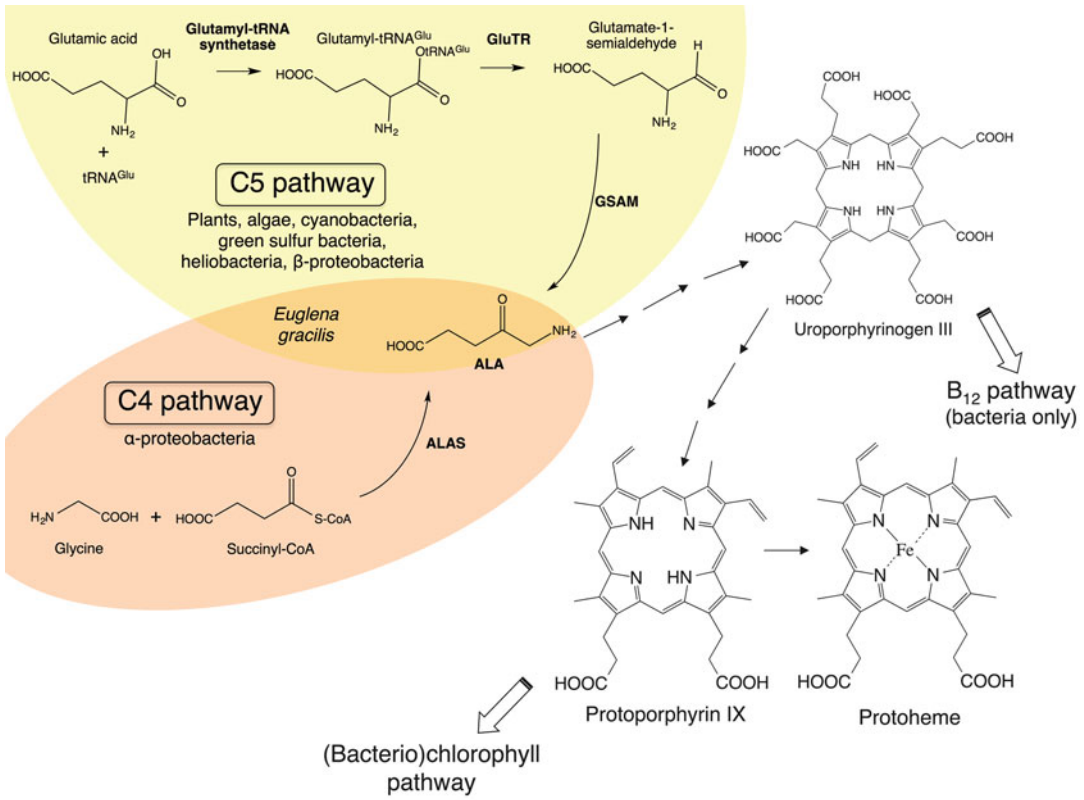
Fig. 25.2. Overview of heme synthesis pathways in photosynthetic organisms. Note that the C4 pathway is also known as the Shemin pathway. The only known organism to harbor both the C4 and C5 machinery is *Euglena gracilis* (Frankenberg et al. 2003). *ALA* δ−aminolevulinic acid, *ALAS* δ−aminolevulinic acid synthase, *GluTR* glutamyl-tRNA reductase, *GSAM* glutamate-1-semialdehyde-2,1-aminomutase.

plants, so these photosynthetic organisms only have to balance the synthesis of two tetrapyrroles instead of three.

Heme and Chl are both involved in redox chemistry as they accept and donate electrons. By definition, cytochromes are proteins containing a bound heme that is used to perform redox chemistry. Heme has the additional characteristic of functioning as a gas carrier as the heme iron bound to a protein such as hemoglobin is capable of forming an axial ligand to diatomic gasses such as O_2, CO, and NO. Chlorophylls are not gas carriers but they do very effectively donate an electron when the tetrapyrrole ring is excited by light. Typically, an electron is donated to a neighboring Chl, pheophytin (a Chl derivative that lacks Mg), or to a ubiquinone. Cobalamin does not undergo re-

dox chemistry like that of heme and Chl, and is instead involved as a cofactor in enzymatic reactions such as the donation of a methyl group or the reduction of nucleotides (Banerjee and Ragsdale 2003).

III. Regulating Heme Biosynthesis

A. Regulation by Heme or Other Tetrapyrrole Precursors

One mechanism of regulating heme synthesis is feedback inhibition in which free heme inhibits the activity of the first enzyme of the pathway, δ−aminolevulinic acid synthase. This enzyme catalyzes the formation of δ−aminolevulinic acid (ALA) from glycine and succinyl-CoA as first established by

Shemin and Russell (1953) and is known as the C-4 pathway (Fig. 25.2). In most characterized species, this enzyme is rate limiting and is feedback-inhibited by free heme (Burnham and Lascelles 1963). Such heme-based regulation was suggested in the purple bacterium *Rb. sphaeroides* as early as 1960, in which excess ALA was observed to decrease the activity of ALA synthase and the downstream enzyme porphobilinogen (PBG) synthase in vivo. Likewise, exogenous heme was shown to specifically decrease ALA synthase activity. On the contrary, PPIX and Mg-PPIX did not exert any influence on these enzymes (Lascelles 1960; Lascelles and Hatch 1969).

Plants, algae, and many bacterial species also synthesize ALA by an alternative route called the C-5 pathway (Beale and Castelfranco 1973; Beale 1978; von Wettstein et al. 1995; Kumar et al. 1996). This alternative route involves several enzymes such as glutamate tRNA synthase where glutamate is coupled to tRNA$_{glu}$ followed by glutamyl-tRNA$_{Glu}$ reductase (GluTR) that catalyzes the reduction of glutamyl-tRNA$_{Glu}$ to a glutamate 1-semialdehyde (Schön et al. 1986). A third enzyme then converts the glutamate 1-semialdehyde into ALA. Early studies in cyanobacteria, green algae (*Chlorella* and *Chlamydomonas*), and plant extracts indicated that the C-5 pathway for ALA production is also inhibited by free heme through a direct interaction with GluTR (Chereskin and Castelfranco 1982; Weinstein and Beale 1985; Huang and Wang 1986a, b; Rieble and Beale 1991; Pontoppidan and Kannangara 1994; Vothknecht et al. 1996).

In addition to feedback control of enzyme activity, the expression of many genes coding for enzymes in the common heme branch of the pathway (*hem* genes) is also regulated through heme availability. In the alga *Chlamydomonas*, Huang and Wang (1986a, b) initially reported that ALA synthesis was regulated in response to heme availability both at the gene expression and enzyme activity levels. Recombinant GluTRs from *Chlorobium vibrioforme* and *Chlamydomonas reinhardtii*

were purified and showed to bind heme. While heme displayed an inhibitory effect on the *Chlorobium* GluTR, this was not the case for the purified *Chlamydomonas* GluTR (Srivastava and Beale 2005; Srivastava et al. 2005). Interestingly, such inhibition was observed in the presence of a crude extract of *Chlamydomonas* in the enzyme reaction mixture (Srivastava et al. 2005). Beside heme, Mg-PPIX the first intermediate of the chlorophyll biosynthesis pathway, was found to activate the transcription of the GluTR-encoding gene, while inhibiting ALA synthesis at the enzyme level (Vasileuskaya et al. 2005; Meinecke et al. 2010; Voss et al. 2011). In purple photosynthetic bacteria, Smart and Bauer (2006) demonstrated that expression of many of the common trunk *hem* genes (*hemA, hemB, hemC, hemE* and *hemH*) was reduced when *Rb. capsulatus* cells were grown in the presence of exogenous heme. Smart and Bauer (2006) further reported the discovery of a heme-binding transcription factor termed HbrL that appears to have a central role in controlling *hem* gene expression in response to heme availability. Sequence analysis of HbrL indicates that it is a member of the LysR family of transcription factors (Smart and Bauer 2006). Spectral analysis of isolated HbrL indicates that it is capable of binding heme (Smart and Bauer 2006).

Studies of *hem* gene expression in *Rb. capsulatus* cells grown in the absence of excess heme, as well as in the presence of an iron chelator that decreases heme levels, show that HbrL functions as an activator of *hemA* (ALA-synthase), *hemB* (PBG synthase), *hemC* (PBG deaminase), *hemE* (uroporphyrinogen decarboxylase), *hemN2/hemZ* (coproporphyrinogen III oxidase), *hemJ* (protoporphyrinogen oxidase), and *hemH* (ferrochelatase) (Smart and Bauer 2006; Zappa and Bauer 2013). In contrast, when cells are grown in the presence of excess heme, HbrL appears to function as a repressor of *hemB* (Smart and Bauer 2006).

In addition to HbrL, there is a second heme-binding transcription factor called PpsR in *Rb. sphaeroides* and the related

CrtJ in *Rb. capsulatus* that is involved in controlling *hem* gene expression in these species (Smart et al. 2004; Yin et al. 2012). PpsR/CrtJ is a well characterized transcription factor that is also involved in redox control of the Mg-tetrapyrrole branch leading from PPIX to BChl (Penfold and Pemberton 1991, 1994; Gomelsky and Kaplan 1995; Ponnampalam and Bauer 1997; Elsen et al. 1998; Ponnampalam et al. 1998). Smart et al. (2004) demonstrated that CrtJ is capable of functioning as a repressor of *hemA*, *hemC* and *hemE* expression, as well as a significant activator of *hemZ* and *hemH* expression in *Rb. capsulatus*. Moskvin et al. (2005) also demonstrated the presence of three PpsR binding site in the divergent *hemC* and *hemE* promoter region of *Rb. sphaeroides*. Recently, it was demonstrated that PpsR from *Rb. sphaeroides* is itself a heme binding transcription factor and that the presence of excess heme in vivo results in decreased ability of PpsR to repress the expression of genes in the Mg-tetrapyrrrole (BChl) branch (Yin et al. 2012). There is no effect of excess heme on the ability of PpsR to repress *hemC* or *hemE,* so it was reasoned that excess heme specifically disrupts the ability of PpsR to bind to a subset of genes leading to increased BChl biosynthesis. Presumably, this would allows the cell to "bleed off" excess PPIX into the Mg-tetrapyrrole (BChl) branch leading to a reduction in heme biosynthesis (Yin et al. 2012).

B. Redox Control of hem Gene Expression

As discussed above, there are a common steps in the heme and BChl biosynthetic pathways, from the synthesis of ALA to PPIX. These two pathways separate at the point of metal insertion (Fe versus Mg) into PPIX (Fig. 25.3). In many species of purple photosynthetic bacteria, the synthesis of the BChl is quite variable as it is highly repressed (>100-fold) in response to the presence of molecular oxygen and to a lesser degree by high light intensity. Thus, there is a considerable flux of tetrapyrrole

intermediates that pass through the common heme/BChl trunk of the tetrapyrrole pathway depending on oxygen tension and light intensity. This has an impact on how the cell must control and coordinate the synthesis of heme with that of BChl and cobalamin as well as the cytochrome and photosystem apoproteins that bind heme and BChl, respectively.

Several studies on the control of heme synthesis in the purple bacteria *Rb. capsulatus* and *Rb. sphaeroides* have shown that expression of *hem* genes is altered in response to oxygen tension. In *Rb. sphaeroides*, aeration of the growth medium was observed to impact negatively the two first enzymes of the heme biosynthesis pathway, ALA synthase and PBG synthase (Lascelles 1960). Neidle and Kaplan (1993a, b) demonstrated that the expression of ALA synthase encoding genes *hemA* and *hemT* was oxygen repressed. In *Rb. capsulatus*, Smart et al. (2004) demonstrated that expression of *hemA* (ALA-synthase), *hemB* (PBG synthase), *hemC* (PBG deaminase), *hemE* (uroporphyrinogen decarboxylase), and *hemN2/hemZ* (coproporphyrinogen III oxidase) increased significantly when *Rb. capsulatus* cells are grown under anaerobic conditions where BChl biosynthesis occurs at a maximum rate. Presumably, this is in response to the shunting of tetrapyrrole intermediates from the common trunk into the Mg-tetrapyrrole branch necessitating that *hem* gene expression be increased to allow increased synthesis of these early intermediates. Interestingly, *hemH* (ferrochelatase) expression was not highly regulated by oxygen, indicating that the pool of PPIX must remain fairly static even under anaerobic conditions where much PPIX is shunted into the Mg-branch.

1. CrtJ/PpsR

One of the regulators identified to be involved in redox control of heme gene expression is CrtJ/PpsR, which as discussed above is also capable of binding heme
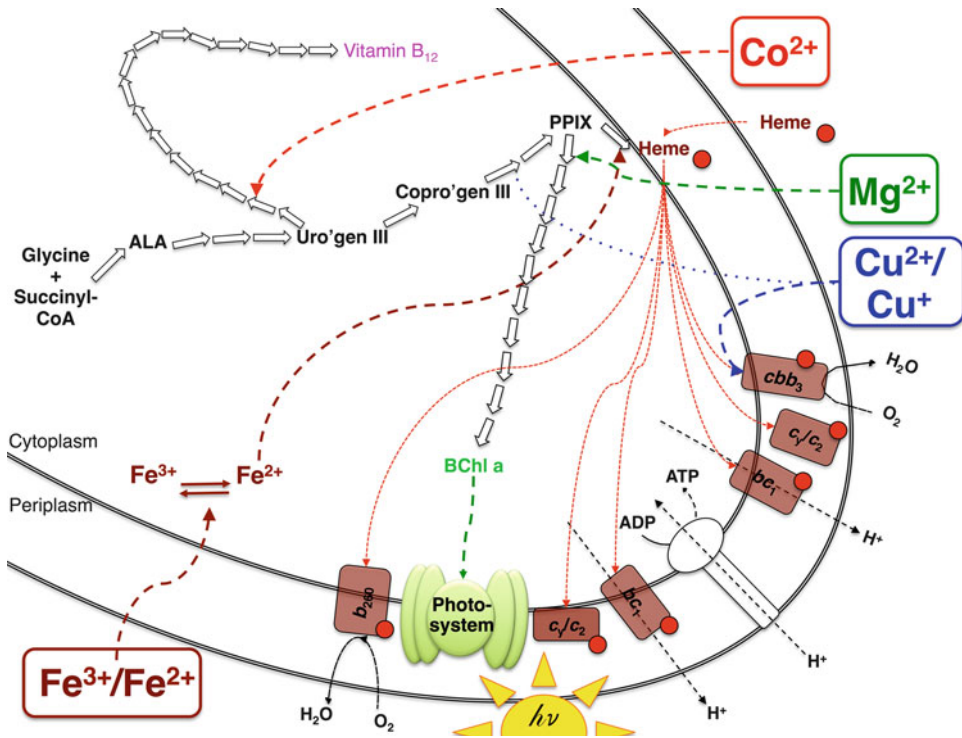
Fig. 25.3. Interdependence of metal import and tetrapyrrole and cytochrome biosynthesis. The trifurcated tetrapyrrole biosynthetic pathway leads to the formation of the Co-, Fe- and Mg-containing tetrapyrroles, namely cobalamin (vitamin B_{12}), heme and BChl, respectively. Cytochromes are dependent on heme synthesis, which itself relies on iron availability, and those represented are involved in the photosynthetic and respiratory electron chains. Copper is a cofactor of respiratory terminal oxidase cbb_3-Cox and was shown to inhibit coproporphyrinogen III oxidase in the tetrapyrrole pathway (*dotted blue line*). *ALA* δ−aminolevulinic acid, b_{260} ubiquinol oxidase, bc_1 cytochrome c oxidoreductase, *BChl a* bacteriochlorophyll a, c_2 cytochrome c_2, c_y cytochrome c_y, $cbb_3$$cbb_3$ cytochrome oxidase, *Copro'gen III* coproporphyrinogen III, *PPIX* protoporphyrin IX, *Uro'gen III* uroporphyrinogen III.

(Smart et al. 2004; Yin et al. 2012). The primary sequence of CrtJ consists of two Per-Arnt-Sim (PAS) domains at the N-terminal followed by a helix-turn-helix DNA binding domain at the C-terminal. In addition to heme binding, CrtJ contains a redox active cysteine (Cys) that is located in the helix-turn-helix DNA binding domain (Masuda and Bauer 2002; Masuda et al. 2002; Cheng et al. 2012). This Cys is capable of forming a disulfide bond with a second Cys in the PAS domain, as well as forming a stable sulfonic acid (Cys-OH) derivative (Masuda et al. 2002; Cheng et al. 2012). Genetic studies suggest that the oxidized Cys-OH form of CrtJ is an aerobic repressor of hemA (ALA synthase) hemC (PBG

deaminase), and hemE (uroporphyrinogen decarboxylase) (Smart et al. 2004). Interestingly, CrtJ appears to be both a strong aerobic and anaerobic activator of hemH, under both aerobic and anaerobic conditions (Smart et al. 2004).

2. RegB-RegA

In addition to CrtJ, the redox responding RegB-RegA two-component signal transduction cascade is known to anaerobically activate *hemA, hemC, hemE, hemZ* and *hemH* expression in *Rb. capsulatus* (Smart et al. 2004) and *hemA* in *Rb. sphaeroides* (Ranson-Olson et al. 2006; Ranson-Olson and Zeilstra-Ryalls 2008). RegA is known to
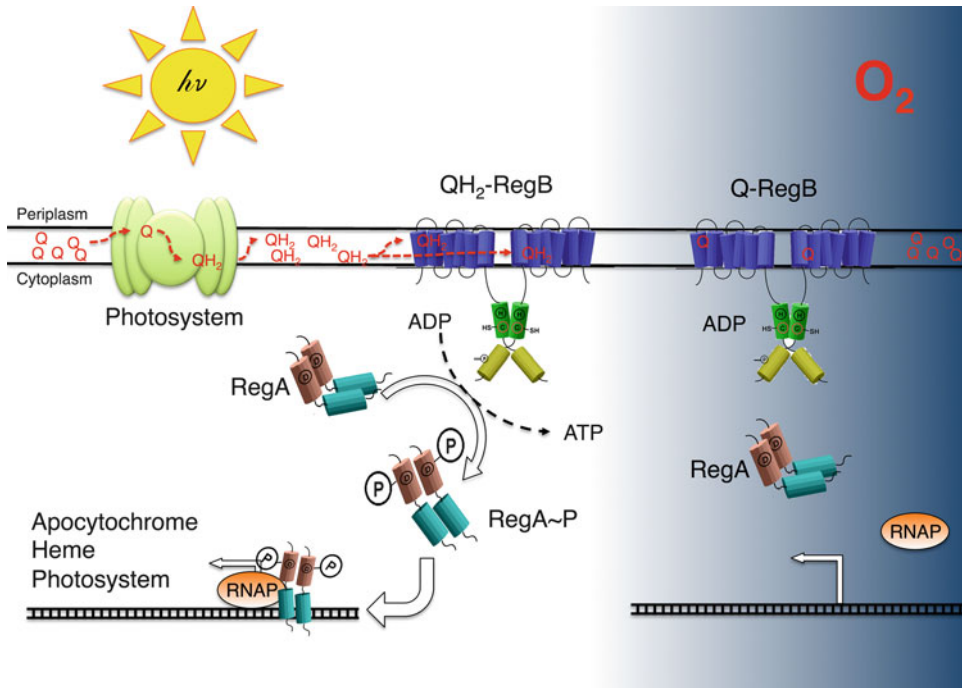
Fig. 25.4. Redox regulation of tetrapyrrole, photosystem and cytochrome biosynthesis by RegBA. The cellular redox state is monitored by the equilibrium between oxidized and reduced ubiquinones, Q and QH_2, respectively. Light energy induces the formation of QH_2. In turn, QH_2-activated RegB triggers the ATP-dependent phosphorylation of RegA. Phosphorylated RegA activates the transcription of a plethora of genes, including ones involved in the synthesis of heme, apocytochrome and photosystem structural protein.

regulate nearly all of the *bch* genes coding for enzymes in the Mg-branch leading to the synthesis of BChl (Willett et al. 2007). RegA thus appears to be a major anaerobic activator of the pathway to ensure increased flux of tetrapyrroles through this pathway under anaerobic conditions where there is maximal synthesis of BChl. As shown in Fig. 25.4 this signal transduction cascade involves the membrane spanning histidine kinase RegB that phosphorylates the DNA binding response regulator RegA. Phosphorylation stimulates the DNA binding activity of RegA (Inoue et al. 1995; Bird et al. 1999). Under anaerobic conditions, RegB kinase activity is active leading to phosphorylation of RegA (Mosley et al. 1994).

RegB has multiple input signals that control its kinase activity in response to environmental redox conditions. One input is the redox state of the ubiquinone pool which is affected by both photosynthesis and respiration (Fig. 25.4). Four membrane-spanning helices of RegB have been shown to interact with both oxidized and reduced forms of ubiquinone with nearly equal low affinities (Swem et al. 2006; Wu and Bauer 2010). It is proposed that a loosely bound ubiquinone to RegB is therefore in equilibrium with the ubiquinone pool. Thus, when the ubiquinone pool is oxidized, as in aerobic respiratory conditions, then the bound ubiquinone would be in an oxidized state. However when the ubiquinone pool is in a reduced state, as would occur under anaerobic photosynthetic growth conditions, then RegB contains bound reduced ubiquinone. Interestingly, the presence of oxidized ubiquinone effectively inhibits the kinase activity of RegB assuring that the kinase is inactive under respiratory growth conditions (Swem et al. 2006; Wu and Bauer 2010). A second level of control is offered by a redox active Cys in RegB that is

known to undergo oxidation in the presence of oxygen to form a stable Cys sulfonic acid derivative (Swem et al. 2003; Wu et al. 2013). Oxidation of this Cys also inhibits the kinase activity of RegB assuring that there is rapid shut off of kinase activity when the cells are exposed to molecular oxygen. Finally, it has also been proposed that a RegB homolog from *Rb. sphaeroides* called PrrB interacts directly with *cbb*$_3$ cytochrome oxidase (*cbb*$_3$-Cox) and that this interaction affects PrrB activity (O'Gara et al. 1998; Oh and Kaplan 1999; Kim et al. 2007).

3. FnrL

In *Rb. capsulatus* and in *Rb. sphaeroides* the redox responding transcription factor FnrL is also thought to have a role in controlling *hem* gene expression. In *Rb. capsulatus,* Smart et al. (2004) identified putative FnrL binding sites in the *hemB* and *hemZ* promoter regions. In *Rb. sphaeroides* there are two promoters identified that control *hemA* expression with a putative FnrL binding site overlapping the start site of the upstream promoter (Zeilstra-Ryalls and Kaplan 1995; Ranson-Olson and Zeilstra-Ryalls 2008). A role of FnrL in controlling *hem* gene expression is intriguing given that the *E. coli* Fnr homolog has an oxygen labile 4Fe-4S cluster that is needed for DNA binding (Bauer et al. 1999). One would presume that, in times of Fe limitation, FnrL would be devoid of this Fe-S cluster leading to an alteration of *hem* gene expression. In *Rubrivivax gelatinosus*, FnrL was shown to control heme synthesis by regulating *hemN* expression (Ouchane et al. 2007) (Table 25.1).

IV. Regulating Cytochrome Apoprotein Synthesis

A. Transcription Control

1. Redox Regulation

Evidence to date indicates that purple photosynthetic bacteria (and by conjecture other photosynthetic organisms) regulate apocytochrome synthesis by both transcriptional and post-transcriptional mechanisms. At the transcription level, several studies in *Rb. capsulatus* and in *Rb. sphaeroides* show that cytochrome encoding gene expression is redox-controlled by many of the same regulators that regulate heme synthesis. For example, as summarized in Fig. 25.3, the RegB-RegA signal transduction cascade, which is a major regulator of heme synthesis, also controls the expression of cytochrome encoding genes in both *Rb. capsulatus* and in *Rb. sphaeroides* (Eraso and Kaplan 1994; Karls et al. 1999; Swem et al. 2001). For example, reporter studies, coupled with DNA binding footprint assays, have demonstrated that phosphorylated RegA binds to the promoter regions of *cycA* (cytochrome c_2), *cycY* (cytochrome c_y), *ccoNOQP* (*cbb*$_3$-Cox), *cydAB* (ubiquinol oxidase) and *petABC* (cytochrome bc_1) in *Rb. capsulatus* (Swem et al. 2001). It has also been demonstrated that *cycA* is directly regulated by PrrA, the RegA homolog in *Rb. sphaeroides* (Eraso and Kaplan 1994; Karls et al. 1999). In *Rb. capsulatus,* each of these cytochromes are involved in either the photosynthetic or respiratory electron transport chain or both. Specifically, two electrons from the photosynthesis reaction center oxi-

Table 25.1. Known transcription factors that affect synthesis of heme and cytochromes.

Transcription factor	Cofactor	Function regulated
RegB-RegA	Ubiquinone, Cu$^+$	Heme, BChl, PS, cytochrome apoprotein synthesis
CrtJ/PpsR	Heme	Heme, BChl, PS, cytochrome apoprotein synthesis
HbrL	Heme	Heme synthesis, iron transport
FnrL	4Fe-4S cluster	Heme synthesis
FixLJ-FiK	Heme	Heme, BChl, PS, cytochrome apoprotein synthesis
FurA	Heme	Heme synthesis, iron transport

dized by light, enter the ubiquinone pool by release of reduced ubiquinone from the reaction center. Electrons from the ubiquinone pool are shuttled back to the reaction center by first passing through the membrane bound cytochrome bc_1 complex and to either cytochrome c_2 or cytochrome c_y that then reduces the oxidized reaction center (Fig. 25.3). When oxygen is present, the electrons preferentially pass from cytochrome c_2 or cytochrome c_y to cbb_3-Cox, essentially bypassing the reaction center and thereby ensuring that respiration occurs preferentially relative to photosynthesis. Ubiquinol oxidase can also reduce oxygen to water by obtaining electrons directly from the ubiquinone pool (Fig. 25.2). Given that photosynthesis adds electrons to the ubiquinone pool, and that respiration removes electrons from the ubiquinone pool, it is not surprising that the ubiquinone pool is significantly more reduced under photosynthetic growth conditions than under respiratory growth conditions (Grammel and Ghosh 2008; Klamt et al. 2008). Thus, controlling RegB kinase activity in response to alterations of the redox state of the ubiquinone pool provides a facile way of monitoring whether oxygen is present or absent as a substrate for respiration (Fig. 25.4).

One interesting aspect of respiratory cytochrome synthesis in *Rb. capsulatus* is that cbb_3-Cox is maximally expressed under semi-aerobic and aerobic growth conditions, whereas ubiquinol oxidase is maximally expressed under anaerobic/semi-aerobic conditions (Swem and Bauer 2002; Swem et al. 2003). It is proposed that cbb_3-Cox has a lower affinity but a higher turnover for substrate oxygen then does ubiquinol oxidase, which is proposed to have a higher affinity for oxygen with a lower substrate turnover (Swem and Bauer 2002). Thus, as cultures go from high to low oxygen tension cbb_3-Cox will be used preferentially followed by ubiquinol oxidase. It is of interest that unphosphorylated RegA appears to function as an aerobic activator of cbb_3-Cox while RegA $\sim$ P appears to function as an anaerobic repressor (Swem and Bauer

2002; Swem et al. 2003). In contrast, both RegA and RegA $\sim$ P function as aerobic and anaerobic activators of ubiquinol oxidase (Swem and Bauer 2002; Swem et al. 2003).

Additional studies on respiratory cytochrome gene expression in *Rb. capsulatus* indicate that ubiquinol oxidase expression is also aerobically repressed by CrtJ and by its coregulator AerR, while cbb_3-Cox is anaerobically activated by FnrL (Swem and Bauer 2002; Swem et al. 2003). Such an FnrL-dependent activation of *ccoNOPQ*, which encodes cbb_3-Cox, was also observed in *Rb. sphaeroides* under microaerophilic conditions (Mouncey and Kaplan 1998). This latter result is supported by the presence of a putative FnrL binding site located between two RegA binding sites in the *Rb. capsulatus ccoNOQP* promoter region, indicating that FnrL has a direct role in controlling cbb_3-Cox biosynthesis (Swem et al. 2003). In addition, FnrL is also absolutely required for anaerobic synthesis of dimethyl sulfoxide (DMSO) reductase that utilizes the alternative respiratory electron acceptors DMSO and trimethylamine oxide (Zeilstra-Ryalls et al. 1997). This requirement of FnrL is of interest given that it has an oxygen labile 4Fe-4S cluster that is needed for DNA binding (Bauer et al. 1999). Thus, the expression of cbb_3-Cox, DMSO reductase and heme biosynthesis are all likely to be impaired under periods of iron stress.

In *Rubrivivax gelatinosus*, deletion of *fnrL* also resulted in the loss of *c*-type cytochromes and PpsR was found to be involved in the expression of the cytochrome *bd* quinol oxidase in aerobic conditions (Ouchane et al. 2007; Hassani et al. 2010b). In this organism, a model was established that described the equilibrium between the use of *bd* quinol oxidase and cbb_3-Cox under aerobic and micro-aerobic conditions, respectively. Such equilibrium is dependent on both FnrL and PprsR (Ouchane et al. 2007; Hassani et al. 2010b).

Another redox control of cbb_3-Cox occurs through the FixLJ-FixK system in *Rhodopseudomonas palustris*, where the oxygen sensor FixL phosphorylates FixJ

that, in turn, activates the transcription factor FixK. FixL uses a heme group to sense O_2 and to subsequently control the phosphorylation of FixJ in an oxygen dependent manner. Phosphorylated FixJ subsequently transcribes FixK which then activates transcription of heme synthesis genes as well as *ccoNOPQ* and *coxBAEFGC*, coding for *cbb$_3$*-type and putative *aa$_3$*-type cytochrome oxidases, respectively. Moreover, the FixK regulon also includes a putative homolog of the response regulator, Irr, that is known to control expression of iron transport genes in response to heme availability (Qi and O'Brian 2002). This complex regulatory cascade further illustrates the interconnection that exists between iron homeostasis, heme biosynthesis, and cytochrome regulation (Rey and Harwood 2010).

Beyond purple bacteria, the control of cytochrome gene expression is widely unstudied. In the cyanobacterium *Synechocystis* sp. PCC 6803, it has been shown that the addition of glucose to the culture results in increased expression of cytochrome *c* oxidase and ubiquinol oxidase (Lee et al. 2007). The mechanism of this regulation has not been studied.

2. Promoter Selectivity

Schilke and Donohue (1992) initially demonstrated that expression of the *cycA* gene coding for cytochrome c_2 was affected by heme availability. They reported that addition of ALA to cells resulted in a five to sevenfold increase in expression of *cycA*. A genetic selection for mutants that were altered in heme regulation of *cycA* expression was subsequently found to result in the isolation of a *Rb. sphaeroides* mutant, ChrR, that has increased *cycA* and *hemA* expression (Schilke and Donohue 1992).

Detailed analysis of the *Rb. sphaeroides cycA* gene has demonstrated that it contains three promoters driving its expression (Karls et al. 1998; MacGregor et al. 1998). The P1 promoter is recognized by the alternative heat shock sigma factor σ^{32} (Karls et al.

1998). The P2 promoter is recognized by the housekeeping sigma factor σ^{70} and is also regulated by PrrA (Karls et al. 1999), and the third promoter P3 is recognized by σ^{E} that is regulated by ChrR (Schilke and Donohue 1992; Newman et al. 1999). Detailed studies of σ^{E} and ChrR show that ChrR is a zinc binding anti-repressor of σ^{E} with the binding of ChrR to σ^{E} affected by singlet oxygen ($^1O_2$). Because ChrR was originally found to respond to a signal from the tetrapyrrole synthesis pathway, this latter involvement of $^1O_2$ is intriguing given that $^1O_2$ is generated as a byproduct of light excitation of Chl.

B. Post-translational Regulation

In addition to transcriptional regulation of cytochrome synthesis, there is considerable evidence that the stability of cytochrome apoproteins is regulated in order to achieve proper assembly of cytochromes with their cofactors (heme and copper) and partner subunits as detailed below. An excellent review on this topic has recently been published by Ekici et al. (2012a) and only highlights of this process will be covered here. Briefly, the biogenesis of *cbb$_3$*-Cox involves a plethora of actors (cofactors, chaperones, subunits, . . .) and mechanisms. Maturation of the catalytic subunit CcoN requires integration of heme *b* and copper co-factors, while CcoO and CcoP are targets of the heme *c* maturation pathway Ccm. The biogenesis involves copper chaperones, disulphide oxidoreductases, and specific membrane lipids (Ekici et al. 2012a). In addition, Oh and Kaplan (2002) initially demonstrated that loss of CcoQ resulted in destabilization of *cbb$_3$*-Cox in *Rb. sphaeroides*. They proposed that CcoQ was involved in protecting the *cbb$_3$*-Cox core complex (CcoN, CcoO and CcoP) from oxidation and loss of heme. In *Rb. capsulatus*, Peters et al. (2008) also demonstrated that loss of CcoQ resulted in instability of *cbb$_3$*-Cox and further showed that CcoQ specifically interacted with CcoP. A second assembly protein called CcoH has also been identified that affects stability

of the CcoP-CcoQ complex (Pawlik et al. 2010). As heme-copper cytochrome CcoN and *c*-type cytochromes CcoO and CcoP follow separate post-translational maturation process, fine-tuned regulation has to occur to obtain a functional cbb_3-Cox complex. On the one hand, strains harboring mutations in CcoN have proven to display abnormal amounts of CcoO and CcoP (Koch et al. 1998). On the other hand, biogenesis of CcoN was shown to still occur in Ccm defective mutants (Ekici et al. 2013).

Finally, the coupling of heme and cytochrome synthesis was further illustrated by a *Rb. capsulatus* strain that lacks *c*-type cytochromes while having a normal content of *b*-type cytochromes. This strain was shown to excrete a massive amount of coproporphyrin and protoporphyrin (1.8 and 0.9 μM, respectively), while synthesizing less BChl. Although the mechanism is not clear, it involves a mutation in the cytochrome *c* maturation system (Biel and Biel 1990). Chlorophyll were also found in purified preparations of cytochrome b_6f in *Chlamydomonas reinhardtii* (Pierre et al. 1997). Interestingly, synthesis of chlorophyll is also found to be necessary to accumulate cytochrome b_6f in both *Chlamydomonas reinhardtii* and *Arabidopsis thaliana* (Pierre et al. 1997; Pontier et al. 2007; Meinecke et al. 2010). Heme and Mg-PPIX were also showed to exert influence on the expression of cytochrome b_5 and a putative cytochrome *c* oxidase assembly protein COX17 (Voss et al. 2011).

V. Metal Homeostasis and Cytochrome Regulation

A. Iron Homeostasis

Harnessed by four linked pyrroles, iron is the key component of heme that provides this tetrapyrrole a capability to accept and donate electrons and gases. As such, iron bound to heme is *the* critical element of cytochromes. Owing to the toxicity of free iron, its acquisition is highly regulated and also has an impact on both heme and cytochrome synthesis. The coupling of iron homeostasis with heme biosynthesis was initially observed in the purple bacterium *Rb. sphaeroides* by Lascelles (1956). In conditions of iron deficiency, *Rb. sphaeroides* was observed to produce limited amounts of heme coupled to an increased accumulation of the heme precursor coproporphyrin (Lascelles 1956; Lascelles and Hatch 1969). More recent studies show that numerous genes coding for iron transporters and heme synthesizing enzymes were simultaneously upregulated in the purple bacterium *Rb. capsulatus* under conditions of iron scarcity (Zappa and Bauer 2013). As mentioned earlier, the LysR-type transcription factor appeared critical in this global activation that couples iron homeostasis and heme synthesis (Zappa and Bauer 2013). Likewise, in the cyanobacterium *Anabaena* sp. PCC 7120, iron deficiency induced the transcription of genes involved in iron homeostasis, heme synthesis and heme degradation. A homologue of the ferric uptake regulator, FurA, was found to be involved in coupling iron homeostasis and heme synthesis in these cyanobacteria (González et al. 2012). This coregulation is, however not universal in all species and nor even at the strain level in cyanobacteria. For example, the transcription of *hemA*, which encodes GluTR, was found to be down-regulated in a MED4 strain of *Prochlorococcus*, while it was stable in the MIT9313 strain under low iron conditions (Thompson et al. 2011).

Interdependence of iron homeostasis with the synthesis of cytochromes involved in photosynthesis has been extensively studied in cyanobacteria. Iron stress in cyanobacteria triggers a number of survival strategies such as the excretion of siderophores to take up iron from the environment, and the reduction of iron demand. The latter strategy consists of replacing an iron-utilizing enzyme by a non-iron utilizing enzyme, e.g. ferredoxin by flavodoxin. Overall, iron-containing enzymes in the photosynthetic electron chain, such as cytochromes, have their cellular level reduced during iron starvation (Ferreira and

Straus [1994]). Under low iron conditions, the transcription of *petA* and *petC,* encoding cytochrome b_6f subunits, is down-regulated in *Prochlorococcus* (Thompson et al. [2011]). Similar observations were made regarding the PetB and PetC subunits of cytochrome b_6f in *Trichodesmium erythraeum* (Shi et al. [2007]). The content of cytochrome b_6f, as observed from the assay of the PetC (Rieske) subunit, was found to be reduced in *Synechococcus elongatus* PCC 7942, and even more in *Synechocystis* sp. PCC 6803, where this results in a dramatic fourfold decrease of the b_6f:PSII ratio (Fraser et al. [2013]). Similarly, cytochrome *f* of *Synechococcus* sp. PCC 7942 was reduced to 40 % of its original content under iron limitation (Sandström et al. [2002]).

B. Copper Homeostasis

Several cytochrome *c* oxidases, such as cbb_3 and aa_3, are both heme and copper containing enzymes. The biogenesis of cbb_3-Cox in *Rb. capsulatus* and *Rb. sphaeroides* has been recently reviewed (Ekici et al. [2012a]). Since proper enzyme activity is dependent on copper availability, this section first focuses on cellular processes that connect copper homeostasis to the regulation of cbb_3-Cox. In both *Rhodobacter* species, cbb_3-Cox apoprotein is the product of the *ccoNOQP* operon, where CcoN is a membrane-embedded catalytic subunit that harbors both heme *b* and a copper center. How the cell imports copper and integrates it into cytochromes is not fully understood, but in the case of cbb_3-Cox, the copper acquisition protein CcoA was recently discovered (Ekici et al. [2012b], [2014]). CcoA is a member of the "Major Facilitator Superfamily" of transporters, members of which have 12 transmembrane helices (Reddy et al. [2012]). Deletion of *ccoA* induces lower cellular copper content and a failure to achieve normal assembly of an active cbb_3-Cox, with such a phenotype being partially alleviated by exogenous copper supplementation (Ekici et al. [2012b]). CcoA showed limited influence on both

transcription and translation of CcoN, which suggests post-translational regulation (Ekici et al. [2012b]). Suppressors of a *ccoA* mutant displaying functional cbb_3-Cox harbor mutations that impair the activity of CopA, a copper efflux transporter (Ekici et al. [2014]). Thus, proper assembly of cbb_3-Cox depends on a copper homeostasis system consisting (at least) of the importer CcoA and the exporter CopA. Moreover, screening for a subset of *ccoA* mutants that do not respond to copper supplementation led to strains harboring mutations in the Ccm cytochrome maturation system. Interestingly, porphyrin secretion that can be observed in Ccm deleted strains seems to be decreased by addition of exogenous copper (Ekici et al. [2013]). Although no clear model exists at this time, these findings indicate an influence of copper on post-translational maturation of both *b*- and *c*-type subunits of cbb_3-Cox. Other cbb_3-Cox maturation components include CcoI and, possibly, CcoG. CcoI is critical for cbb_3-Cox biogenesis and is a P-type ATPase that transports copper across the cytoplasmic membrane to the periplasm. It takes part in the insertion of the copper ion into CcoN, although the detailed mechanism remains to be elucidated (Ekici et al. [2012a]). The role of CcoG is still unclear, partially due to lack of a defined phenotype of the CcoG mutant. However, involvement in redox balance of Cu^+/Cu^{2+} between copper import (CcoA) and export (CcoI) has been hypothesized (Ekici et al. [2012a]).

In addition to the copper transport proteins described above, the putative copper chaperone SenC in *Rb. capsulatus*, is involved in maturation of cbb_3-Cox (Lohmeyer et al. [2012]). SenC is a membrane-anchored protein that was shown to bind copper. Moreover, mutants lacking SenC are defective in cbb_3-Cox, with this phenotype capable of being rescued by copper supplementation. SenC interacts with the subunits CcoP and CcoH (Lohmeyer et al. [2012]). Interestingly, the impact of a SenC deletion on cbb_3-Cox maturation is pronounced at the usual and optimum growth temperature (35 °C), but not at 20 °C. Finally, whereas SenC seems

to affect the assembly of cbb_3-Cox post-translationally, the transcription of *senC* itself depends on the presence of the copper efflux pump CcoI (Lohmeyer et al. 2012). Interestingly, PrrC, the SenC homologue in *Rb. sphaeroides*, does not appear to be involved in cbb_3-Cox assembly (Eraso and Kaplan 2000). Finally, a *periplasmic Cu_A-c*haperone (PCu$_A$C) homologue was identified in *Rb. capsulatus,* despite this species not harboring a aa_3-type Cu_A-containing cytochrome oxidase (Ekici et al. 2012a; Lohmeyer et al. 2012). A potential role in trafficking copper between CcoI and CcoN has been suggested, although no evidence has been provided (Lohmeyer et al. 2012).

A model was recently proposed to describe how purple bacteria manage the toxicity of copper with its requirement for cytochrome *c* oxidases. In *Rubrivivax gelatinosus,* two Cu^{2+}-P1-type ATPases were characterized: CtpA (a homologue to *Rb. capsulatus* CcoI and *Rb. sphaeroides* RdxI), and CopA. Interestingly, CtpA was shown necessary for assembly of both cytochrome oxidases cbb_3 and $caa_3,$ while CopA does not take part in cytochrome biogenesis but alleviates copper toxicity (Hassani et al. 2010a; Azzouzi et al. 2013). In copper replete conditions, a *copA* mutant was shown to excrete coproporphyrin III, and have less cytochrome *c* oxidase. In the absence of CopA, copper seems to deregulate the synthesis of heme at the HemN step, which results in a decrease in cytochrome oxidase. The mechanism of copper toxicity on the HemN enzyme may involve the destabilization of the 4Fe-4S cluster. Such a mechanism highlights the delicate equilibrium of copper necessity in cytochrome assembly versus its toxic impact on heme precursor synthesis (Hassani et al. 2010a; Azzouzi et al. 2013). Moreover, CopA homologues are present in *Rb. capsulatus* and *Rb. sphaeroides* so this model could be extended to other purple bacteria (Ekici et al. 2012b; Azzouzi et al. 2013; Ekici et al. 2014).

C. Iron and Copper Homeostasis Interplay with Cytochrome Regulation

Cyanobacteria display a useful example of the influence of iron and copper homeostasis on the synthesis/stability of cytochromes in the photosynthetic electron transport chain. Indeed, cytochrome b_6f transfers electrons to PSI through either cytochrome c_{553} (cytochrome c_6) or plastocyanin, which utilizes copper as a co-factor. Under iron stress, cyanobacterial siderophore production also lowers copper availability, since iron binding siderophores also display a high affinity for copper (Ferreira and Straus 1994). However, unlike ferrisiderophores, the Cu^{2+}-siderophore complexes are not imported into the cell, hence generating copper limiting conditions. As a result, copper limitation is induced under iron stress, resulting in the preferential use of cytochrome c_6 over plastocyanin. This situation, starting with iron limitation and ending with the preferential use of an iron-using cytochrome over a copper-using protein, is counterintuitive. However, the rationale underlying these observations is that the pool of cytochrome c_6 is limited. Such an "imperfection" in the iron-sparing mechanism may be a small price to pay for an effective siderophore machinery that enables both iron scavenging and alleviation of copper toxicity. Interestingly, some cyanobacteria have lost the plastocyanin homologue in the process of natural selection and rely solely on cytochrome c_6, even during iron starvation (Ferreira and Straus 1994). Moreover, the Fe^{3+} transporter FutA$_2$ in *Synechocystis* sp. PCC 6803 was shown to be involved in this plastocyanin-cytochrome c_6 switch. Indeed, the *futA$_2$* deleted mutant showed low cytochrome *c* activity and fails to produce cytochrome c_6 instead of plastocyanin (Waldron et al. 2007).

Copper interference with cytochrome regulation takes place also at the aforementioned switch between plastocyanin and cytochrome c_6 in cyanobacteria. Indeed, such

a switch depends not only on iron but also on copper availability, as extracellular copper levels were shown to regulate transcription of both electron transporters (Zhang et al. 1992). In *Synechocystis* sp. PCC 6803, two Cu^{2+} transporters of the P1-ATPase family were described, as required for an efficient switch: CtaA and PacS, involved in cellular import and compartmentalization into thylakoids, respectively (Tottey et al. 2001). A copper chaperone, Atx1, acting with PacS, is also involved (Tottey et al. 2002).

VI. Concluding Remarks

Cytochromes are major electron carriers in photosynthetic organisms responsible for moving electrons among various components of the photosynthetic apparatus as well as to terminal electron acceptor complexes such as cytochrome oxidases. As such, they are, in large part, responsible for keeping a proper redox poise in cells that are continually challenged by a highly varying input of electrons from photosynthesis as well as from cellular metabolic pathways.

Cytochromes use iron bound to heme and, in some cases one or more bound coppers, as redox active centers to accept and donate electrons (Fig. 25.1). Both of these metals are toxic when not bound to cytochrome apoproteins. The same toxicity occurs when heme is not bound to cytochrome apoprotein. Not surprisingly, a high level of coordination occurs between the import of iron and copper for the synthesis of heme and cytochrome apoproteins. When iron, copper or heme synthesis is impaired, then the synthesis of cytochrome apoproteins is also inhibited.

Clearly the synthesis of cytochrome apoproteins depends on highly regulated events, and a molecular understanding of this regulation remains incomplete. Evidence is accumulating that there is a complex connection in the coregulation of cytochrome apoproteins with enzymes involved in iron transport and heme biosynthesis. It is intriguing that the redox state of the ubiquinone pool, which is highly affected by the activity of cytochromes, is a major input to the control of the RegB-RegA signal transduction cascade. This signaling cascade is responsible for controlling expression of genes that code for enzymes in heme biosynthesis and cytochrome apoproteins in anoxygenic phototrophs. The interconnections of iron transport with heme and cytochrome synthesis is further highlighted in anoxygenic bacteria where the DNA-binding transcription factors FnrL, PpsR and HbrL, which control iron transport, heme synthesis and apocytochrome synthesis, themselves bind either iron or heme as a cofactor. A major challenge will be to extend similar studies on the regulation of cytochrome biosynthesis to eukaryotic algae and plants where additional cellular processes such as organelle compartmentalization add further layers of complexity.

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Part VI

**Assembly of Cytochrome Complexes
and Super-Complexes**

Cofactor Assembly of Cytochrome bc_1-b_6f Complexes

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Summary

Cytochromes bc_1 and b_6f are enzyme complexes catalyzing essential electron transfer reactions in bacterial and organellar energy-transducing membranes. Electron transport relies on heme and Fe-S redox cofactors contained in the three catalytic subunits common to bc_1 and b_6f complexes: a *b*-type cytochrome (cytochrome *b*), a *c*-type cytochrome (cytochrome c_1 or *f*) and a [2Fe-2S]-containing protein (Rieske). Maturation of the *b*-type and *c*-type cytochromes requires conversion of the subunit from the apoform to the holoform by the incorporation of two hemes (b_L and b_H) in cytochrome *b* of the bc_1 complex, three hemes (b_L, b_H and c_i) in cytochrome b_6 of the b_6f complex, and a single heme (*c*) in both cytochromes c_1 and *f*. Assembly of heme *c* requires the formation of two thioether bonds at a C*XX*CH motif in cytochrome c_1 and *f* on the *p*-side, and for c_i, a single thioether bond in cytochrome b_6 on the *n*-side of the membrane, respectively. It is not known how non-covalently bound hemes (b_L and b_H) are inserted into cytochrome *b* and b_6, but covalent linkage of heme is known to be a catalyzed process. The chemistry of thioether linkage formation appears, on the surface, to be simple (i.e. addition of a sulfhydryl to the α carbon of the vinyl group of heme). However, experimental investigations in bacteria, mitochondria and plastids have revealed an unsuspected biochemical complexity for this process. At least six different pathways for thioether bond formation have been described. Four of these, System I, II, III (on the *p*-side) and IV (on the *n*-side), are defined by prototypical components and have been extensively studied.

I. Introduction

Cytochrome bc_1 and cytochrome b_6f complexes (hereafter referred to as bc_1 and b_6f) are multimeric enzymes responsible for electron transfer reactions in bacterial and eukaryotic energy-transducing membranes (Dibrova et al. 2013; Hasan et al. 2013). Cytochrome bc_1 complexes are quinol-cytochrome *c* oxido-reductases that function

in aerobic respiration in diverse bacterial phyla and all mitochondria, with the exception of some protozoan parasites and one photosynthetic alveolate (Nawathean and Maslov 2000; Stechmann et al. 2008; Flegontov et al. 2015). In photosynthetic bacteria, bc_1 are also key enzymes in anoxygenic photosynthesis (see Chap. 10). On the other hand, cytochrome b_6f complexes are essential to oxygenic photosynthesis and operate as quinol-plastocyanin (or cytochrome c_6) oxido-reductases in all cyanobacteria and plastids of both algae and land plants (see Chap. 9). In cyanobacteria, b_6f is also required for the respiratory electron transfer chain (see Chap. 17). Variations of the typical bc_1/b_6f enzymes also exist in several eubacterial and archeal lineages. Such a variant is the *bc* complex, an enzyme related to the b_6f, and

present in both anoxygenic phototrophic and non-phototrophic bacteria of the Firmicutes phylum (see Chap. 3).

Common to bc_1 and b_6f are three catalytic subunits, a b-type cytochrome, a c-type cytochrome (c_1 or f), and a Rieske iron-sulfur protein (ISP), which harbor the prosthetic groups required for catalysis (Dibrova et al. 2013; Hasan et al. 2013). In addition to the catalytic subunits, bc_1 and b_6f contain subunits which do not participate directly in electron transfer, but are required for assembly/activity of a functional complex. While mitochondrial bc_1 contains additional seven or eight subunits that are absent in bacterial bc_1 complexes (see Chap. 10), the four additional subunits found in the chloroplast b_6f have counterparts in the cyanobacterial complex (see Chap. 9). The biogenesis of bc_1 and b_6f is an intricate process, requiring the synthesis of the structural subunits, the maturation of the redox subunits by attachment/incorporation of the prosthetic groups and the stepwise integration of the different subunits in the membrane. In this chapter, we will focus on the pathways controlling prosthetic group attachment to the catalytic subunits of bc_1 and b_6f, with emphasis on the heme attachment reaction.

II. Prosthetic Groups in bc_1 and b_6f

A. Common and Unique Prosthetic Groups in bc₁/b₆f Complexes

Iron-sulfur (Fe-S) cluster and heme (ferroprotoporphyrin IX) are the prosthetic groups common to both bc_1 and b_6f complexes and are present in the three conserved catalytic subunits (Dibrova et al. 2013; Hasan et al. 2013). The membrane-anchored Rieske subunit, present in both bc_1 and b_6f, contains a single [2Fe-2S] cluster in a domain facing the p-side[1] of the membrane

(Dibrova et al. 2013; Hasan et al. 2013). Rieske ISPs are characterized by unique ligand coordination of their Fe-S cluster, with one of the two iron centers being coordinated by two cysteine residues and the other by two histidine residues (Schmidt and Shaw 2001). In bc_1/b_6f, cytochromes b and b_6 are membrane-embedded proteins containing two b-hemes, b_L and b_H, on the p- and n-side of the membrane, respectively. Both hemes of cytochromes b and b_6 are coordinated by two conserved histidines in a four-helix bundle. Cytochromes c_1 and f are membrane-anchored, c-type cytochromes, also generically referred to as cytochromes c. Cytochromes of the c-type are a large family of hemoproteins with one or several heme moieties covalently attached to a $CX_nC(H/K)$[2] motif, known as the heme-binding site (Thony-Meyer 1997). Axial ligands of covalently linked heme in c-type cytochromes are provided by the H/K residue in the heme-binding site and an amino acid in the protein, usually a methionine residue (Thony-Meyer 1997). Histidine in the heme-binding site is the most common axial ligand and lysine is only found in nitrate-reducing enzymes (Einsle et al. 1999). In cytochrome c_1 and cytochrome f, a single heme c is attached on the p-side of the membrane. This attachment occurs via two thioether bonds between the vinyl-2 and -4 groups of heme and the sulfhydryl groups on the first and second cysteines occurring in a CXXCH motif of the protein. A notable exception occurs in the phylum Euglenozoa where the vinyl-4 of heme is linked to a single cysteine contained within a FXXCH motif in mitochondrial cytochrome c_1 (Priest and Hajduk 1992).

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[1]p- and n-sides refer to the positive and negative side of the energy-transducing membrane system, respectively. The p-side corresponds to the bacterial

perisplasm, thylakoid lumen and mitochondria IMS while the n-side is the bacterial cytoplasm, plastid stroma and mitochondrial matrix.

[2]Where X can be any amino-acid except cysteine. The number of intervening residues (n) is usually 2, with the rare exception of some bacterial cytochromes where n = 3, 4 or 15 (Jungst et al. 1991; Jentzen et al. 1998; Aragão et al. 2003; Hartshorne et al. 2007).

Another type of covalent linkage of a single heme, referred to as heme c_i (or c_n), has been found in cytochrome b_6. This heme is covalently linked via one thioether bond between the α carbon of the vinyl-2 group of heme and a cysteine residue (Kurisu et al. 2003; Stroebel et al. 2003). Heme c_i was also detected as covalently linked to the cytochrome b subunit of the bc complexes in *Bacilli* and *Heliobacteria* representatives of the Firmicutes phylum (Kutoh and Sone 1988; Yu and Le Brun 1998; Ducluzeau et al. 2008). Unlike heme c in cytochromes c_1 and f, heme c_i is not attached to a recognizable consensus motif and is localized on the n-side of the membrane (de Vitry 2011). Because b-type and c-type cytochromes refer to cytochromes with non-covalently and covalently bound heme(s), respectively, cytochrome b_6, which was initially defined as a b-type based on b_L and b_H hemes is now also referred to as a c-type cytochrome because of the presence of heme c_i (Thony-Meyer 1997; Kuras et al. 2007).

In addition to Fe-S and heme, subunit IV of the b_6f contains two non-covalently bound pigments, one molecule of chlorophyll a (Huang et al. 1994; Pierre et al. 1997) and one molecule of β-carotene (Zhang et al. 1999), which are absent in the bc_1. The typical carotenoid in b_6f is 9-*cis*-β-carotene but other carotenoids, such as 9-*cis*-α-carotene and echinenone have been found in some algal and cyanobacterial enzymes (Boronowsky et al. 2001; Li et al. 2005). The function of chlorophyll a and β-carotene remains enigmatic as light is not required for electron transfer catalyzed by the b_6f complex.

B. Incorporation of Prosthetic Groups in bc$_1$ and b$_6$f Subunits

Maturation of the redox subunits of the bc_1 and b_6f requires apo- to holoform conversion of the subunit by the incorporation of one or several prosthetic groups. Failure to incorporate the prosthetic group yields a bc_1/b_6f assembly defect as the apoform of the subunit is usually unstable and degraded. One notable exception is the Rieske ISP in *Saccharomyces cerevisiae*, whose apoform was shown to assemble in the bc_1 in the absence of Fe-S cluster incorporation (Graham and Trumpower 1991).

While the requirement for catalysis in the covalent attachment of heme has long been acknowledged, it is not known if incorporation of non-covalently bound cofactors, such as b-hemes into apocytochrome b/b_6, chlorophyll a and β-carotene into subunit IV and the Fe-S cluster in the Rieske ISP are also enzymatically assisted in vivo. The fact that these cofactors are retained by non-covalent interactions, and were shown, in some cases, to be correctly inserted into their corresponding apoprotein(s) in vitro, has led to the assumption that incorporation of non-covalently bound cofactors is not a catalyzed reaction in vivo (Robertson et al. 1994; Holton et al. 1996; Gubernator et al. 2006). The recent discovery that different assembly factors of mitochondrial bc_1 associate with cytochrome b intermediates containing either b_L only or b_L and b_H suggests that incorporation of non-covalent heme might also be an assisted process (Hildenbeutel et al. 2014). Another example, highlighting the role of factors in assisting the delivery of non-covalently bound heme, is that of NO synthase. Its biogenesis requires thioredoxin, the Hsp90 chaperone, and glycerolphosphate-dehydrogenase for insertion of b-heme into the apoenzyme (Chakravarti et al. 2010; Ghosh et al. 2011; Hannibal et al. 2012). The concept of protein-assisted incorporation of non-covalently bound prosthetic group extends to molecules other than heme, as shown by the description of dedicated carriers facilitating Fe-S cluster insertion in a subset of apoprotein target(s) (Balk and Schaedler 2014). Hence, it is possible that incorporation of the Fe-S cluster in the apoform of bc_1/b_6f Rieske ISP also requires dedicated factor(s).

III. Pathways for the Covalent Attachment of Heme

A. Six Distinct Pathways for Heme Attachment in bc_1/b_6f

Covalent heme attachment to apocytochromes c_1 and f is a post-translational modification, occurring on the p-side of the energy-transducing membrane of bacteria, mitochondria and plastids (Kranz et al. 2009; Allen 2011; Mavridou et al. 2013; Verissimo and Daldal 2014). In cyanobacteria and plastids, heme c_i is formed by covalent linkage of heme to apocytochrome b_6 on the n-side of the thylakoid membrane. By analogy, it is reasonable to assume that heme c_i in Firmicute bc is also attached on the cytoplasmic side of the membrane (de Vitry 2011). Heme attachment can take place independently of the presence of other bc_1/b_6f complex subunits but the steady-state level of the holoprotein is usually dependent upon association/assembly with other subunits (Kuras et al. 1997; Zara et al. 2007).

At least three distinct mechanisms for the covalent attachment of heme to the apoforms of cytochromes c_1 and f, the so-called Systems I, II and III, have been described (Kranz et al. 2009; Allen 2011; Mavridou et al. 2013; Verissimo and Daldal 2014; Babbitt et al. 2015). All of these systems are not unique to the maturation of cytochrome c_1 and f, but some also control the heme ligation reaction to multiple apocytochrome c substrates in the bacterial periplasm, soluble apocytochrome c in the mitochondrial IMS (intermembrane space) and soluble apocytochrome c_6 in the thylakoid lumen of green algae. A fourth pathway for covalent heme attachment, System IV, is solely dedicated to the attachment of heme c_i to apocytochrome b_6, and hence is specific to the biogenesis of the b_6f (de Vitry 2011).

Because each system can be recognized on the basis of unique assembly factors, the distribution of the different maturation pathways was analyzed in the three domains of life. While the occurrence of a heme attachment pathway cannot be entirely predicted, a few generalities about the distribution of the three systems in energy-transducing membranes have been noted (Bertini et al. 2007; Allen et al. 2008a; Giegé et al. 2008; Allen 2011; Babbitt et al. 2015). While all bacteria utilize System I or System II, archaea have so far been found to have only System I. All plastids appear to use System II and mitochondria possess System I or System III.

Mitochondrial cytochromes c and c_1, with a heme attached via a single thioether bond, were found to occur in all three major taxonomic groups of the Euglenozoa (diplonemids, kinetoplastids and euglenids), even in the absence of any component of the known c-type cytochrome maturation system. This was taken as an indication that a novel pathway for cytochrome c maturation (also referred to as System V) must operate in mitochondria of organisms belonging to this phylum (Allen et al. 2008a; Allen 2011). Similarly, as no prototypical component of System IV can be detected in the genomes of Firmicutes, a novel pathway for thioether bond formation in cytochrome b, System VI, was postulated (Allen 2011; Mavridou et al. 2013). Note that because Systems V and VI are yet to be experimentally investigated, their designations as novel systems still await confirmation.

B. Biochemical Requirements for Thioether Bond Formation

The biochemical requirements to complete the covalent attachment of heme on the p-side are presumed to be universal and common to all energy-transducing membranes (Hamel et al. 2009; Bonnard et al. 2010). Covalent heme attachment is dependent on several steps including: (1) transport of the apocytochrome c and heme substrates to the p-side of the energy-transducing membrane, (2) maintenance of both the sulfhydryls of the $CX_nC(H/K)$ motif and the heme iron in a reduced form, and (3) formation of two thioether linkages between the vinyl-2 and -4 of heme, with the N- and C-terminal cysteines of the heme-binding motif, respectively.

1. Transport of Pre-apocytochrome c and Heme

The transport of bacterial and organellar apocytochromes *c* from the site of their synthesis (cytoplasm or plastid stroma) to their final sub-cellular location (periplasm, mitochondrial IMS or thylakoid lumen) has been examined in many experimental systems. In most cases, this requires transport of both the heme and the protein via independent pathways, and pre-apoprotein cleavage prior to heme attachment.

Most *c*-type cytochromes are synthesized in a precursor form with appropriate targeting sequences that direct them, as unfolded polypeptides across at least one biological membrane, via general translocation routes such as Sec in bacteria, Toc/Tic/Sec in the plastid and Tom/Tim in the mitochondria (Thöny-Meyer and Künzler 1997; Nakamoto et al. 2000; Hamel et al. 2009). The presequences are cleaved during translocation to yield the apoform of the protein, which is typically the substrate for the heme attachment reaction. Although less common, heme attachment has been found to occur first as a prerequisite for processing of the pre-cytochrome c_1 in *S. cerevisiae* mitochondria (Zollner et al. 1992; Steiner et al. 1996).

The final two steps of heme synthesis, catalyzed by protoporphyrinogen oxidase and ferrochelatase, take place on the *n*-side of the membrane (Ajioka et al. 2006). Ferrochelatase is required for the incorporation of reduced iron in protoporphyrin IX and seems to be membrane-associated, facing the *n*-side (Ajioka et al. 2006). This orientation implies that a mechanism for transmembrane "delivery" of heme from the *n*-side to the *p*-side must operate, since mature heme is produced on the *n*-side. Exceptions occur in organisms where heme is not synthesized in the mitochondria. Such organisms are either parasites relying on dietary heme, like helminths and some trypanosomes, or eukaryotes producing heme within the plastid (Atteia et al. 2005; Hamza and Dailey 2012; Koreny et al. 2013). In both cases, heme is assumed to be transported from the cytoplasm or the plastid to the mitochondrial IMS. With the exception of System II, where a transmembrane heme delivery route was shown to be required for cytochrome *c* assembly (see Sect. III.D.2), the pathways controlling heme distribution from its site of synthesis are unknown.

2. Maintenance of Sulfhydryls and Heme Iron in the Reduced Form

In vitro, uncatalyzed conversion of apocytochrome *c* to its holoform can be achieved in the presence of heme, provided that a reducing agent is added to the reaction (Daltrop et al. 2002; Daltrop and Ferguson 2003, 2004). In the absence of a reductant, the apocytochrome *c* heme-binding site is disulfide-bonded and heme attachment does not proceed. This indicates that reduced apocytochrome *c* is the only competent substrate for the heme ligation reaction. In the case of heme, the need of reduced iron for thioether bond formation is less intuitive and was deduced from reconstitution experiments (Barker et al. 1993; Daltrop et al. 2002). In these experiments, iron in the ferric form was postulated to initiate a radical-based chemistry, yielding side products with incorrectly attached heme. Hence, the initial oxidation state of the heme iron, when it is presented to the apoprotein, is critical to the formation of the thioether bond linkage. Mechanisms for heme reduction have been postulated for System I (see Chap. 27 by Khalfaoui-Hassani et al. in this volume), System II (see Sect. II.D.2), and System III (see Sect. III.E.2). There are known mechanisms for maintaining the heme-binding site sulfhydryls in a reduced form in Systems I and II (see Sects. III.C.2 and III.D.3), but not in System III. It is likely that System V does not require a mechanism to maintain sulfhydryls in a reduced form, considering that there is a single thioether bond forming cysteine in the A/F*XX*CH heme-binding motif (Allen et al. 2008b).

3. Stereospecific Formation of Thioether Bond Formation

Chemical reaction of the heme-binding site sulfhydryls with the vinyl side chains of heme can yield eight possible combinations. Yet only one combination is found in all cytochromes *c*, where the α carbons of vinyl-2 and -4 of heme are linked to the amino (N)- and carboxyl (C)-terminal cysteines in the C*(X)$_n$*CH/K motif (Barker and Ferguson 1999). This stereospecificity of heme attachment also extends to mitochondrial cytochrome *c* of Euglenozoa, where the cysteine in the A/F*XX*CH motif is forming a thioether bond with the α-carbon of the vinyl-4 of heme (Vilmos et al. 2009). The mechanisms controlling this stereoselectivity in the heme ligation reaction still remain undeciphered.

C. System I or CCM Pathway (Cytochrome C Maturation)

1. Overview of the CCM Pathway in Bacteria

The most complex "CCM" (for *C*ytochrome *C M*aturation) pathway, is currently the best characterized in terms of identity of the components, their biochemical activities and the sequence of events (Kranz et al. 2009; Sanders et al. 2010; Verissimo and Daldal 2014). The CCM was initially discovered in α- and γ-proteobacteria. Additionally, it was also assumed to operate in the mitochondria of vascular plants and some protozoa (e.g. *Paramecium* spp.), based on the occurrence of mitochondria-encoded Ccm-like proteins (Allen et al. 2003). Extensive functional studies in various microbial models, including *Escherichia coli* and *Rhodobacter capsulatus*, led to the identification of multiple cytochrome *c* assembly factors that can be categorized in three functional modules (Fig. 26.1): (1) heme handling/delivery, (2) preparation of apocytochrome *c* substrate via maintenance of the heme-linking sulfhydryls in a reduced form, and (3) thioether bond formation. The

bacterial CCM pathway is detailed in the Chap. 27 by Khalfaoui-Hassani et al. in this volume. In the following section, we review the findings of the mitochondrial CCM pathway from experimental investigation in the *Arabidopsis* plant model (Fig. 26.1).

2. A Variant of Bacterial System I in Land Plant Mitochondria

(1) Heme Handling and Delivery. In bacteria, the heme relay pathway includes several steps in the periplasm: (i) loading of heme onto CcmC and transfer onto heme chaperone CcmE, (ii) formation of holoCcmE by covalent attachment of heme to a conserved histidine, and (iii) release of holoCcmE from interaction with CcmC by the ABC-transporter CcmAB and CcmD (see the Chap. 27 by Khalfaoui-Hassani et al.). In land plant mitochondria, the components controlling the delivery and relay of heme can be recognized on the basis to their sequence similarity to bacterial CcmABCE, with the exception of CcmD (Giegé et al. 2008). CcmD, a small membrane-anchored protein, is part of the bacterial CcmABC-holoCcmE complex and is required for the release of holoCcmE (Feissner et al. 2006a; Richard-Fogal et al. 2008, 2009). It is likely that CcmD cannot be identified in plants because there is little conservation of its primary sequence in System I in bacteria and archaea.

Bacterial CcmC is a polytopic membrane HHP (Heme Handling Protein). HHPs are heme-interacting proteins containing heme-coordinating residues (such as histidine) and hydrophobic amino acids that can interact with the porphyrin ring (such as tryptophan) (Li et al. 2011). Bacterial CcmC displays a characteristic tryptophan-rich "WWD" heme-binding motif, flanked by two conserved histidines which are essential for heme loading onto CcmE (Schulz et al. 1999, 2000; Richard-Fogal and Kranz 2010). Compatible with a function in mitochondrial cytochrome *c* assembly, the tryptophan-rich "WWD" domain and flanking histidines of
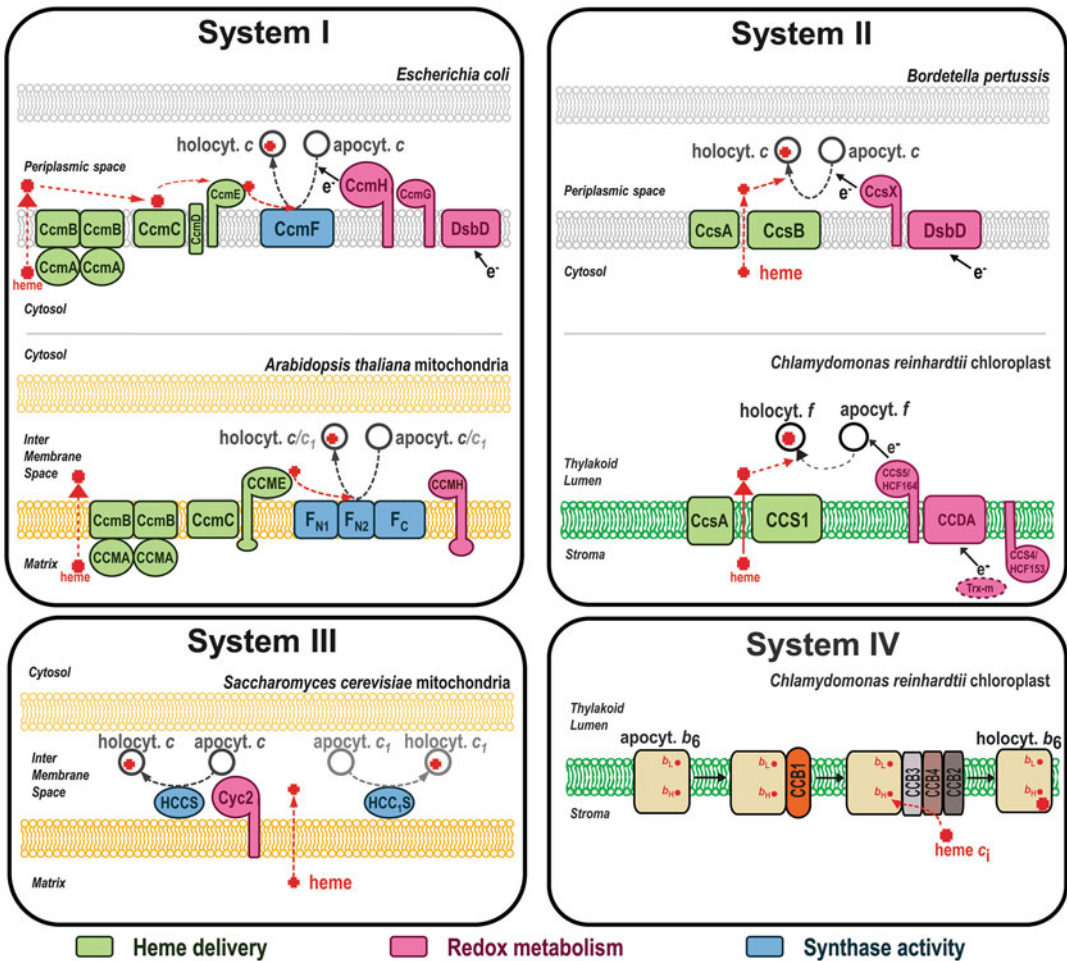
Fig. 26.1. Overview of pathways (Systems I, II, III and IV) for covalent heme attachment. The figure is adapted from Hamel et al. 2009. Bacterial membranes are shown in *gray*, thylakoid membranes are shown in *green* and mitochondrial membranes are shown in *orange*, the outer membrane (*light orange*) and the inner membrane (*dark orange*). For Systems I, II and III, the components shown in *green* are involved in heme handling/delivery, the ones in *pink* are involved in redox metabolism, and the *blue* components are postulated to have a holocytochrome *c* synthase activity. For System II, CcsA and CcsB/CCS1 display heme handling/delivery but also act as holocytochrome *c* synthase. System I operates in mitochondria of land plants and some protists, and Gram^{-} (α, γ) bacteria. System II occurs in chloroplasts, Gram^{+} and Gram^{-} (β, δ, ε) bacteria, aquificales and cyanobacteria. System III is restricted to mitochondria of fungi, animals, apicomplexan parasites, *red* and *green* algae and diatoms. CcdA or DsbD occur in bacterial System I and in System II and are Thiol-disulfide oxido-reductases (TDORs) of the DsbD family. Cyc2p is restricted to organisms where both HCCS and HCC$_1$S are present. In animals, only one holocytochrome *c* synthase, HCCS is present. For System IV, a simplified pathway for the CCB-dependent heme attachment is shown (adapted from Saint-Marcoux et al. 2009).

plant CcmC are predicted to be exposed to the mitochondrial IMS (Giegé et al. 2008). Plant CcmC is mitochondria-encoded and detected in mitochondrial membrane fractions (Raczynska et al. 2006). A severe reduction of CcmC abundance, in a mutant deficient for the processing of the *ccmC* transcript, has no impact on the activities of the bc_1 and cytochrome *c* oxidase complexes (Jonietz et al. 2011). However, the abundance of mitochondrial holocytochrome c_1 and *c* was not documented and the role of CcmC

in cytochrome c maturation remains to be demonstrated.

Arabidopsis CCME is a nuclear-encoded peripheral protein of the mitochondrial inner membrane with its heme-binding domain exposed to the mitochondrial IMS (Spielewoy et al. 2001). When expressed in the *E. coli* periplasm, mitochondrial CCME inserts correctly in the membrane but is unable to substitute for the function of bacterial CcmE in cytochrome c maturation (Spielewoy et al. 2001). However the fact that mitochondrial CCME binds heme covalently, via its conserved histidine when expressed in bacteria, was taken as an indication that its function as a heme chaperone is probably conserved (Spielewoy et al. 2001).

Nuclear-encoded CCMA, the ATP binding component of the ABC-transporter, localizes to the mitochondrial matrix, and was shown to be associated with the inner membrane (Rayapuram et al. 2007). In agreement with its sequence similarity to bacterial CcmA, a recombinant form of mitochondrial CCMA exhibits ATPase activity (Rayapuram et al. 2007). Interaction of CCMA at the inner membrane with CcmB, the mitochondria-encoded membrane component of the ABC transporter, is supported by yeast two-hybrid (Y2H) experiments (Rayapuram et al. 2007). ATP/Mg^{2+} was shown to facilitate CCMA dissociation from the mitochondrial membrane, most likely because ATP hydrolysis induced conformational changes that modify the interaction with the transmembrane domains of CcmB (Rayapuram et al. 2007). Mitochondrial CCMA occurs in a 480 kDa complex that does not contain other Ccm proteins such as CcmF, CCME or CCMH (see below) (Rayapuram et al. 2007). It is likely that this complex contains CcmB but this could not be tested due to the lack of an antibody against this protein.

(2) Preparation of the Apocytochrome c Substrate. Maintenance of the heme-linking cysteines in a reduced form is essential for covalent attachment of heme to apocytochrome c. Bacterial CcmH is a membrane-anchored, periplasm-facing thiol-disulfide reductase proposed to be involved in maintaining the heme-binding motif of apocytochromes c in a reduced form (Sanders et al. 2010; Verissimo and Daldal 2014). However, its mechanism of action still remains obscure (Verissimo and Daldal 2014) (see the Chap. 27 by Khalfaoui-Hassani et al.). In plants, the ortholog of CcmH is a nuclear-encoded, integral inner membrane protein with an IMS-facing domain containing a RC*XX*C redox motif (Meyer et al. 2005). Y2H experiments show an interaction between the IMS-facing domain of mitochondrial CCMH and apocytochrome c. Moreover, in vitro redox assays with a recombinant form of mitochondrial CCMH show that the RC*XX*C motif can reduce a disulfide in a model apocytochrome c peptide (Meyer et al. 2005). The thioredoxin-like CcmG and a TDOR (Thiol-disulfide oxido-reductase) of the DsbD family (see Fig. 26.1), which are proposed to maintain bacterial CcmH in a reduced form, cannot be identified in land plant genomes (Giegé et al. 2008; Sanders et al. 2010; Cho and Collet 2013). Hence, it is unclear how mitochondrial CCMH is maintained reduced in the IMS (Giegé et al. 2008; Bonnard et al. 2010).

(3) Thioether Bond Formation. CcmF was long considered to be the component catalyzing thioether bond formation but experimental evidence for this claim was only provided recently (Ren et al. 2002; Sanders et al. 2008; Richard-Fogal et al. 2009; San Francisco et al. 2014). Bacterial CcmF is a heme-handling protein (HHP) with a "WWD" domain and two flanking histidines on the p-side and two transmembrane histidines facing the n-side of the membrane (Richard-Fogal et al. 2009; San Francisco et al. 2011). The histidines on the p-side are proposed to bind the CcmE-attached heme while histidines on the n-side are ligands of a structural b-heme. The transmembrane b-heme was proposed to be involved in transferring electrons from the cytoplasm to the periplasmic side in order to maintain the heme substrate in a reduced form. *Arabidopsis* CcmF is encoded by the mitochondrial genome in the form of three

genes ($ccmF_{N1}$, $ccmF_{N2}$, $ccmF_C$), each of which encodes an integral inner membrane protein with similarity to a corresponding domain of bacterial CcmF (Giegé et al. 2004; Rayapuram et al. 2008). Histidines on the p-side are in $CcmF_{N1}$ and $CcmF_{N2}$ while the n-side histidines are provided by $CcmF_{N2}$ and $CcmF_C$. The occurrence of $CcmF_{N1}$, $-F_{N2}$, $-F_C$ in a mitochondrial membrane complex and Y2H-based interaction of $CcmF_{N2}$ with $CcmF_{N1}$ and $CcmF_C$ suggest that all three CcmF proteins constitute a functional unit in plant mitochondria (Giegé et al. 2004; Rayapuram et al. 2008). $CcmF_{N2}$ also contains the WWD domain that was shown to face the IMS and interact with apoforms of both cytochrome c and c_1 in an Y2H assay (Rayapuram et al. 2008). $CcmF_{N2}$ interacts with CCMH in Y2H assays and both proteins are also detected in a complex at the inner membrane (Rayapuram et al. 2008). It is likely that mitochondrial CcmF and CCMH form a complex controlling the heme ligation reaction, similar to the bacterial CcmFH, which was shown to carry the cytochrome c synthase activity (San Francisco et al. 2014). A role for *Arabidopsis* $CcmF_C$ in c-type cytochrome maturation was evidenced through the identification of a splicing defect of the $ccmF_C$ transcript that results in decreased accumulation of holoforms of cytochromes c and c_1 (Francs-Small et al. 2011). Similarly, an editing defect at a single position in the $ccmF_N$ transcript results in loss of $CcmF_N$ and severe reduction of mitochondrial c-type holocytochromes in maize (Sun et al. 2015).

D. System II or CCS (Cytochrome C Synthesis) Pathway

1. Discovery of System II in Plastids

System II was originally discovered in the green alga *Chlamydomonas reinhardtii* through genetic studies of the *ccs* mutants (*ccs* for *cytochrome c synthesis*), a class of photosynthesis-minus mutants specifically deficient for the accumulation of the holoforms of plastid *c*-type cytochromes (Howe and Merchant 1992; Howe et al. 1995; Xie et al. 1998). The *ccs* mutants are deficient for the thylakoid membrane-bound *c*-type cytochrome *f*, which is required for cytochrome b_6f assembly, and also for soluble cytochrome c_6, the functional replacement of plastocyanin in Cu-deficient conditions (see the Chaps. 30 and 31 by Bendall and Howe, and Diaz-Moreno et al. in this volume). All *ccs* mutants display a block in the conversion of apo- to holoform of plastid *c*-type cytochromes because the heme attachment to apocytochromes, a chemical reaction taking place in the thylakoid lumen, is compromised (Howe and Merchant 1993, 1994; Xie et al. 1998). It is also expected that lumen resident cytochrome c_{6A}, the novel *c*-type cytochrome conserved in the green lineage (see the Chap. 33 by Howe et al.) is not assembled in the *ccs* mutants. However, this could not be determined because its holoform could not be detected in wild-type strains.

At least 7 loci, plastid *ccsA* and nuclear *CCS1* to *CCS6* were uncovered through genetic analysis of *ccs* mutants (Howe and Merchant 1992; Xie et al. 1998; Dreyfuss and Merchant 1999; Page et al. 2004). Sequence analysis of the cloned *ccsA* and *CCS1* genes (Xie and Merchant 1996; Inoue et al. 1997) revealed that the predicted protein products were novel integral membrane proteins co-occurring in cyanobacteria, the majority of the Gram-positive bacteria (e.g. *Bacillus subtilis*), proteobacteria of the β- (e.g. *Bordetella pertussis*), δ − (e.g. *Desulfovibrio desulfuricans*), and ε groups (e.g. *Helicobacter pylori*, *Wolinella succinogenes*), and aquificales (Simon and Hederstedt 2011). Forward and reverse genetics studies in several bacterial and cyanobacterial models have confirmed the roles of CcsA and CCS1-like proteins in the assembly of *c*-type cytochromes. This led to the definition of a distinct pathway for thioether bond formation in apocytochrome *c* (Tichy and Vermaas 1999; Beckett et al. 2000; Le Brun et al. 2000; Kern et al. 2010a, b). A representation of bacterial and plastid System II components is shown on Fig. 26.1.

2. A Transmembrane Heme Channel with Cytochrome c Synthase Activity

While CcsA was recognized as a member of the HHP family, based on the presence of the "WWD" domain and conserved histidines also found in System I CcmC and CcmF, there was no motif in CCS1 suggestive of a biochemical function in thioether bond formation (Inoue et al. 1997; Goldman et al. 1998; Hamel et al. 2003). Functional investigation of plastid CcsA and CCS1 proteins established that they are polytopic membrane proteins with functional domains exposed to the lumen and essential histidinyl residues on both the lumen and stromal side of the thylakoid membrane (Dreyfuss et al. 2003; Hamel et al. 2003). CcsA and CCS1 require each other for accumulation in vivo and were therefore proposed to function as a transmembrane heme delivery system from stroma to lumen in a complex, possibly with the products of some of the other *CCS* loci (Dreyfuss et al. 2003; Hamel et al. 2003). Experimental support for the biochemical activity of CcsA and CCS1 came with the discovery that naturally occurring Ccs1-CcsA fusion proteins called CcsBA, from several ε-proteobacteria, could assemble reporter cytochrome(s) *c* in an *E. coli* strain lacking all of the Ccm components (Feissner et al. 2006b; Frawley and Kranz 2009; Goddard et al. 2010; Kern et al. 2010a; Richard-Fogal et al. 2012). The finding that CcsBA could substitute for all Ccm components indicated that the fusion protein was necessary and sufficient for thioether bond formation. The function of CcsBA as a transmembrane heme delivery system was confirmed from spectroscopic analysis of the purified CcsBA protein. This analysis revealed that heme can be trapped at an external binding domain where periplasmic localized histidines, provided by the CcsA moiety, act as axial ligands (Frawley and Kranz 2009). Histidines in transmembrane domains (TMD) of CcsB and CcsA, close to the cytoplasmic surface of the membrane, were postulated to act as an entry site for heme. This conclusion was based on the fact that heme was no longer detected at the external binding domain when these residues are mutated. Imidazole-dependent rescue of the cytochrome *c* synthase activity of CcsBA carrying mutations in the TMD histidines also supported their implied involvement in providing a route for heme transport from the cytoplasm to the periplasm (Frawley and Kranz 2009). The mechanism by which heme is maintained reduced prior to the ligation reaction remains to be established. However, heme is bound to CcsBA in the oxidized form when either one of the heme-interacting histidines facing the periplasm is mutated (Frawley and Kranz 2009). This observation led to the proposal that histidine ligands act by protecting reduced heme from oxidation.

3. Operation of a Transmembrane Disulfide Reducing Pathway

(1) A Two-Component Redox Pathway in Bacteria. In System II, the involvement of thiol-based chemistry in the heme attachment reaction was first demonstrated in bacteria through the discovery of redox factors controlling cytochrome *c* assembly. Genetic screens in System II bacterial models led to the identification of a thiol-disulfide oxidoreductase (TDOR) from the DsbD family at the plasma membrane and a membrane-anchored, periplasm-facing thioredoxin-like ResA/CcsX, which are required for the covalent linkage of heme to apoforms of cytochromes *c* (Schiott et al. 1997a, b; Beckett et al. 2000; Erlendsson et al. 2003). Loss of function of the redox cytochrome *c* assembly factors can be bypassed by provision of exogenous reduced thiols in the medium, a finding that led to the proposal that these components function in a disulfide reducing pathway (Beckett et al. 2000; Erlendsson and Hederstedt 2002; Erlendsson et al. 2003; Feissner et al. 2005; Small et al. 2013). One interpretation is that the thiols act as a substitute for the reducing activity of the assembly factors. A possible mechanism is that the thiols reduce a disulfide in the apocytochrome *c* heme-binding motif to free sulfhydryls. In

agreement with this view is the fact that the redox cytochrome *c* assembly factors are no longer required for heme attachment when the components of the disulfide forming pathway are inactivated (Erlendsson and Hederstedt 2002; Erlendsson et al. 2003; Small et al. 2013). One likely explanation is that disulfide bonds in apocytochromes *c* are formed by the disulfide forming pathway during their translocation to the periplasm. When the disulfide forming pathway no longer operates, sulfhydryls remain reduced and apocytochrome *c* is in the competent form to be acted upon by heme. So it appears that in bacteria the disulfide reducing pathway, in the context of thioether bond formation, is only needed to counter sulfhydryl oxidation of the heme-binding cysteines. In System I bacteria, a similar transmembrane disulfide reducing pathway (consisting of the thioredoxin-like CcmG and a TDOR) is also required for cytochrome *c* assembly (Fig. 26.1). As in System II, this pathway is only necessary for holocytochrome *c* maturation when the disulfide bond forming enzymes operate (Monika et al. 1997; Deshmukh et al. 2000, 2003; Bardischewsky and Friedrich 2001; Turkarslan et al. 2008).

In vivo, the disulfide bond in the heme-binding site of apocytochrome *c* is reduced to sulfhydryls by action of the thioredoxin-like ResA/CcsX. This is supported by extensive biochemical and structural studies of *B. subtilis* ResA, which reveal that the enzyme undergoes drastic conformational change and consequent increase in reactivity upon binding of oxidized apocytochrome *c* (Crow et al. 2004; Colbert et al. 2006; Lewin et al. 2006, 2008; Hodson et al. 2008). In the periplasm, ResA is maintained reduced by the activity of a TDOR of the DsbD family, which conveys the reducing power across the cytoplasmic membrane via thiol-disulfide exchange reactions. The primary source of electrons for this pathway is provided in the form of NADPH, via TrxA, the cytoplasmic thioredoxin (Möller and Hederstedt 2008).

(2) A Bacterial-Like Redox Pathway in Plastids. In plastids, the implication of thiol-based chemistry in the assembly of plastid cytochrome *c* was inferred from (1) the observation that the *Chlamydomonas ccs4* and *ccs5* mutants could be phenotypically rescued by application of exogenous reductants, and (2) the occurrence in *Arabidopsis* of CCDA, another TDOR of the DsbD family at the thylakoid membrane, and HCF164, a membrane-anchored thioredoxin-like protein facing the lumen (Lennartz et al. 2001; Page et al. 2004; Motohashi and Hisabori 2010). Loss of function of CCDA and HCF164 yields a b_6f assembly defect, an expected phenotype if heme attachment to apocytochrome *f* is compromised. However, this phenotype could not be attributed to a specific defect in cytochrome *c* assembly because the heme attachment reaction was not monitored in the *ccda* and *hcf164* mutants in *Arabidopsis* (Lennartz et al. 2001; Page et al. 2004). Molecular identification of the *Chlamydomonas CCS5* gene showed that the corresponding gene product is the ortholog of *Arabidopsis* HCF164. CCS5 was shown to interact with plastid apocytochromes *c* in a Y2H assay and a recombinant form of the molecule was active in reducing a disulfide-bonded *CXX*CH motif in a soluble form of apocytochrome *f* in vitro (Gabilly et al. 2010). Based on these findings, a role for CCS5/HCF164 as an apocytochrome *c* disulfide reductase in the heme attachment reaction was formulated. Oxidation of apocytochrome *c* *CXX*CH motif in the thylakoid lumen remains to be demonstrated, but the fact that the *ccs5*-null mutant is rescued in vivo by application of exogenous thiols is in agreement with the proposed disulfide reductase activity of CCS5/HCF164 (Gabilly et al. 2010). It is possible that, as in bacteria, apocytochrome *c* is a target of the disulfide bond forming pathway operating in the thylakoid lumen, but this remains to be experimentally tested (Karamoko et al. 2011; Lu et al. 2013).

The identification of CCS4 is intriguing, because this component does not display any motif suggesting a role in thiol-based redox chemistry (Gabilly et al. 2011). CCS4 displays limited similarity to *Arabidopsis*

HCF153, a thylakoid membrane-anchored protein required for the assembly of the cytochrome b_6f complex (Lennartz et al. 2006). However, the activity and site of HCF153 action in the assembly process has so far remained undeciphered. The thiol-dependent photosynthetic rescue of the *ccs4* mutant, and the suppression of the *ccs4* phenotype by ectopic expression of CCDA, confirms the activity of CCS4 in a disulfide-reducing pathway for cytochrome *c* assembly (Gabilly et al. 2011). Moreover, the CCDA-dependent suppression of *ccs4* solidifies the placement of CCDA in plastid cytochrome *c* maturation, as earlier studies in *Arabidopsis* supported, but did not establish the requirement of plastid CCDA in the conversion of apo- to holocytochromes *c* (Page et al. 2004). By analogy to the bacterial disulfide reducing pathway, the proposed model is that CCDA and CCS5/HCF164 define a trans-thylakoid pathway for the delivery of reductants from stroma to lumen via thiol-disulfide exchanges (Page et al. 2004; Motohashi and Hisabori 2006, 2010; Gabilly et al. 2010, 2011). The involvement of CCS4 in the disulfide bond reducing pathway remains unclear, but a role in regulating the activity/stability of CCDA was postulated (Gabilly et al. 2011). The source of reductant is expected to originate from the stroma and *in organello* experiments support the role of the stromal thioredoxin Trx-*m* as a possible electron donor to both CCDA and HCF164 (Motohashi and Hisabori 2006, 2010).

4. Additional System II Components in Plastids?

Saturating screens in System II bacteria have established the involvement of four assembly components CcsA, Ccs1/CcsB, a TDOR of the DsbD family and the thioredoxin-like ResA/CcsX (Beckett et al. 2000; Le Brun et al. 2000). In plastids, at least four additional CCS factors were revealed through genetic studies of *Chlamydomonas ccs* mutants, out of which, CCS3 and CCS6 still remain uncharacterized at the molecular level (Xie et al. 1998; Page et al. 2004). The *CCS2* locus was cloned and the corresponding gene shown to encode a plastid-resident OPR (octotricopeptide repeat) protein (Cline et al., unpublished). Because all the OPR proteins characterized to date regulate translation, stability or maturation of chloroplast transcripts (Auchincloss et al. 2002; Balczun et al. 2005; Merendino et al. 2006; Eberhard et al. 2011; Rahire et al. 2012; Kleinknecht et al. 2014; Marx et al. 2015; Wang et al. 2015), the CCS2 protein is probably not directly involved in the heme attachment reaction. One possible function is that it controls the translation of the plastid-encoded *ccsA* transcript. It is plausible that cytochrome *c* maturation in the thylakoid lumen is more complicated than in bacteria and relies on additional components. The discovery that CCS4 functions in the disulfide reducing pathway and appears to be restricted to photosynthetic eukaryotes supports this view (Karamoko et al. 2013). The fact that the *Chlamydomonas CCS3, CCS4, CCS5* and *CCS6* loci are only defined by single alleles suggests that screens for plastid cytochrome *c* deficient mutants are not saturated and that additional CCS loci could be uncovered (Howe and Merchant 1992; Xie et al. 1998; Dreyfuss and Merchant 1999; Page et al. 2004).

E. System III or HCCS (HoloCytochrome C Synthase) Pathway

1. Discovery of HCCS, the Signature Component of System III

System III, the first pathway for thioether bond formation to be described is defined by a single component, the prototypical HCCS or holocytochrome *c* synthase. This enzyme is responsible for the terminal step in the holocytochrome *c* synthesis pathway, that is, the formation of the thioether bonds in apocytochromes *c* and c_1 (Allen 2011; Babbitt et al. 2015). The HCCS pathway seems to be restricted to mitochondria of fungi, animals, apicomplexan parasites, red and green algae, and some protists (e.g. *Dictyostelium discoideum*), as gauged

from genome sequence analysis (Giegé et al. 2008; Allen 2011; Mavridou et al. 2013; Babbitt et al. 2015). Remarkably, the existence of two distinct mitochondrial enzymes, HCCS and HCC_1S, catalyzing heme attachment onto apocytochrome c and c_1, respectively, was first discovered more than 30 years ago from studies of holocytochrome c reconstitution using apoprotein, heme and mitochondrial extracts from fungi (Korb and Neupert 1978; Basile et al. 1980; Hennig and Neupert 1983; Visco et al. 1985). Identification of the HCCS and HCC_1S-encoding genes came from later genetic studies of mutants deficient in mitochondrial cytochromes c and c_1 in fungi *S. cerevisiae*, *Neurospora crassa*, and *Candida albicans*, and these organisms have remained the focus of attention for the analysis of System III (Dumont et al. 1987; Nargang et al. 1988; Drygas et al. 1989; Zollner et al. 1992; Cervera et al. 1998). Genetic studies in *S. cerevisiae* established that apocytochrome c can only be assembled by HCCS, while apocytochrome c_1 is a substrate of both HCCS and HCC_1S (Bernard et al. 2003). The holocytochrome c_1 synthase activity of HCCS is weak but can be enhanced if the substrate-enzyme interactions are modified via over-expression of the HCCS-encoding gene or point mutations in either HCCS or apocytochrome c_1 (Bernard et al. 2003). However, animals are the only System III organisms with a single HCCS, which was shown to act on both apocytochrome c and c_1 substrates (Schwarz and Cox 2002; Bernard et al. 2003).

Both HCCS and HCC_1S are peripherally bound to the mitochondrial inner membrane and face the IMS, the compartment where the heme attachment reaction takes place (Dumont et al. 1991; Steiner et al. 1995; Bernard et al. 2005). All HCCSs are related in sequence (less than 20 % amino acid sequence identity in pairwise comparisons) and display zero to four typical HRMs (heme regulatory motif), also present in several other heme-binding proteins, such as the transcription factor Hap1p and HO (Heme oxygenase)

(Li et al. 2011). In intact or detergent-solubilized mitochondria, HCCSs were shown to interact with apocytochromes c (Nicholson et al. 1988; Nicholson and Neupert 1989; Mayer et al. 1995) and also with heme via the HRM (Steiner et al. 1996). Nevertheless, the HRMs are not strictly essential for formation of holocytochrome c and c_1, suggesting that other domains of the HCCS must be important for their enzymatic activity (Steiner et al. 1996; Moore et al. 2011).

Because mitochondrial cytochrome c could be correctly matured in the bacterial periplasm or cytoplasm upon expression of HCCS alone, and did not require co-expression of any other assembly factors, HCCSs were proposed to participate in the biogenesis of c-type cytochromes by catalyzing thioether bond formation (Dumont et al. 1988; Pollock et al. 1998; Sanders and Lill 2000; Richard-Fogal et al. 2012).

However, despite the fact that HCCSs were the first c-type cytochrome assembly factors to be discovered, the specific enzymatic function of the proteins had remained elusive for a long time until recently. An important breakthrough in detailing the enzymology of HCCS came with the purification and spectroscopic analysis of the human enzyme co-expressed with its cognate apocytochrome c substrate in the bacterial cytoplasm (San Francisco et al. 2013; Babbitt et al. 2014a, b). Furthermore, the use of mutant forms of the enzyme and apocytochrome substrate enabled the trapping of intermediate complexes, which led to the delineation of possible steps in the heme attachment reaction. The first step in the HCCS-dependent heme attachment reaction is the binding of heme to the enzyme via two domains (I and II) containing conserved residues. One of these, a histidine in domain II, acts as an axial ligand of heme. Formation of the heme-HCCS complex triggers the recognition of the apocytochrome c substrate. The determinants for substrate recognition by HCCS were established using cytochrome c and found

to lie within a 9 residue sequence upstream the CXXCH motif (Stevens et al. 2011; Asher and Bren 2012; Verissimo et al. 2012; San Francisco et al. 2013). It is likely that the sequence requirements for recognition of cytochrome c_1 are similar but this awaits experimental testing.

The heme-HCCS-apocytochrome c together forms a ternary complex, where heme is coordinated by one histidine in domain II and another histidine in the CXXCH motif on apocytochrome c (San Francisco et al. 2013). The mechanism for thioether bond formation is still obscure, but bis-histidinyl coordination of heme in the heme-HCCS-apocytochrome c complex is postulated to provide the ideal geometry for stereospecific thioether bond formation. In addition, the observation that some residues in HCCS control the retention of holocytochrome c in HCCS suggests that a mechanism for releasing the mature holoform from the enzyme is probably required for optimal cytochrome c synthesis (San Francisco et al. 2013; Babbitt et al. 2014a, b). Interestingly, none of the domains defined as important for the HCCS enzymatic activity include the HRMs initially postulated to be required for HCCS function (Allen 2011). In HO, another enzyme containing HRM motifs, the HRM cysteine sulfhydryls can switch between an oxidized or reduced form depending on the cellular redox state. Furthermore, thiol-disulfide interconversion at the HRMs was shown to regulate the heme-binding activity of HO and it is possible HRMs in HCCSs perform the same function (Yi and Ragsdale 2007; Yi et al. 2009).

2. Missing Components in System III?

System III appears deceptively simple in terms of composition, in contrast to the complexity of Systems I and II, because extensive genetic screens in *S. cerevisiae* and *N. crassa* have so far revealed only HCCS and HCC$_1$S as assembly factors for mitochondrial cytochromes c (Lang and Kaudewitz 1982; Dumont et al. 1987; Drygas et al. 1989; Sherman 1990; Zollner et al.

1992). Important future avenues for research include investigation into the mechanisms of heme delivery to the mitochondrial IMS. Furthermore, by analogy to System I and II, mechanisms for maintenance of the sulfhydryls and chemical reduction of heme prior to the ligation reaction must also operate. There is evidence that both of these are still biochemical requirements of the pathway from *in organello* reconstitution of holocytochromes c and c_1. In the former instance, the CXXCH sulfhydryls were shown to be maintained reduced by an unknown mechanism until the heme ligation reaction (Nicholson et al. 1987). In the latter instance, both pyrimidine and flavin nucleotides were shown to be required for chemical reduction of heme iron (Basile et al. 1980; Nicholson and Neupert 1989; Nicholson et al. 1989; Tong and Margoliash 1998).

One candidate protein for the reduction of heme is Cyc2p, a mitochondrial protein first discovered in a screen for cytochrome c deficient mutants in yeast and initially assigned a function in the import of apocytochrome c (Dumont et al. 1993; Pearce et al. 1998; Sanchez et al. 2001). Cyc2p is an inner membrane anchored, IMS-facing, NAD(P)H-dependent flavoprotein and was re-isolated as a factor enhancing the holocytochrome c_1 synthase activity of HCCS through a multicopy suppressor screen (Bernard et al. 2003, 2005). An in vivo indication that Cyc2p controls a reductive step in the heme attachment reaction is the finding that the requirement for its function can be bypassed by exogenous reductants (Corvest et al. 2012), similar to what was observed for plastid and bacterial cytochrome c assembly mutants deficient in the disulfide reducing pathway (Deshmukh et al. 2000, 2003; Bardischewsky and Friedrich 2001; Turkarslan et al. 2008; Gabilly et al. 2010, 2011). However, although redox titrations of Cyc2p flavin indicate that reduction of a disulfide at the CXXCH site of apocytochrome c is a thermodynamically favorable reaction, Cyc2p does not act as an apocytochrome c CXXCH disulfide

reductase in vitro. Instead, Cyc2p is able to catalyze the NAD(P)H-dependent reduction of hemin in vitro, indicating a possible role in reducing the heme iron prior to its attachment (Corvest et al. 2010, 2012). Y2H analysis showing that Cyc2p interacts with HCCS and also with apocytochromes c and c_1 led to the proposal that Cyc2p, possibly in a complex with HCCS, reduces the heme iron prior to co-factor attachment to the apoforms of cytochrome c and c_1. Operation of a distinct heme reductase dedicated to the HCC_1S enzyme was postulated based on the fact that Cyc2p is only required for the HCCS-dependent assembly of cytochrome c and c_1 and does not control the HCC_1S-catalyzed heme attachment to apocytochrome c_1 (Corvest et al. 2010). Because Cyc2p appears to be restricted to fungi, additional mechanism(s) of heme reduction in System III mitochondria must exist in other organisms.

F. System IV or CCB (Cofactor Assembly on Complex C Subunit B)

1. A Multi-step Pathway for Heme c_i Formation in the b_6f Complex

Covalently attached heme in cytochrome b_6 in the b_6f and cytochrome b in the bc of Firmicutes was suspected from spectroscopic measurement and the observation that heme/cytochrome b association was unusually resistant to denaturing treatment (Lavergne 1983; Joliot and Joliot 1988; Kuras et al. 1997; Yu and Le Brun 1998) However, b_L or b_H heme was believed to be attached covalently at the time and the discovery of an additional heme (heme c_i) bound via a single thioether bond in the X-ray structures of chloroplast and cyanobacterial b_6f came as a surprise (Kurisu et al. 2003; Stroebel et al. 2003). Heme c_i is located in close vicinity to heme b_H and unlike heme c in canonical c-type cytochromes, does not appear to have any amino acid axial ligands (Kurisu et al. 2003; Stroebel et al. 2003; de Vitry et al. 2004). Heme c_i has a unique axial ligand, a water molecule (or

possibly a hydroxide ion) also interacting with a propionate group of heme b_H. A pathway for apo- to holocytochrome b_6 conversion was deduced from the analysis of *Chlamydomonas* strains depleted for heme or carrying mutations of the histidines coordinating b_L and b_H hemes (Kuras et al. 1997). While apocytochrome b_6 could be immunodetected in heme-deprived cells and mutants unable to ligate b_L, a distinct immunoreactive species of cytochrome b_6 was present in mutants altered for b_H ligation. The same species is also detected when the thioether bond forming cysteine in cytochrome b_6 is mutated (de Vitry et al. 2004). This indicates that loss of b_H ligation prevents formation of heme c_i and, reciprocally, that, in the absence of covalent attachment of heme, heme b_H is presumably no longer retained. Spectroscopic analysis of the purified b_6f complex altered for b_H ligation showed that heme b_L was present while hemes b_H and c_i were lost (Malnoë et al. 2011). Collectively, these results demonstrate that holocytochrome b_6 assembly proceeds through a b_L-containing intermediate, followed by incorporation of b_H and formation of heme c_i.

2. Identification of Novel Proteins Required for Heme c_i Assembly

The accumulation of a b_L-containing intermediate was exploited as a signature to identify assembly factors controlling heme c_i formation, referred to as CCB (for Cofactor assembly of complex C subunit B[3]) (Kuras et al. 1997; de Vitry et al. 2004). In *Chlamydomonas*, four *CCB* loci were defined from the genetic analysis of b_6f-deficient mutants that no longer assemble holocytochrome b_6 but still accumulate the b_L-containing cytochrome b_6 intermediate (Gumpel et al. 1995; Kuras et al. 1997, 2007). The *ccb* mutants are unable to grow photosynthetically but still retain low levels of b_6f (Lyska et al. 2007; Saint-Marcoux

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[3]B is for petB (or cytochrome b_6) of the cytochrome b_6f complex (complex C).

et al. 2009). Wild-type levels of b_6f lacking heme c_i can be restored in *ccb* mutants with suppressor mutations attenuating the activity of the thylakoid FtsH protease (Malnoë et al. 2014). This result demonstrates that heme c_i is not absolutely required for complex assembly/activity and that b_6f lacking heme c_i becomes prone to proteolytic degradation. The photosynthetic growth of the suppressed strains is light-sensitive, an indication that the presence of heme c_i is required for optimal activity of the b_6f complex (Malnoë et al. 2014).

The *CCB* genes were cloned and the corresponding gene products were shown to be novel thylakoid transmembrane proteins conserved in all organisms performing oxygenic photosynthesis (Kuras et al. 2007). In *Arabidopsis*, loss of function of CCB proteins yields a defect in heme c_i formation (Lyska et al. 2007; Lezhneva et al. 2008). In cyanobacteria, *ccb* mutants are impaired for photosynthesis, but the function of CCB in heme c_i biogenesis could not be ascertained because the impact on the b_6f complex was not examined (Ishikawa et al. 2009).

The following pathway of action for the CCB machinery can be deduced from genetic and biochemical studies (Fig. 26.1). Interaction of CCB proteins with cytochrome b_6 is supported by BN-PAGE and co-immunoprecipitation experiments (Saint-Marcoux et al. 2009). Because subunit IV is not detected in association with the CCB factors, it was deduced that unassembled cytochrome b_6, presumably a form containing both b_L and b_H hemes, is the substrate of the CCB machinery. The detection of a CCB1/cytochrome b_6 complex in *ccb2*, *ccb3* and *ccb4* mutants indicates that this complex is the first intermediate in the pathway and may chaperone the cytochrome b_6 substrate before the heme ligation step. This step is believed to be catalyzed by a CCB2/CCB4/CCB3 complex, also detected in association with cytochrome b_6 in a mutant altered for the thioether bond-forming cysteine. The presence of a stable CCB2/CCB4 heterodimer and a CCB3/cytochrome b_6 complex indicates

that these might also be intermediates in the assembly pathway. A model was postulated where CCB3/cytochrome b_6 is the second intermediate in the pathway and recruits the CCB2/CCB4 complex to form the CCB3/CCB2/CCB4 heme ligation complex (Fig. 26.1). Although the biochemical activity of the CCB factors remains to be elaborated, it is reasonable to postulate that some of the protein domains must interact with both heme and cytochrome b_6. Moreover a mechanism for maintaining heme in a reduced form must exist, considering that the redox state of the heme iron is critical for the chemistry of thioether bond formation. Typical heme-binding motifs found in System I and II cytochrome c assembly factors, or conserved histidines, are not present in the CCB factors (Kuras et al. 2007). Occurrence of conserved, heme-interacting residues, such as tryptophan, tyrosine and/or phenylalanine, in regions exposed to the n-side of the membrane speaks to a possible heme handling/relay function, but this remains to be experimentally tested.

Acknowledgments

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Biogenesis of Cytochrome *c* Complexes: From Insertion of Redox Cofactors to Assembly of Different Subunits

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Summary

Cytochromes (cyts) are ubiquitous heme containing proteins that are key components of energy transduction pathways. They participate in a wide variety of electron transfer reactions, which are essential for cellular processes responsible for chemical energy (ATP) production. The *cbb₃*-type cyt *c* oxidase (*cbb₃*-Cox) provides an excellent model to study biogenesis of membrane-integral, oligomeric cyt *c* complexes. Its subunits contain three hemes *c*, two hemes *b* and a copper (Cu_B) atom as cofactors that use distinct insertion processes. In cyts *c*, heme *b* is covalently ligated (referred to as heme *c*) via a complex maturation process that involves in some species up to nine components (Ccm-System I). In

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addition to the cyts c, many cyt c containing complexes carry other cofactors, and insertion of these cofactors requires additional biogenesis components besides the Ccm-system I. In the case of cbb_3-Cox, the mechanisms underlying incorporation of hemes b into the catalytic subunit are not well understood. However, remarkable progress was achieved recently on how the single Cu_B atom at the catalytic heart of this heme-copper oxidase is acquired. Finally, insertion of the cofactors must be temporally and spatially coordinated with the assembly of the subunits in order to yield a functional cbb_3-Cox enzyme. In this chapter, we discuss the biogenesis of cbb_3-Cox from the insertion of its catalytic heme-copper (Cu_B) center and maturation of its c-type cyts to the assembly of its mature subunits, mainly focusing on studies carried out with the anoxygenic phototrophic bacterium *Rhodobacter capsulatus*.

I. Introduction

A vital characteristic of all living organisms is the ability to produce energy-rich molecules (ATP) required for central biological processes, such as biosynthesis and transport of molecules as well as cell motility and thermogenesis. Among the heme-containing proteins, cytochromes (cyts) are evolutionarily conserved components of biological energy transduction (Moore and Pettigrew 1990). They are ubiquitous to almost all forms of major electron transport pathways, including photosynthesis (Ps) and respiration (Res), and are crucial for energy production and cell viability (Pettigrew and Moore 1987). In the 1920s, Keilin classified cyts based on the spectroscopic properties of their different heme cofactors (Keilin 1925) [See Chap. 1 by Bendall]. Heme b (protoporphyrin IX-Fe) is an organic molecule composed of an iron (Fe) atom ligated to four nitrogen atoms of a porphyrin ring. Often, the side chains of specific amino acid residues (His or Met) from the polypeptide provide axial ligands to penta- or hexa-coordinate the Fe^{2+} (reduced) or Fe^{3+} (oxidized) ion. In cyts b, non-covalently bound hemes b are frequently coordinated by His, and rarely by other residues (Moore and Pettigrew 1990). Similarly, cyts a, d and o are also coordinated

to the corresponding polypeptides with one or two axial ligands. Their heme cofactors are derivatives of heme b that are chemically modified at different positions of the porphyrin ring. Both the hemes a and o contain a hydroxyl ethyl farnesyl group at position C3, and heme a contains an additional formyl group at position C18 of the porphyrin ring. Heme d has two hydroxyl groups at positions C12 and C13 (see e.g., Sanders et al. 2009 for detailed description). In contrast to the cyts a, b, d and o, all cyts c invariably contain a heme b cofactor that is stereo-specifically ligated to the polypeptide by two (rarely one) thioether bonds. These bonds are formed between the positions C3 (vinyl-2) and C8 (vinyl-4) of the porphyrin ring and the thiol groups of Cys_1 and Cys_2 located at a conserved heme-binding motif (C_1XXC_2H) within the apocytochromes (apocyts) c (Bowman and Bren 2008). The His residue of this motif axially coordinates the heme-iron together with another Met or His residue (Moore and Pettigrew 1990).

Over the last few decades, *Rhodobacter* (*R.*) species have emerged as model organisms for studying the structure, function and biogenesis of the cyts c and cyt c complexes (Sanders et al. 2009). For instance, the facultative anaerobic phototrophic α–proteobacterium *R. capsulatus* produces a variety of soluble and membrane-bound cyts c (Fig. 27.1), including the cyts c_1, c_2, c', c_y, c_o (CcoO), c_p (CcoP) and DorC in order to sustain its versatile modes of growth (Daldal et al. 1987; Zannoni and Daldal 1993; Gray et al. 1994; Koch et al. 1998). Of these proteins, cyt c_1 is a carboxyl (C)-terminally membrane-anchored subunit of cyt bc_1

Abbreviations: Ccm – Cytochrome c maturation system I; Cox – Cytochrome c oxidase; Cyt – Cytochrome; HCO – Heme-copper terminal oxidase; HHP – Heme handling protein; HMBD – Heavy metal binding domain; MFS – Major facilitator superfamily; Ps – Photosynthesis; Qox – Quinol (hydroquinone) oxidase; Res – Respiration
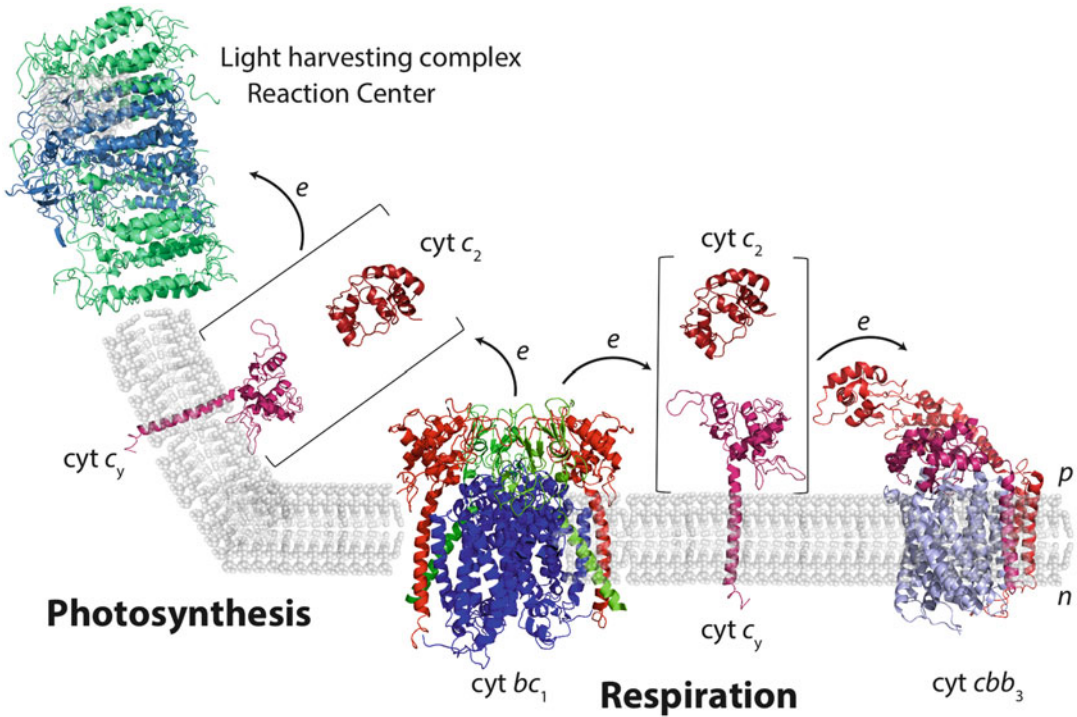
Fig. 27.1. 3D structures of *R. capsulatus* cyts *c* and *b* containing cyt complexes involved in photosynthesis and respiration. The structures shown are the *R. sphaeroides* light harvesting and reaction center (4JCB), *R. capsulatus* cyt bc_1 (1ZQT), *P. stutzeri* cyt cbb_3-Cox (3MK7) and *R. capsulatus* cyt c_2 (1C2R), while that of cyt c_y is a hypothetical homology model. The membrane-anchored cyt c_y and the soluble cyt c_2 carry electrons between the cyt bc_1 and the reaction center during photosynthesis, and between the cyt bc_1 and the cbb_3-Cox during respiration. Cyt *c* subunits are shown in different tones of red.

complex (cyt bc_1 or respiratory complex III) that is required for Ps growth (Daldal et al. 1987), whereas the cyts CcoO and CcoP are subunits of the cbb_3-type cyt *c* oxidase (cbb_3-Cox), which carries out aerobic and microaerophilic Res growth (Gray et al. 1994; Koch et al. 1998). The periplasmic small cyt c_2 together with the amino (N)-terminally membrane-anchored cyt c_y play critical roles as electron carriers during both Ps and Res (Fig. 27.1) (Jenney and Daldal 1993; Jenney et al. 1994; Hochkoeppler et al. 1995). Under anaerobic conditions in the presence of dimethylsulfoxide (DMSO), or trimethylamine N-oxide (TMAO) (McEwan et al. 1985) *R. capsulatus* relies on the membrane-bound pentaheme cyt *c* DorC, which receives electrons from the membrane quinone/quinol (Q/QH_2) pool and reduces DMSO to dimethyl sulfide (DMS), or TMAO to trimethylamine (TMA) (Shaw et al. 1999a, b).

The apocyts *c* are usually synthesized in the cytoplasm, translocated across the membrane, and matured to holocyts *c* in the periplasm. In Gram-negative bacteria, the periplasmic maturation process involves an elaborate pathway, referred to as cyt *c* maturation system I (Ccm-system I) (Sanders et al. 2010). It includes the translocation and delivery of heme *b*, chaperoning and thio-oxidoreduction of the apocyts *c* and heme ligation steps. Unlike the electron carrier cyts *c* (e.g., cyt c_2 and cyt c_y), some cyts *c* (e.g., cyt c_1, CcoO and CcoP) associate together with other membrane bound proteins to form oligomeric enzyme complexes. Often, these enzymes also contain other cofactors whose biogenesis involves additional components along with the Ccm-system I. Thus, multifaceted interactions between multiple maturation components render biogenesis of cyt *c*

complexes highly complex. Coordinated incorporation of specific cofactors to specific subunits is needed to ensure the production of functional and stable multi-cofactor carrying complexes (Ekici et al. 2012a). As an example, the bacterial cyt bc_1 contains in addition to cyt c_1 two other catalytic subunits, which are the Rieske iron-sulfur (Fe-S) protein and cyt b (Iwata et al. 1995; Berry et al. 2004). The Fe-S protein is a membrane-anchored protein with a 2Fe-2S cluster as a redox cofactor, whereas cyt b is a multi-span integral membrane protein that carries two hemes b (b_L and b_H). Similarly, heme-copper oxidase (HCO) superfamily members, to which respiratory cyt c oxidase (Cox) belongs, are multi-cofactor containing complexes that have copper (Cu) atoms. The mitochondrial enzyme aa_3-Cox (respiratory complex IV) has a bi-metallic center with two Cu atoms (Cu_A) in its subunit II (Cox2), and two hemes a (a and a_3) in its subunit I (Cox1). Heme a_3 forms with a third Cu atom (Cu_B) the heme-Cu_B catalytic center where O_2 is reduced to H_2O (Iwata et al. 1995; Svensson-Ek et al. 2002). The bacterial cbb_3-Cox has a similar heme-Cu_B active site, but it contains two hemes b (b and b_3) instead of the hemes a of aa_3-Cox. In cbb_3-Cox, the Cu_A binuclear center is replaced by three hemes c of CcoO and CcoP (Buschmann et al. 2010). In addition, this enzyme is not essential for aerobic respiration of *R. capsulatus* as this species also contains a cyt bc_1-independent and unrelated O_2 reductase (probably a bb- or bd-type quinol oxidase (Qox) devoid of heme c) (Marrs and Gest 1973; Daldal et al. 2001). This feature renders the cyts c non-essential in this species, thus permitting genetic analyses.

The cbb_3-Cox provides a fascinating paradigm for investigating the biogenesis of cyt c complexes due to the presence of two axially coordinated hemes b, three covalently ligated hemes c and a single Cu atom (Cu_B) as its cofactors (Sanders et al. 2009; Ekici et al. 2012a) (Fig. 27.1). Of the subunits of cbb_3-Cox, CcoO and CcoP are c-type cyts that use Ccm-system I present in various organisms. CcoN contains the heme-Cu_B catalytic site and its maturation relies on homologues of the eukaryotic components dealing with Cu trafficking and assembly into the mitochondrial type aa_3-Cox as well as on specific bacterial components. In this chapter, we focus on the structure and subunit composition of cbb_3-Cox, and on the insertion of its Cu atom and maturation of its cyts b and c, using as an example *R. capsulatus* enzyme where most of the studies were conducted.

II. The *cbb₃*-Cox

A. Distribution and Role of cbb₃-Cox

The cbb_3-Cox enzymes are widespread in the bacterial kingdom (Ducluzeau et al. 2008). They are commonly found in the α-proteobacteria (e.g., *Rhodobacter* species) and the *cytophaga*, *flexibacter* and *bacteroides* (CFB) groups. The genes encoding cbb_3-Cox are also found in the last mitochondrial ancestor *Midichloria mitochondrii* (Sassera et al. 2011), but so far, no cbb_3-Cox related sequences have been identified in Archaea or Eukaryota (Hemp and Gennis 2008). Structural genes of cbb_3-Cox were first identified in the facultative symbiotic N_2-fixing *Rhizobiacaea* (Kahn et al. 1989; Preisig et al. 1993) and due to their importance for symbiotic N_2 fixation, they were initially termed *fixNOQP* (Preisig et al. 1993). For example, in *Bradyrhizobium japonicum* expression of cbb_3-Cox is required for symbiotic N_2 fixation under very low O_2 conditions in soybean root nodules (Preisig et al. 1993). Subsequent studies identified the cbb_3-Cox structural genes in non-symbiotic bacteria such as *R. capsulatus,* and referred to them as *ccoNOQP* (Garcia-Horsman et al. 1994; Gray et al. 1994; Thony-Meyer et al. 1994).

The cbb_3-Cox enzymes are present in many microaerophilic pathogenic bacteria, and are implicated in host colonization. Moreover, in some species like *Campylobacter jejuni* (Parkhill et al. 2000), *Helicobacter*

pylori (Tomb et al. 1997; Alm et al. 1999), *Neisseria meningitidis* and *Neisseria gonorrhoea* (Li et al. 2010) cbb_3-Cox is the only Cox enzyme. It is suggested that its higher O_2 affinity (compared to aa_3-Cox) allows these pathogens to colonize low O_2 containing tissues (Pitcher and Watmough 2004). For example, *C. jejuni* mutants lacking cbb_3-Cox are unable to colonize the chick caecum (Weingarten et al. 2008), which is primarily colonized by obligate anaerobic bacteria. Thus, cbb_3-Cox was proposed to keep *C. jejuni* cytoplasmic O_2 tensions low enough to protect its O_2 sensitive metabolically important enzymes (Woodall et al. 2005; Weingarten et al. 2008). This so called "respiratory protection" phenomenon was first described in *Azotobacter vinelandii* as a way of using respiration to protect the O_2 sensitive nitrogenase from deleterious effects of O_2 (Jones et al. 1973). Similarly, facultative anaerobic phototrophs rely on cbb_3-Cox to eliminate O_2 and reduce their environment in order to establish productive anaerobic Ps growth (Marrs and Gest 1973) (Fig. 27.1). For example, the terminal oxidases (including cbb_3-Cox) of the purple non-sulfur phototrophic bacterium *Rubrivivax gelatinosus* are crucial for anoxygenic Ps gene expression (Hassani et al. 2010b).

B. Subunit and Cofactor Composition of cbb₃-Cox

The three dimensional (3D) structure of cbb_3-Cox from *Pseudomonas stutzeri* was solved at a resolution of 3.2 Å (Fig. 27.2a) (Buschmann et al. 2010), revealing that even though the HCO enzymes are diverse in terms of their subunit and cofactor compositions, the overall architecture of the membrane-embedded part of cbb_3-Cox is similar to that of *R. sphaeroides* aa_3-Cox (Svensson-Ek et al. 2002) and *Thermus thermophilus* ba_3-Cox, (Soulimane et al. 2000). The cbb_3-Cox enzymes often have four structural subunits, CcoN (subunit I), CcoO (subunit II), CcoP (subunit III) and CcoQ (subunit IV) that are encoded by

the *ccoNOQP* operon (Ducluzeau et al. 2008). The catalytic subunit CcoN has 12 membrane-spanning helices. It forms a "clamshell"-like structure, with its N- and C-termini located close to each other on the cytoplasmic side of the membrane (Buschmann et al. 2010). CcoN contains a low-spin heme b (b) and a high-spin heme b (b_3) associated with a single Cu atom (Cu_B) to form the catalytic center that binds and reduces O_2 to H_2O (Fig. 27.2b). The Cu_B atom is coordinated with three His residues, and one of them is covalently ligated to a nearby, possibly redox-active, Tyr residue as in other Cox enzymes (Buse et al. 1999; Hosler et al. 2006). Two additional calcium atoms with a proposed stabilizing role are found in the structure of CcoN. One of these atoms interacts directly with the heme b_3-Cu_B catalytic center (Fig. 27.2b), while the second one is located between the loops of CcoN at the edge of the structure. CcoO and CcoP are mono- and di-heme containing c-type cyts, respectively, and have one or two N-terminally located transmembrane (TM) helices as membrane anchors (Buschmann et al. 2010).

The outer heme c of CcoP whose pyrrole rings C and D are solvent-exposed, is thought to accept electrons from a soluble or a membrane-bound electron donor (e.g., cyt c_2 or cyt c_y in *R. capsulatus*), and transfers them to the inner heme c of CcoP. Electrons are then routed to heme c of CcoO, which convey them to heme b and subsequently to the heme b_3-Cu_B center of CcoN. CcoO makes strong contacts with CcoN α-helices, specifically via a calcium atom that coordinates the heme b and heme b_3 at the binuclear catalytic center via its Ser residue (Ser102 for *P. stutzeri*) (Fig. 27.2b). O_2 binds to heme b_3 and is reduced via the electrons coming from heme b_3, Cu_B, and possibly the redox-active Tyr residue (Tyr251 for *P. stutzeri*) (Buschmann et al. 2010, 2014) (Fig. 27.2b).

CcoQ is a small, single TM helix containing protein that has no cofactor. Earlier, it was considered to play a regulatory role (Oh and Kaplan 2002), which remains to be proven. This subunit is absent in some cbb_3-
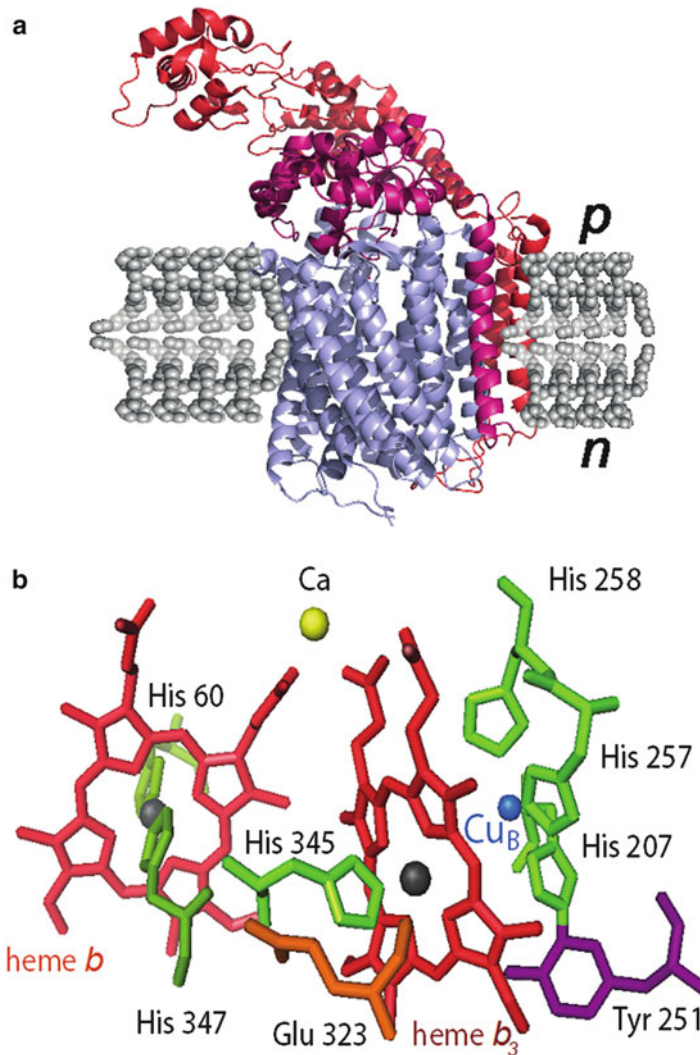
Fig. 27.2. Structure and subunit composition of *cbb₃*-Cox. (**a**) 3D structure of *cbb₃*-Cox of *P. stutzeri* (3MK7) as shown in Fig. 27.1. The 12 TM helix-containing CcoN subunit is shown in *blue* whereas the monoheme CcoO and the diheme CcoP are in *magenta* and *red*, respectively. The CcoQ subunit is not found in the structure of *cbb₃*-Cox. (**b**) The heme *b* and heme *b₃*-Cu$_B$ binuclear center of the catalytic subunit CcoN of *cbb₃*-Cox. The color scheme is as follows: Heme *b*, *light red* and heme *b₃*, *dark red*; His, *green*; Tyr, *violet*; Glu, *orange*; copper – *blue*; hexa-coordinate heme iron, *light grey*; penta-coordinate heme iron, *dark grey*; and calcium, *yellow*. Note the covalent bond between His 207 and Tyr 251.

Cox, and is not essential for the activity of *cbb₃*-Cox in *R. sphaeroides* (Oh and Kaplan 1999), *R. capsulatus* (Peters et al. 2008) and *B. japonicum* (Zufferey et al. 1996), leaving its function undefined. Although a gene encoding *ccoQ* is present in the genome of *P. stutzeri*, curiously, CcoQ is not found in the 3D structure of *cbb₃*-Cox (Buschmann et al. 2010). Nevertheless, CcoQ appears to be a *bona fide* subunit of *cbb₃*-Cox as it co-purifies with the other subunits in *B. japonicum* (Zufferey et al. 1998). Moreover, it forms with CcoN, CcoO and CcoP an active complex of ~230-kDa in blue-native poly-acrylamide gels (BN-PAGE) in *R. capsulatus* (Kulajta et al. 2006) and can be cross-linked

to CcoP, suggesting its close vicinity to this subunit in cbb_3-Cox (Peters et al. 2008).

C. Maturation of CcoN

CcoN is probably co-translationally inserted into the *R. capsulatus* cytoplasmic membrane (Koch et al. 2003; Koch 2007), and its hemes b, b_3 and Cu_B cofactors are deeply buried within the protein. Incorporation of these cofactors into CcoN seems to be critical for proper folding and stabilization of cbb_3-Cox. How, and in which order, these cofactors are inserted into CcoN is unknown, although a co-translational insertion process for hemes a and Cu cofactors for aa_3-Cox has been proposed (Khalimonchuk et al. 2005).

1. Hemes b of cbb₃-Cox

Available structures of soluble and membrane-bound heme b containing proteins show that this cofactor is typically buried within the cognate protein without significant surface exposure (Smith et al. 2010). How the non-covalently attached heme groups are incorporated into their cognate proteins is still a largely unexplored process. Heme insertion into soluble cyts b was initially considered to occur spontaneously, but recent studies indicate that accessory proteins are required in vivo for efficient heme b incorporation into proteins. For example, insertion of the heme b cofactor into the NO synthase requires thioredoxin, Hsp90 chaperone, and glycerolphosphate-dehydrogenase (Chakravarti et al. 2010; Ghosh et al. 2011; Hannibal et al. 2012).

The process of non-covalently attached heme b (or $a,$ or o) insertion into the HCO enzymes is mainly examined for the aa_3-Cox of *P. denitrificans* and *R. sphaeroides*, and bo_3-Qox of *E. coli*. In the case of bo_3-Qox, use of [55]Fe-labeled heme showed that these cofactors were inserted at a late step during assembly (Palombo and Daley 2012). It is suggested that the heme-binding pocket within the catalytic subunit CyoB is only formed upon its interaction with a subcomplex containing the CyoD and CyoC

subunits, which do not contain any cofactor (Stenberg et al. 2007). However, it remains unknown whether any dedicated accessory protein(s) is involved in heme b or heme o_3 insertion into bo_3-Qox. This process seems to be different in the case of the aa_3-Cox, which requires Surf1 for the insertion of heme a_3 (Hannappel et al. 2012). Surf1 (called Shy1 in yeast) is a well-conserved membrane protein of about 30 kDa that binds heme via conserved His and Trp residues (Bundschuh et al. 2009). It consists of two TM domains, connected via a large loop facing the inter-membrane space in mitochondria or the periplasmic space in bacteria (Hannappel et al. 2012). In humans, Surf1 mutations are frequent causes of Leigh syndrome, a fatal neurological disorder associated with severe Cox deficiency (Tiranti et al. 1998; Zhu et al. 1998). Interestingly, insertion of the low-spin heme a into *R. sphaeroides* aa_3-Cox does not seem to require Surf1 (Smith et al. 2005). As heme a_3 and Cu_B insertion are suggested to occur simultaneously in the mitochondrial aa_3-Cox (Cobine et al. 2006), Surf1 might be required for coordinating heme a_3 and Cu_B insertions. In bacteria, heme a and Cu_B insertions might be less tightly coupled, as heme a_3 insertion does not seem to require prior assembly of Cu_B in *R. sphaeroides* aa_3-Cox (Hiser et al. 2000). Likewise, Cu_B insertion occurs even if heme a_3 insertion fails due to the absence of Surf1 (Smith et al. 2005), suggesting that alternative pathways might exist. In agreement with this possibility, *Bacillus subtilis*, which has no Surf1 homologue, contains two HCO enzymes that contain heme a (Hill and Andrews 2012). Interestingly, bacteria like *R. capsulatus* or *H. pylori* that have cbb_3-Cox as their sole HCO do not have a Surf1 homologue. It remains unknown whether dedicated assembly proteins are absolutely required for heme b and/or b_3 insertion into cbb_3-Cox.

A possible candidate for heme b insertion into cbb_3-Cox might be CcoS, which is encoded by the *ccoGHIS* gene cluster located immediately downstream of the *ccoNOQP* operon (Koch et al. 1998;

Ducluzeau et al. 2008). CcoS is a small protein (56 amino acid residues) with a single TM helix. It has no recognizable secondary structure motif, and is resilient to mutations of its highly conserved amino acid residues (Pawlik et al. unpublished). Its exact function is unclear, and its overall biochemical properties (e.g., helical content or hydrophobicity) may be more important than its primary sequence. *R. capsulatus* mutants lacking CcoS assemble a completely inactive cbb_3-Cox (i.e., the 230 kDa complex in BN-PAGE) due to the absence of heme b and a functional heme b_3-Cu_B binuclear center in CcoN (Koch et al. 2000; Kulajta et al. 2006). Absence of CcoS has no effect on CcoO and CcoP maturation via Ccm-System I. Whether Cu_B is inserted into cbb_3-Cox in the absence of CcoS is unknown, but available data suggest that Cu insertion is needed for CcoN stability (Kulajta et al. 2006; Ekici et al. 2013), suggesting that CcoS might be involved in heme b insertion into this subunit.

2. Cu_B Biogenesis of cbb_3-Cox

Cu is required as a cofactor for multiple cupro-enzymes, including various types of HCOs, Cu-Zn superoxide dismutases, multicopper oxidases and type II NADH dehydrogenases. However, free Cu is toxic for cells due to its chemical reactivity, thus cells control Cu homeostasis by monitoring its acquisition, trafficking, storage, and delivery to target proteins. *R. capsulatus* proteins that are known to be involved in this process so far are listed in Table 27.1.

a. Cu Acquisition and Transport

During the last decade, Cu transport and delivery to HCOs was intensely studied, mainly motivated by Cu toxicity and Cu related diseases associated with Cox enzymes. In eukaryotic cells, Cu enters the cytoplasm via dedicated Cu importers called Ctr (Lutsenko and Petris 2003; Hardman et al. 2006; van den Berghe et al. 2007). However, bacteria have no homologues of Ctr proteins, leaving undefined how Cu enters the bacterial

cytoplasm (Rensing and Grass 2003). A major facilitator superfamily (MFS)-type Cu transporter, CcoA was recently identified in *R. capsulatus* by analyses of a mutant that exhibited Cu-dependent cbb_3-Cox assembly and activity defects (Ekici et al. 2012b). CcoA is an integral membrane protein with 12 TM helices arranged into two subdomains that are separated by a large cytoplasmic loop, and contains the characteristic DRX-GRR motifs of the MFS-type transporters. CcoA also contains additional putative Cu binding Mets (MX_xM) motifs (Khalfaoui-Hassani et al. 2016) as found in the Ctr-type eukaryotic Cu importers. The idea that CcoA is responsible for Cu transport was initially suggested by lower cellular Cu contents in *R. capsulatus* mutants lacking CcoA as compared with wild type strains (Ekici et al. 2012b). Additional studies, including decreased radioactive ^{64}Cu uptake ability (Ekici et al. 2014), and inter species heterologous complementation (Beaudoin et al. 2013), established that CcoA is a bacterial cytoplasmic Cu importer, which is required for cbb_3-Cox production (Fig. 27.3). Interestingly, CcoA homologues are also present in eukaryotic organisms. For example, its *Schyzosaccharomyces pombe* homologue Mfc1 is located at the forespore membrane of this species, and is exclusively expressed during meiosis (Beaudoin et al. 2011). When Mfc1 is expressed in *S. pombe* mutants lacking the plasma membrane Cu importers Ctr4 and Ctr5, it complements the defective ability of these mutants to grow on respiratory carbon sources and to import ^{64}Cu (Beaudoin et al. 2011). Similarly, *R. capsulatus* CcoA also complements *S. pombe* mutants lacking Ctr4 and Ctr5, restoring their ability to grow on non-fermentable carbon sources and to import ^{64}Cu (Beaudoin et al. 2013). These findings established that the MFS-type transporter CcoA is a cytoplasmic Cu importer in *R. capsulatus*.

In bacteria, well-known cytoplasmic Cu exporters are members of the P1B-type ATPase superfamily that use ATP hydrolysis for Cu transport across the cytoplasmic membrane (Solioz and Vulpe 1996; Palmgren and

Table 27.1. Components involved in the biogenesis of the *R. capsulatus* CcoN subunit of cbb_3-Cox.

Cu protein	Structural features	Proposed function
CcoI (FixI/CtpA/CopA2)	Eight TM helices with ATP-binding, phosphorylation and phosphatase domains. HMBD (CXXXC) and CPC are the Cu binding site	P1B-type ATPase that transports Cu to Cox and/or to other Cu containing proteins across the cytoplasmic membrane
CopA (CopA1)	Eight TM helices with ATP-binding, phosphorylation and phosphatase domains. HMBD (CXXXC) and CPC are the Cu binding site	P1B-type ATPase that exports Cu out of the cytoplasm to control Cu amounts in the cytoplasm
CcoA (*S. pombe* Mfc1 homologue)	Twelve TM helices with conserved AVYGRR and AFRGRE motifs. Mets (MXXM) motifs are its putative Cu binding sites	MFS-type transporter that imports Cu into the cytoplasm, and is required for cbb_3-Cox production
Sco (Sco1/Sco2/SenC/PrrC)	Single TM helix with a thioredoxin fold and a conserved Cu binding motif comprising the CXXXC sequence and a His ligand	Cu chaperone required for Cu_A and/or Cu_B insertion into various Cox enzymes. It might also affect oxidative stress and gene regulation
PCuAC (Pcc$_A$)	Has a potential Cu binding motif $MX_{10}MX_{21}HXM$. Cu binds via one His and three Met residues	Periplasmic chaperone proposed to donate Cu to Sco protein (like eukaryotic Cox17)
CcoG (FixG)	Five TM helices with two putative ferredoxine-like 4Fe-4S cluster binding motifs	Proposed to affect intracellular Cu^{1+}/Cu^{2+} redox equilibrium
CcoS (FixS)	Small, single TM membrane protein (56 amino acids long) with no recognizable motif	Proposed to be involved in b/b_3 hemes and/or Cu_B insertion in cbb_3-Cox

Axelsen 1998; Rensing and Grass 2003). The recent 3D structure of *Legionella pneumophila* P1B-type transporter CopA revealed the direction of Cu transport, and locations of the characteristic motifs of this superfamily (Gourdon et al. 2011), including the ATP binding, phosphorylation and phosphatase domains. CopA also contains an N-terminal heavy metal binding domain (HMBD) with conserved metal binding motifs (CXXC), and a membrane integral Cu binding site (CPX). The HMBD domain is thought to interact with specific cytoplasmic Cu chaperones via a docking platform as a Cu entry point (Gourdon et al. 2011). Cu transport by P1B-type transporters is from the cytoplasmic face of the membrane where ATP hydrolysis occurs to the other side of the lipid bilayer. Hence, in Gram-negative bacteria, CopA-like transporters seem mainly involved in Cu efflux from the cytoplasm. Earlier, the *Synechocystis* PCC 6803 P1B-type transporters CtaA and PacS were reported as Cu transporters to the cytoplasm and chloroplast stroma, respectively (Tottey et al. 2001, 2002). Whether they are indeed Cu importers deserves further studies.

Various Cu specific P1B-type transporters play distinct cellular functions depending on their efflux rates. For instance, *Pseudomonas aeruginosa* (Gonzalez-Guerrero et al. 2010), *R. capsulatus* (Koch et al. 2000; Ekici et al. 2014) and *Rubrivivax gelatinosus* (Hassani et al. 2010a; Azzouzi et al. 2013) express at least two homologous P1B-type ATPase involved in Cu export. The high flux *P. aeruginosa* CopA1 [homologue of *R. capsulatus* (Ekici et al. 2014) and *R. gelatinosus* CopA (Hassani et al. 2010a)] is involved in Cu detoxification, whereas the low flux CopA2 [homologue of *R. capsulatus* CcoI (Koch et al. 2000) and *R. gelatinosus* CtpA (Hassani et al. 2010a)] is dedicated to supply Cu to cbb_3-Cox (Fig. 27.3).
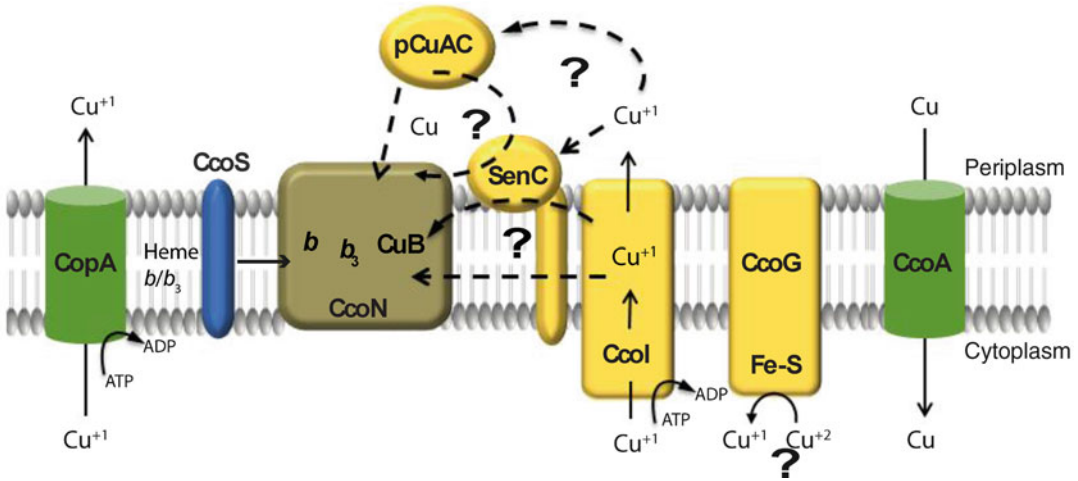
Fig. 27.3. A working model for maturation of the heme b_3-Cu$_B$ catalytic center of *R. capsulatus* CcoN subunit of *cbb$_3$*-Cox. Cu (possibly Cu^{2+}) is imported into the cytoplasm via a major facilitator superfamily (MFS)-type transporter CcoA. In the cytoplasm, Cu^{2+} is thought to be reduced to Cu^{1+} by the ferredoxin-like protein CcoG via its 4Fe-4S clusters, and exported across the cytoplasmic membrane via the P1B-type ATPase CcoI (shown on the right). CcoI delivers Cu to CcoN either during its co-translational insertion through the membrane (arrow in the membrane) or from the periplasm either directly, or via putative Cu chaperones like the membrane-anchored SenC and the periplasmic pCuAC. In all instances, the exact delivery route of Cu is not known, as indicated? with CcoS is proposed to be involved in incorporation of heme *b* and heme *b$_3$* to CcoN. In order to maintain cytoplasmic Cu homeostasis and avoid toxicity while providing Cu to cuproproteins, the P1B-type transporter CopA (a high flux Cu exporter which is a homologue of the low flux CcoI) extrudes excess of Cu from the cytoplasm using ATP (shown on the *left*).

In the absence of these transporters (i.e., CopA2 or CcoI or CtpA) cells contain no *cbb$_3$*-Cox, indicating a role of these transporters in Cu$_B$ incorporation into CcoN (Preisig et al. 1996; Koch et al. 1998, 2000). However, it is unknown whether Cu is inserted into CcoN without complete export across the membrane by a low flux Cu-transporter during its co-translational membrane insertion, or via specific periplasmic chaperone(s) that may differ among species (Fig. 27.3). Moreover, *R. gelatinosus* CtpA also supplies Cu to cupro-enzymes other than *cbb$_3$*-Cox, like *caa$_3$*-Cox and N$_2$O reductase (Hassani et al. 2010a), suggesting a broader role for these transporters. The findings that the cytoplasmic Cu importer CcoA and the P1B-type Cu exporter CcoI are both required for *R. capsulatus cbb$_3$*-Cox biogenesis indicate that Cu needs to be imported into, and then exported from, the cytoplasm in order to be incorporated into the CcoN subunit of *cbb$_3$*-Cox. Currently,

R. capsulatus components involved in cytoplasmic Cu trafficking need further studies.

Two different ways of overcoming *cbb$_3$*-Cox deficiency induced by the absence of CcoA have been observed. First, phenotypic suppression of mutants lacking CcoA can be achieved by supplementation of the growth medium with exogenous μM amounts of Cu^{2+}. This finding suggests that a mechanism(s) independent of CcoA can also supply Cu to *cbb$_3$*-Cox when Cu is not scarce (Ekici et al. 2012b). Second even when Cu is not abundant, bypass suppressors of *R. capsulatus* mutants lacking CcoA overcome *cbb$_3$*-Cox deficiency by increasing cellular Cu content (Ekici et al. 2012b, 2014). These suppressor mutations occur at a unique ten consecutive cytosine base pair repeat in *copA* gene, inactivating the high flux P1B-type transporter CopA (Ekici et al. 2014). *R. capsulatus* mutants lacking CopA alone have no effect on *cbb$_3$*-Cox biogenesis, but are hypersensitive to Cu. Indeed, a double mutant

lacking both CcoA and CopA restores cbb_3-Cox production at the expense of cellular Cu hypersensitivity. Thus, homeostatic balance between the Cu importer CcoA and the Cu exporter CopA is crucial to ensure safe delivery of Cu to target cuproteins like cbb_3-Cox (Ekici et al. 2014).

b. Copper Insertion

Once imported into the cytoplasm, Cu is trafficked to various cuproteins, including HCO enzymes. Although how Cu is specifically delivered to cbb_3-Cox is not yet established, analyses of mutants lacking cbb_3-Cox activity implicated into this process several gene products. The specificity of Cu delivery to the different Cu accepting proteins (e.g., Cu_A and Cu_B of aa_3-Cox) is thought to be associated with specific putative Cu chaperones, as discussed below.

Sco (SenC/PrrC) Chaperones
Sco1 and Sco2 proteins are well studied in yeast and mammalian cells, and are mainly involved in Cu_A insertion into the subunit II of aa_3-Cox. Although both of these proteins were identified in yeast, only Sco1 participates in Cu_A formation in this species (Schulze and Rodel 1988; Krummeck and Rodel 1990), whereas Sco1 and Sco2 are both required for Cox biogenesis in humans (Leary et al. 2004). Mutations in either of these proteins cause clinical disorders manifested as fatal encephalomyopathies (Shoubridge 2001; Stiburek et al. 2009). Homologues of the Sco proteins were also identified in a number of bacteria. The 3D structure of *B. subtilis* Sco1 depicts it as a membrane-anchored protein with a single TM helix (Balatri et al. 2003). Its periplasmic domain contains a thioredoxin fold, a Cys-rich Cu binding motif and a His ligand (Hill and Andrews 2012). Like their eukaryotic counterparts, bacterial Sco proteins seem to be specific to the insertion of Cu_A of aa_3-Cox. For example, a *B. japonicum* mutant lacking Sco1 has no aa_3-Cox in its cytoplasmic membrane (Buhler et al. 2011), while in species like *R. sphaeroides*

(PrrC) (Eraso and Kaplan 2000), *Neisseria meningitides,* or *Neisseria gonorrhoeae* (Eraso and Kaplan 2000; Seib et al. 2003), absence of Sco activity has no effect on cbb_3-Cox (due the absence of CuA). In addition, some Sco homologues were shown to have a thiol:disulfide oxidoreductase activity, possibly important for Cu insertion into the target proteins (McEwan et al. 2002; Hill and Andrews 2012).

Interestingly, genome surveys using bioinformatic approaches identified genes encoding for Sco proteins in a number of bacteria that also lack aa_3-Cox, suggesting that in these cases, the role(s) of Sco proteins might extend beyond the Cu_A insertion into this enzyme. Functions like Cu metallochaperoning for enzymes other than Cox (Banci et al. 2007), oxidative stress response (Seib et al. 2003) and photosynthetic gene regulation (Eraso and Kaplan 2000) were proposed for these proteins. Moreover, in some species like *R. capsulatus* (Swem et al. 2005) and *P. aeruginosa* (Frangipani and Haas 2009), absence of the Sco homologue SenC also impairs cbb_3-Cox biogenesis in a manner commensurate with the availability of exogenous Cu (Fig. 27.3). Recent chemical cross-linking experiments in *R. capsulatus* indicated that SenC can be located near CcoP and CcoH proteins that are associated with cbb_3-Cox (Lohmeyer et al. 2012). Finally, transcription of *senC* is enhanced by Cu addition in the absence of CcoI, linking it to Cu delivery to cbb_3-Cox (Lohmeyer et al. 2012). Overall, the precise role(s) of SenC during cbb_3-Cox biogenesis still remains unclear.

PCu$_A$C (Pcc$_A$) Chaperone
The gene for the periplasmic Cu_A chaperone PCu$_A$C, which has a potential metal binding motif, was first identified near that of Sco1 in *Deinococcus radiodurans* (Banci et al. 2005). In this species, PCu$_A$C binds Cu^{1+} through one His and three Met residues, donates Cu to Sco1, like the eukaryotic Cu chaperone Cox17. In *T. thermophiles*, PCu$_A$C selectively inserts Cu^{1+} into the subunit II of ba_3-Cox to assemble a Cu_A site with the

Cys ligands presumably reduced by Sco1 thiol:disulfide oxidoreductase activity prior to Cu insertion (Abriata et al. 2008). The role(s) of PCu_AC together with and without PrrC (SenC) was also examined in *R. sphaeroides*, which produce both aa_3-Cox and cbb_3-Cox (Thompson et al. 2012). It was proposed that PrrC (SenC) might capture and deliver Cu to Cu_A of the aa_3-Cox and to Cu_B of the cbb_3-Cox (Fig. 27.3), while the predominant role of PCu_AC may be to capture and deliver Cu to PrrC (SenC) and Cox11 involved in the maturation of aa_3-Cox Cu_B center, similar to Cox17 in mitochondria (Khalimonchuk et al. 2005). Homologues of PCu_AC, called Pcc1, are also found in species that lack aa_3-Cox like *R. capsulatus*. Unlike *D. radiodurans*, the structural gene of *R. capsulatus* Pcc1 is not located next to that of *senC*, but its product contains similar conserved metal-binding motifs. In the absence of Pcc_A, *R. capsulatus* cbb_3-Cox activity is reduced, but can be restored by addition of exogenous Cu (Trasnea et al. 2015).

CcoG Protein

CcoG is encoded by the *ccoGHIS* cluster located downstream of the *ccoNOQP* operon. It is an integral membrane protein with five predicted TM helices and has two putative 4Fe-4S cluster-binding motifs, but whether it contains any iron-sulfur cluster(s) is unknown. *R. capsulatus* mutants lacking CcoG exhibit a substantial amount of cbb_3-Cox activity in a growth phase dependent manner (Koch et al. 2000) (Fig. 27.3), and its absence seems to affect cbb_3-Cox activity mainly during the early phases of Res growth. It has been proposed that CcoG might affect the intracellular Cu^{1+}/Cu^{2+} redox equilibrium (Preisig et al. 1996), and its role might be partially redundant with some other component(s) especially in the absence of O_2. In addition, a mutant lacking both CcoG and CcoA is phenotypically similar to a mutant lacking only CcoA (Ekici et al. 2012a, b), suggesting that CcoG acts downstream of CcoA.

D. Maturation of CcoO and CcoP

The CcoO and CcoP subunits of cbb_3-Cox are matured through the Ccm-system I pathway that operates independently from CcoN maturation (Ekici et al. 2013). This process, which is present virtually in all organisms, occurs post-translationally, and is responsible for the covalent ligation of heme *b* to apocyts *c* to produce mature holocyts *c*. The Ccm pathway relies on major cellular functions like protein translocation, extra-cytoplasmic protein folding and degradation, redox homeostasis as well as heme synthesis, acquisition, and insertion into target proteins. Four variants of the cyt *c* biogenesis process (System I–IV) were identified in different organisms, and in spite of their different degrees of complexity, they all share common characteristics (Kranz et al. 2009; Sanders et al. 2010; Allen 2011; de Vitry 2011; Simon and Hederstedt 2011; Stevens et al. 2011). Both apocyts *c* and hemes are independently translocated across a membrane into a cellular compartment where dedicated chaperones and enzymes perform heme ligation. Apocyts *c* are translocated into the periplasm through the Sec pathway in Gram-negative bacteria (Natale et al. 2008; Facey and Kuhn 2010). These processes are coordinated spatially and temporally to minimize cytotoxic effects of free heme and proteolytic degradation of apocyts *c* (Goldman et al. 1996; Moore and Helmann 2005). Thioether bond formation requires reduced heme-Fe^{2+} and thiol groups of the apocyt *c* heme-binding site Cys residues (Kranz et al. 1998; Sanders et al. 2010). Finally, mature cyts *c* are assembled into their respective cyt *c* complexes. Here, we focus on *R. capsulatus* cyt *c* biogenesis (Ccm-System I) pathway, which is similar to that found in α- and γ-proteobacteria, archaea, and mitochondria of plants and red algae. Different components involved in this process are listed in Table 27.2.

The Ccm-System I displays broad substrate specificity and requires the presence of a C_1XXC_2H heme-binding site for efficient heme attachment (Allen et al. 2002, 2006,

Table 27.2. Components involved in biogenesis of *R. capsulatus* CcoO and CcoP subunits of cbb_3-Cox (Ccm-System I).

Ccm protein	Structural features	Proposed function
CcmA	Peripheral membrane protein with ATP binding domain Walker A (GX_4GKS/T) and Walker B motifs ($R/KX_3GX_3LX_3D$)	Part of CcmABCD, a putative ABC-type transporter; required for ATP-dependent "release" of holoCcmE from the CcmCDE complex
CcmB	Six TM helices and a conserved FXXDXXDGSL motif	Part of the CcmABCD, a putative ABC-type transporter; required for the "release" of holoCcmE from the CcmCDE complex
CcmC	Six TM helices with a tryptophan-rich (WGXF/Y/WWXWDXRLT) motif and two conserved His residues	Part of the CcmABCD, a putative ABC-type transporter; loads heme to apoCcmE and provides axial ligands to the heme-iron; acts as a CcmE-specific heme lyase
CcmD	Small single TM helix protein with no conserved domains	Part of the CcmABCD, a putative ABC-type transporter; improves CcmC activity; required for the release of holoCcmE from the CcmCDE complex
CcmE	Single TM helix with a periplasmic domain containing a conserved HXXXY motif	Heme chaperone binding covalently vinyl-2 of heme via a conserved His residue; delivers heme to apocyts c
CcmF	Eleven TM helices with a tryptophan-rich (WGGXWFWDP-VEN) motif and four conserved His residues	Part of the CcmFHI heme ligation complex; interacts with apocyts c and holoCcmE; suggested to reduce heme of holoCcmE for its transfer to apocyts c
CcmG	Single TM helix with a periplasmic domain containing a thioredoxin motif (CXXC)	Binds poorly apocyts c; involved in thioreduction of apocyts c either directly, or indirectly via CcmH, or by resolving a CcmH-apocyt c mixed-disulfide bond
CcmH	Single TM helix with a periplasmic domain containing a thioredoxin-like motif (LRCXXC)	Part of the CcmFHI heme ligation complex; interacts with apocyts c and suggested to reduce its disulfide bond to enable stereo-specific heme ligation
CcmI	Two TM helices linked by a cytoplasmic loop with a leucine-zipper-like motif; has a large periplasmic domain containing three TPR repeats	Part of the CcmFHI heme ligation complex; is an apocyt c chaperone and binds the C-terminal portion of apocyt c via its periplasmic domain

2009). It ligates heme b to a variety of substrates, including natural and artificial cyts c (Sanders and Lill 2000) with single or multiple heme binding sites (Pitts et al. 2003), and even short peptides of a few residues (Braun and Thony-Meyer 2004; Lee et al. 2005). Below, we describe the Ccm proteins that are grouped into three functional modules with different roles. Then we discuss a working model for the mechanism of stereo-specific heme ligation, and the possible occurrence of a multi subunit Ccm supercomplex (dubbed a "Ccm machine").

1. Ccm-System I: Functional Organization

R. capsulatus Ccm-System I has nine specific membrane-bound components (CcmABCDEFGHI) that can be grouped into three functional modules (Fig. 27.4). These three modules accomplish the (1) transport and relay of heme b (Module 1), (2) preparation and chaperoning of ligation-competent apocyts c (Module 2), and (3) ligation of heme-apocyt c (Module 3) to yield holocyts c.
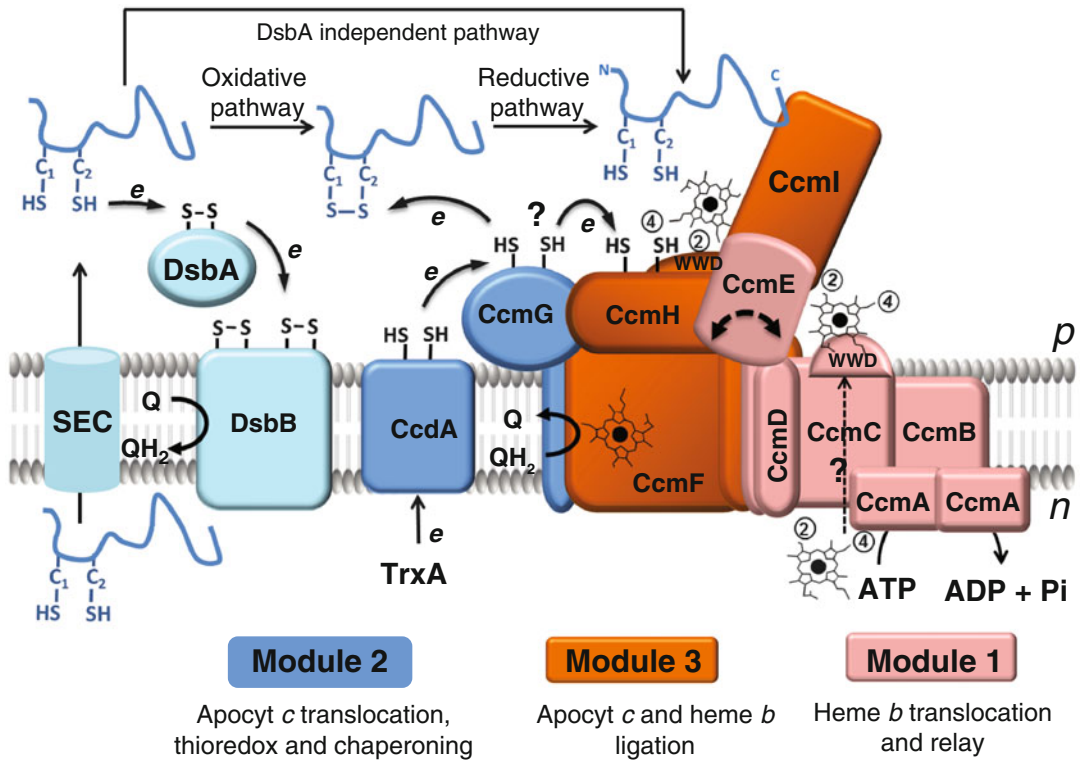
Fig. 27.4. Modular organization of cyt *c* maturation (Ccm) system I in *R. capsulatus*. Three membrane-integral functional modules carry out Ccm in a coordinated manner: Module 1 (in *pink*) is responsible for heme *b* translocation across the membrane and holoCcmE production. The ATP-binding CcmA and its partners CcmB, CcmC, CcmD and the heme chaperone CcmE form an ABC-type transporter in a putative stoichiometry of CcmA$_2$BCDE. Heme *b* is transported to the periplasm, and forms a non-covalent complex with CcmCD and CcmE. Oxidized heme-Fe^{3+} binds covalently via its vinyl-2 to a conserved His residue at the CcmE heme-binding site. ATP hydrolysis by CcmA is postulated to induce a conformational change (indicated by the *arrow* in CcmE) that renders CcmE ready to deliver heme *b* to the apocyt *c* substrates. Module 2 (in *dark blue*) carries out the thioreduction and chaperoning to the heme ligation site of the newly Sec-translocated apocyts. The periplasmic disulfide bond formation components DsbA and DsbB (*light blue*) form a disulfide bond at the heme-binding sites of the apocyts *c* to avoid their degradation, and the membrane-anchored thioredoxin-like CcmG reduces this intramolecular disulfide bond to allow heme ligation. CcdA receives electrons from the cytoplasmic thioredoxin TrxA and regenerates CcmG, completing the thioredox loop. DsbA, DsbB and CcdA are not exclusively involved in Ccm as they also play critical roles in other cellular processes. Module 3 (in *orange*) is composed by CcmFHI that forms the heme ligation complex. CcmI traps the C-termini of the apocyt *c* substrates while CcmF holds the heme delivered by holoCcmE, facilitating its covalent ligation to the apocyts *c* heme binding sites. The specific role of CcmH is still elusive, and suggested to ensure stereo-specificity of the thioether bonds during heme ligation.

a. Module 1. CcmABCD and CcmE: Heme *b* Translocation and Relay to Module 3

Module 1 has five (CcmABCD and CcmE) components of which CcmABCD are homologous to the ABC (ATP-binding cassette) transporters, and are involved in conveying heme *b* to the periplasm and

loading it on the heme-chaperone CcmE (Fig. 27.4). Of these proteins, CcmA is a peripheral membrane protein with a typical ATP-binding region containing Walker A and B domains (Walker et al. 1982). CcmB and CcmC are integral membrane proteins with six TM helices each, and together with CcmA form a putative ABC-type transporter complex with a stoichiometry of CcmA$_2$BC

(Goldman et al. 1997; Page et al. 1997; Goldman and Kranz 2001; Feissner et al. 2006b; Rayapuram et al. 2007). CcmC has a hydrophobic tryptophan rich (WWD) motif and two conserved His residues exposed to the periplasm (Goldman et al. 1998; Richard-Fogal et al. 2009; Richard-Fogal and Kranz 2010). It belongs to the heme handling protein (HHP) family (Lee et al. 2007) and is the only protein necessary for loading heme b on apoCcmE to produce holoCcmE (Schulz et al. 1999). CcmD is a small polypeptide (about 60 residues) with a single TM helix and a hydrophilic C-terminal domain (Ahuja and Thony-Meyer 2005; Richard-Fogal et al. 2008) that enhances CcmC activity for holoCcmE synthesis (Feissner et al. 2006a; Richard-Fogal et al. 2008). CcmE is a member of the oligobinding family of proteins (Arcus 2002), has a single TM helix, and a large periplasmic β barrel core domain ending with a flexible C-terminus (Arnesano et al. 2002; Enggist et al. 2002; Aramini et al. 2012). It covalently and transiently binds vinyl-2 of heme b via its conserved His residue within the HXXXY domain (Schulz et al. 1998; Lee et al. 2005; Harvat et al. 2009). Mutation of this His residue prevents holoCcmE formation, and blocks cyts c production (Schulz et al. 1999; Enggist et al. 2003). In spite of being the only protein necessary for holoCcmE formation, CcmC lacks any obvious heme delivery path, leaving the mechanism by which heme reaches the periplasm unknown. Once heme b is in the periplasm, it interacts with the WWD domain of CcmC (Ren and Thony-Meyer 2001) and the heme-binding region of apoCcmE (Enggist et al. 2003; Harvat et al. 2009). Heme is ligated covalently to the CcmE His residue, and CcmC provides the heme-Fe axial ligands (two His residues) in a CcmCD-heme-CcmE complex that contains oxidized heme (Fe^{3+}) (Richard-Fogal et al. 2009; Richard-Fogal and Kranz 2010). The catalytic mechanism behind the formation of this His-heme unusual covalent bond between CcmE and heme is not yet established (Lee et al. 2005; Kranz et al. 2009). How heme b is delivered from holoCcmE to apocyts c also remains unclear. Current models propose that ATP hydrolysis by CcmAB triggers the release of holoCcmE from the stable CcmCD-heme-CcmE complex (Feissner et al. 2006a). During this process, a Tyr residue at the heme-binding domain HXXXY of CcmE replaces the heme-Fe axial ligands provided by CcmC (Uchida et al. 2004) to render holoCcmE proficient for Ccm.

b. Module 2. CcmG and CcmH: Apocyt c Thioredox and Relay to Module 3

The thiol:disulfide oxidoreductases CcmG and CcmH are specific components of module 2 that work together with the DsbA-DsbB oxidative protein-folding pathway. CcmG and CcmH are responsible for maintaining the apocyt heme-binding site Cys thiol groups in the reduced state. CcmH is also a component of Module 3, as part of the heme ligation complex together with CcmF and CcmI (see below) (Fig. 27.4). Apocyts c have processed signal sequences, and are translocated to the periplasm through the Sec pathway where the Cys residues of their heme-binding motifs undergo oxidation by the DsbA-DsbB (Hamel et al. 2009; Kranz et al. 2009; Bonnard et al. 2010; Sanders et al. 2010). DsbA has a highly reactive thioredoxin-like (CXXC) domain and acts upon a wide variety of substrates (Shouldice et al. 2011). DsbB is an integral membrane protein with two thioredoxin-like motifs that reoxidizes DsbA by shuttling the reducing power to the membrane Q/QH_2 pool, and eventually to the electron transport chain (Inaba and Ito 2008). This disulfide bond formation pathway is critical for folding of several proteins, providing resistance to proteolysis, and inducing conformational changes required for their functions and regulations (Depuydt et al. 2011). The Cys residues at the apocyts c heme-binding site need to be reduced for covalent heme ligation hence CcmG and probably CcmH are critical for keeping the apocyts ligation-competent. CcmG is a single TM helix protein with a *bona fide* thioredoxin fold with a CXXC

motif. Its active site is unusually acidic and has a groove that might be required for its interaction with CcdA or its substrate(s) (Edeling et al. 2002; Stirnimann et al. 2005). CcdA has six TM helices and two redox-active Cys residues, reduces CcmG (Deshmukh et al. 2000; Fabianek et al. 2000; Katzen et al. 2002; Stirnimann et al. 2006), and is regenerated by the cytoplasmic thioredoxin TrxA (Deshmukh et al. 2000; Katzen et al. 2002; Stirnimann et al. 2005). In *E. coli*, DsbD (the homologue of CcdA) is a larger membrane protein containing three distinct domains, each with a pair of Cys residues (Stewart et al. 1999; Stirnimann et al. 2006). Besides their involvement in cyt *c* biogenesis, CcdA and DsbD are thiol:disulfide oxidoreductases that act on numerous other substrates (Stirnimann et al. 2006), and involved in the expression of virulence genes or sporulation in some organisms (Han and Wilson 2013). The Cys residues from the CXXC motif in both CcdA and CcmG are essential for the Ccm pathway (Monika et al. 1997; Fabianek et al. 1998; Turkarslan et al. 2008). Addition of exogenous thiol compounds can compensate for the Ccm deficiency in CcmG and CcdA mutants (Deshmukh et al. 2000; Bardischewsky and Friedrich 2001; Erlendsson and Hederstedt 2002; Turkarslan et al. 2008). Similarly, addition of oxidants (e.g., Cu^{2+} or diamide) was shown to improve production of cyts *c* in DsbA-DsbB mutants (Metheringham et al. 1995; Sambongi and Ferguson 1996; Erlendsson and Hederstedt 2002; Deshmukh et al. 2003; Turkarslan et al. 2008; Mavridou et al. 2012a). Interestingly, absence of DsbA or DsbB suppresses Ccm deficiency of CcdA-null (Erlendsson and Hederstedt 2002; Deshmukh et al. 2003) or CcmG-null mutants (Turkarslan et al. 2008), indicating that thio-reduction of the apocyts becomes unnecessary in the absence of their thio-oxidation (Turkarslan et al. 2008; Mavridou et al. 2012a). Besides being involved in thio-reduction, CcmG also participates in chaperoning the apocyts *c* (Turkarslan et al. 2008).

In *R. capsulatus*, CcmH has a single TM helix and a periplasmic domain with an unusual three helix-bundle fold with a conserved LRCXXCQ motif (Fig. 27.4) (Di Matteo et al. 2007; Ahuja et al. 2008; Zheng et al. 2012). *E. coli* CcmH is a fusion protein between this redox-active domain and a C-terminal extension homologous to CcmI (see below) as found in *R. capsulatus* and *P. aeruginosa* (Fabianek et al. 1999; Zheng et al. 2012). Both Cys residues of CcmH are important for Ccm but not essential under all conditions (Fabianek et al. 1999; Robertson et al. 2008). Remarkably, no thioredox compensation occurs in mutants lacking both DsbA and CcmH, suggesting an additional role for CcmH during Ccm (Turkarslan et al. 2008). Currently, the exact sequence of events during thio-reduction of apocyts *c* is not clear. Initially, a linear dithiol-disulfide cascade with CcmG reducing CcmH, followed by CcmH reducing the oxidized apocyts *c* substrates was proposed (Monika et al. 1997; Fabianek et al. 1999; Setterdahl et al. 2000; Reid et al. 2001; Schoepp-Cothenet et al. 2001; Meyer et al. 2005; Zheng et al. 2012). However, the unusual thioredoxin-fold and the redox properties of CcmH together with the fact that *P. aeruginosa* CcmG does not reduce in vitro the disulfide bond of CcmH but can reduce a 5-thio-2-nitrobenzoic acid (TNB)-CcmH mixed disulfide (Di Matteo et al. 2010), lead to alternative proposals (Verissimo and Daldal 2014). CcmH forms first an inter-molecular mixed-disulfide bond with apocyts *c* that is subsequently resolved by CcmG (Fabianek et al. 1999; Reid et al. 2001; Di Matteo et al. 2007, 2010). Establishing the exact sequence of thioredox reactions between CcmG, CcmH and the apocyts *c* is required for understanding the mechanism of heme ligation.

c. Module 3. CcmFHI: Apocyt *c* and Heme Ligation

Heme-apocyt *c* ligation *per se* is attributed to Module 3, which in *R. capsulatus* contains at least the CcmF, CcmH and CcmI forming a

membrane-integral complex (Sanders et al. 2008). In *E. coli* the CcmFH complex is a functional homologue of *R. capsulatus* CcmFHI (Ren et al. 2002). CcmI has two distinct domains with different functions (Ritz et al. 1993; Delgado et al. 1995; Lang et al. 1996; Cinege et al. 2004). The membrane embedded N-terminal CcmI-1 domain has two TM helices and a cytoplasmic loop with a leucine zipper-like motif. The CcmI-2 domain is a large periplasmic C-terminal extension decorated with three tetratricopeptide repeats (TPR) (Lang et al. 1996; Sanders et al. 2007; Verissimo et al. 2011), which are ubiquitous protein-protein interaction domains (D'Andrea and Regan 2003). In *R. capsulatus*, the CcmI-1 domain is required for the production of all cyts *c* whereas CcmI-2 is unnecessary for the C-terminally membrane-anchored cyt c_1 (Lang et al. 1996). It is now established that CcmI is an apocyt *c* chaperone that binds the C-terminal helix of apocyt c_2 mainly via its periplasmic CcmI-2 domain (Han et al. 2008; Verissimo et al. 2011; Di Silvio et al. 2013). In addition, genetic data indicate that the CcmI-2 domain and CcmG can functionally substitute for each other during Ccm (Deshmukh et al. 2002; Sanders et al. 2005, 2007). CcmF is a large integral membrane protein that contains heme *b* (Richard-Fogal et al. 2009). It belongs to the HHP family and has a periplasmic WWD motif like CcmC (Lee et al. 2007). There are four conserved His residues in CcmF: two of them ligate its heme *b* cofactor (San Francisco et al. 2011), and the remaining two coordinate heme *b* of holoCcmE to form a stable CcmF-heme-CcmE complex (Richard-Fogal et al. 2009; San Francisco and Kranz 2014) at least in the absence of CcmGH. CcmF can be reduced by QH_2 in vitro and is proposed to reduce the oxidized heme-iron (Fe^{3+}) in holoCcmE. However, mutations at the proposed QH_2 binding site in CcmF do not abolish cyt *c* production (Mavridou et al. 2013), leaving unknown how holoCcmE heme is reduced. Recent reports indicate that under optimized conditions CcmFH together with CcmG are sufficient for cyts

c production in *E. coli* (San Francisco et al. 2014). Although this is surprising because CcmABCDE are normally required for Ccm, it strengthens the proposal that *E. coli* CcmFH (or CcmFHI in other species) are responsible for heme ligation *per se* (San Francisco et al. 2014).

2. Do the Ccm Components form a Membrane-Integral Maturase Supercomplex?

The *E. coli* Ccm-System I components form a single operon, whose products were initially thought to co-localize in the inner membrane, forming a large "maturase" complex (Thony-Meyer 1997). However, in other organisms, these components are dispersed among multiple genomic loci (Beckman et al. 1992; Lang et al. 1996; Deshmukh et al. 2000). Regardless of the genomic distributions, the use of genetic strategies allowed accumulation and isolation of different intermediate complexes from dodecylmaltoside (DDM) solubilized membrane fractions. A CcmE-heme-apocyt *c* intermediate, with heme being covalently bound to both CcmE (via His) and apocyt *c* (via Cys), was isolated in the absence of CcmFHG (Mavridou et al. 2012b). In addition, apoCcmE devoid of heme was shown to interact with apocyt *c* in vitro, recognizing its heme-binding site (Verissimo et al. 2013). Similarly, CcmE is known to form a complex with CcmCD (Richard-Fogal et al. 2009; Richard-Fogal and Kranz 2010) and with CcmF in the presence of heme (San Francisco and Kranz 2014). *R. capsulatus* apoCcmE forms a ternary complex with apocyt c_2 and CcmI in vitro (Verissimo et al. 2013). Furthermore, using DDM-dispersed membrane fractions, apoCcmE was shown to interact directly with CcmI and CcmH (Verissimo et al. 2013). Additionally, CcmI, CcmH and CcmF co-purify with apocyt c_2, and CcmG with CcmI (Verissimo and Daldal unpublished), in agreement with the observation that CcmFHG are sufficient for cyts *c* production (San Francisco et al. 2014). Altogether, these findings suggest

that the heme ligation complex CcmFHI also contains CcmE and CcmG, and raise the possibility of the occurrence of a CcmABCD-CcmE-CcmFHI-CcmG super-complex (Ccm machine) that may catalyze the entire Ccm process in *R. capsulatus* (Verissimo and Daldal 2014) (Fig. 27.4). If so, then the single TM domain subunits like CcmD might be involved in the assembly, stabilization and regulation of this large supercomplex (Zickermann et al. 2010), by mediating tight associations between CcmABCDE and CcmFHIG complexes. In support of this proposal, co-localization of *R. capsulatus* heme ligation components CcmFHI was reported in high molecular weight (∼800 kDa) chromatography column fractions (Sanders et al. 2008). Similar large entities containing different Ccm-System I components were also observed in *A. thaliana* (∼500 kDa) (Meyer et al. 2005), and wheat (∼700 kDa) mitochondria (Giege et al. 2004).

Large supercomplexes composed of several proteins interacting weakly with each other are difficult to isolate, and often require specific lipid dispersion conditions using mild detergents (e.g., digitonine or amphipoles) to maintain them together (Krause 2006). The intermediate complexes cited above were isolated in the presence of the non-ionic detergent DDM, known to disrupt membrane supercomplexes (e.g., mitochondrial case) (Krause 2006). Thus, whether the Ccm components form alto-gether a highly stable membrane supercom-plex needs further studies. If a Ccm machine exists, then CcmE might act as a heme-shuttle interacting simultaneously with the heme handling (CcmABCD) and the heme ligation (CcmFHI) complexes (Fig. 27.4). The occurrence of such an apparatus may facilitate concerted availability of heme and apocyt *c* substrates, enhancing efficiency of the maturation process. This macromolecular organization is attractive for the maturation of multiheme cyts *c*, where several hemes have to be ligated successively. In this case, CcmI may keep the apocyts *c* trapped in the Ccm machine while the other Ccm

components ensure stereo-specific ligation and axial coordination of heme cofactors.

In the light of the new findings and the possible occurrence of a Ccm machine, we refined our heme ligation model (Sanders et al. 2010; Verissimo and Daldal 2014). In the absence of heme, apoCcmE may be associated more closely with CcmI-CcmH than CcmCD. Once an apocyt *c* is available, CcmI (which is together with CcmFH) traps its C-terminus while apoCcmE recognizes its heme-binding site. To ensure stereo-specific heme ligation, a mixed disulfide bond be-tween the apocyt *c* Cys_1 and oxidized CcmH is formed, leaving Cys_2 available. When heme *b* reaches the periplasm it is oxidized (Fe^{3+}), and binds covalently (through its vinyl-2) to the His residue of CcmE to form a CcmE-heme-CcmCD complex. CcmC then provides a heme platform via its WWD domain and its His residues axially ligate heme-iron. Following ATP hydrolysis by CcmAB, the occurrence of a conformational change in CcmC was suggested to render holoCcmE competent for interaction with other heme ligation components. CcmE then forms a CcmE-heme-CcmF complex, with CcmF providing the heme scaffold and the His axial ligands. Upon reduction of the heme-iron (Fe^{2+}) (possibly via the heme cofactor of CcmF) the heme *b* vinyl-4 forms the first thioether bond with the reduced Cys_2 thiol of apocyt *c*. Next, CcmG (or possibly the free Cys of CcmH) resolves the mixed disulfide between CcmH and apocyt *c* Cys_1, rendering it able to interact with heme *b*. This leads to the formation of the second thioether bond between vinyl-2 and apocyt Cys_1 and release of heme from holoCcmE. We note that at present many of the steps of this model, and their chronological orders are hypothetical, and multiple alternative possi-bilities are plausible. Yet, the in vivo trapped CcmCD-heme-CcmE (Richard-Fogal et al. 2009), CcmE-heme-apocyt *c* (Mavridou et al. 2012b) and CcmF-heme-CcmE (San Francisco and Kranz 2014) intermediates, as well as the in vitro observed CcmI-apocyt c_2–apoCcmE (Verissimo et al. 2013), CcmHI-apoCcmE   (Verissimo et al. 2013)

complexes are consistent with this working model which undoubtedly will be elucidated by future studies.

E. Assembly of cbb₃-Cox

In *R. capsulatus* membranes separated by BN-PAGE, cbb_3-Cox is visualized as an active complex of ∼230 kDa, which contains all four structural subunits (CcoNOQP) (Kulajta et al. 2006) (Fig. 27.5). The putative assembly factor CcoH, encoded by the *ccoGHIS* cluster, is also associated with this complex, suggesting that it may be a more permanent component of cbb_3-Cox (Pawlik et al. 2010). Using similar BN-PAGE analyses, two inactive assembly intermediates of ∼210 kDa and <70 kDa were also detected in *R. capsulatus* membranes. The ∼210 kDa complex contains CcoN, CcoO and CcoH, but lacks CcoP, whereas the <70 kDa complex contains CcoP, CcoQ and CcoH (Kulajta et al. 2006; Pawlik et al. 2010). Although not essential for activity, CcoQ is required for full activity of cbb_3-Cox enzyme, and chemical cross-linking

data show that CcoQ and CcoP interact with each other (Peters et al. 2008). Available data suggest that assembling together the CcoNOH and CcoQPH subcomplexes, possibly via CcoH dimerization, might yield the active cbb_3-Cox (Fig. 27.5) (Pawlik et al. 2010).

CcoO found in the 210 kDa, and CcoP in the <70 kDa complexes contain their covalently attached heme groups as indicated by their peroxidase activity (Kulajta et al. 2006), suggesting that cyt *c* maturation occurs prior to their assembly into cbb_3-Cox. Recent findings indicate that a defective Ccm-System I does not abolish membrane insertion and stability of CcoN (Ekici et al. 2013), although whether this subunit contains its cofactors is not known. Thus, maturation of CcoO and CcoP through the Ccm machinery and that of CcoN via its dedicated components (e.g., CcoA, CcoI, SenC, Pcc_A and CcoS) are two independent events that are coordinated temporally and spatially for successful production of an active cbb_3-Cox.

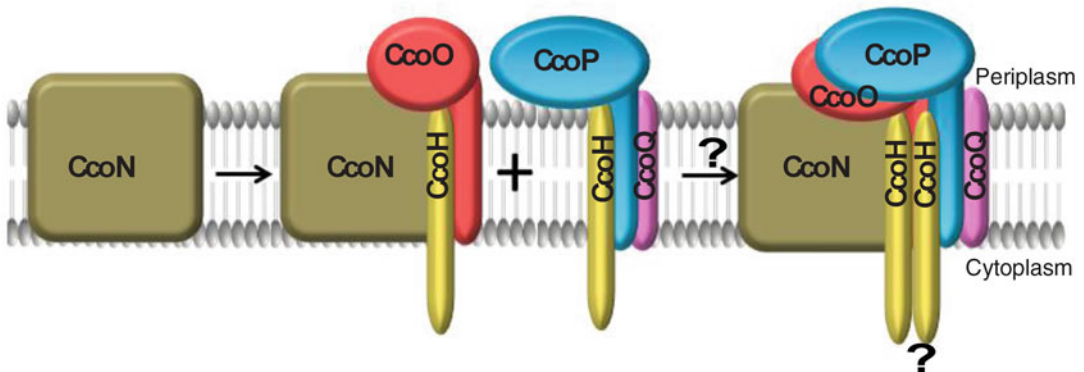The unexpected finding that CcoH is associated with the assembly intermediates of

Fig. 27.5. Assembly model of cbb_3-Cox in *R. capsulatus*. Insertion of CcoN into the membrane is independent of the maturation of the other cbb_3-Cox subunits, but when heme *b*, heme b_3 and Cu_B are incorporated is unknown. The assembly of an active cbb_3-Cox enzyme (the 230 kDa active complex revealed by BN-PAGE) proceeds via two inactive subcomplex intermediates. The first subcomplex contains CcoN, CcoO and CcoH (the 210 kDa inactive complex revealed by BN-PAGE) while the second subcomplex contains CcoP, CcoQ and CcoH (the 70 kDa inactive complex revealed by BN-PAGE). Considering that CcoH is associated with both subcomplex intermediates the assembly of cbb_3-Cox is thought to occur via a fusion between CcoNOH and CcoQPH possibly facilitated by CcoH. Whether CcoH is associated to cbb_3-Cox as a dimer is not known. The cyt *c* CcoO and CcoP subunits present in the subcomplex intermediates contain their hemes *c*, further indicating that their maturation occurs prior to their assembly to cbb_3-Cox.

cbb_3-Cox suggests that this protein behaves like a *bona fide* subunit rather than an assembly factor of *R. capsulatus* enzyme. This notion is also supported by the observation that CcoH stability depends on the presence of cbb_3-Cox. Interestingly, CcoH of *R. capsulatus* is a homologue of yeast (Rcf1 and Rcf2) and mammalian (HIG2A) putative supercomplex assembly factors (Strogolova et al. 2012). Yeast Rcf1 binds to the Cox13 subunit of mitochondrial aa_3-Cox as a late assembly factor and affects its dimerization. Yeast cells lacking Rcf1 have reduced amounts of supercomplexes formed by the associations of cyt bc_1 and aa_3-Cox and produce ROS (Vukotic et al. 2012). Similarly, depletion of HIG2A (a mammalian homologue of CcoH) alters supercomplex organization in mouse myoblast cells (Chen et al. 2012). The 3D reconstitutions of bovine supercomplexes suggest that these CcoH homologues might fill up a gap between cyt bc_1 and aa_3-Cox, as seen by cryo-electron microscopy and electron tomography techniques (Dudkina et al. 2011). Whether CcoH affects supramolecular organizations of cyt bc_1 and cbb_3-Cox in *R. capsulatus* membranes is an attractive possibility that deserves future studies. Identification of putative respiratory supercomplexes and their regulation in response to changing physiological conditions is clearly important for our complete understanding of energy transduction pathways.

Acknowledgments

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Assembly of Transmembrane *b*-Type Cytochromes and Cytochrome Complexes

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Summary

Cytochromes are involved in charge-transfer reactions, and many cytochromes contain a transmembrane domain and are part of membrane-localized electron transfer chains. Protoporphyrin IX (heme *b*) is the first heme product in the tetrapyrrole/heme biosynthesis

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pathway. In contrast to *c*-type cytochromes, there is no need for a specialized machinery catalyzing covalent attachment of the heme molecule to a *b*-type apo-cytochrome, nor is the cofactor further modified, as in *a*-, *d*- and *o*-type cytochromes. Thus, formation of a holo-cytochrome is relatively simple for *b*-type cytochromes, and this class of proteins probably represents the most ancient members of transmembrane cytochromes. However, assembly of individual transmembrane *b*-type cytochromes as well as of larger cytochrome complexes involves multiple steps, which have to be tightly controlled and aligned: the apo-protein as well as the heme cofactor needs to be synthesized, targeted to, and integrated into a membrane prior to holo-cytochrome formation. Spontaneous folding and assembly of individual transmembrane *b*-type cytochromes involves folding of the polypeptide chain and formation of a heme-binding cavity, which allows specific and tight binding of the cofactor. Additional biogenesis steps are eventually required for maturation of transmembrane *b*-type cytochrome complexes.

I. Introduction

Heme proteins are a class of soluble or membrane-bound proteins that carry one or multiple heme cofactors (Reedy and Gibney 2004). They are involved in binding and transport of small molecules, such as oxygen or nitric oxide, as well as in catalytic reactions, such as in case of oxygenases, catalases or peroxidases. When heme proteins are involved in charge-transfer reactions, the proteins are called cytochromes, a term introduced almost 100 years ago by Keilin (1925). However, it is noteworthy that some proteins, such as cytochrome monooxygenases of the cytochrome P-450 type, have obtained their name by accident, and these proteins are not involved in electron transfer reactions (Abramson et al. 2000; (NC-IUB) (1992)). Many cytochromes contain a transmembrane (TM) domain and are part of membrane-localized electron transfer chains (Koch and Schneider 2007; Thöny-Meyer 1997). Here, the individual cytochromes are typically

subunits of larger multi-subunit TM protein complexes.

Assembly of TM cytochromes involves multiple steps, which have to be tightly controlled and aligned (Fig. 28.1). The apo-protein as well as the heme cofactors need to be synthesized, targeted to, and integrated into a membrane prior to holo-cytochrome formation. Furthermore, as holo-cytochromes are typically part of larger cytochrome complexes, additional biogenesis steps are eventually required for their maturation.

Tetrapyrroles, such as hemes and chlorophylls, are synthesized by two common pathways. The initial precursor 5-aminolevulinic acid (ALA) is built from glycine and succinyl-CoA in animals, fungi, yeast and some bacteria, whereas plants, algae and most bacteria synthesize ALA from glutamate (Mochizuki et al. 2010). Eight ALA molecules assemble to the tetrapyrrole uroporphyrinogen III, which is subsequently converted to protoporphyrin IX. Incorporation of iron, catalyzed by the ferrochelatase, results in formation of Fe-protoporphyrin IX (heme *b*), whereas incorporation of magnesium by the magnesium-chelatase results in formation of Mg-protoporphyrin IX, which in plants is subsequently converted in multiple steps into chlorophyll (Mochizuki et al. 2010). In animals and fungi, the steps of heme biosynthesis, starting from ALA,

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Abbreviations: b_{559} – *b*-type heme with a reduced α-band peak at 559 nm; GET – Guided entry of tail-anchored proteins; RNC – Ribosome-nascent-chain complex; SRP – Signal recognition particle; SR – SRP-receptor; TM – Transmembrane; TIM – Translocase of the inner mitochondrial membrane; TOM – Translocase of the outer mitochondrial membrane
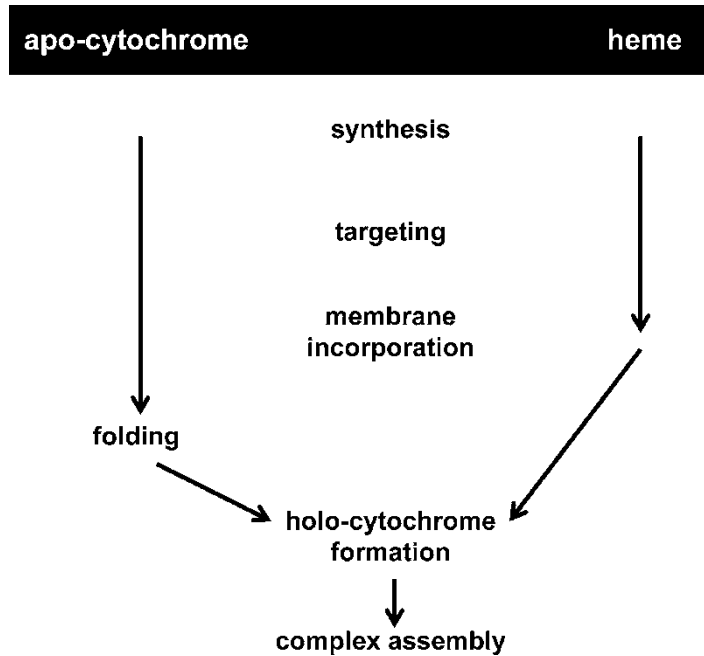
Fig. 28.1. Multiple-step assembly of TM cytochromes and cytochrome complexes. Prior to the formation of a TM holo-cytochrome, the apo-protein as well as the heme cofactors need to be synthesized, targeted to, and integrated into a membrane. When holo-cytochromes are part of larger cytochrome complexes, additional biogenesis steps are required for their maturation. All steps need to be tightly controlled and aligned.

are localized within the cytoplasm, whereas the later steps of the heme biosynthesis are localized within mitochondria. In plants, heme biosynthesis takes place almost exclusively in plastids and only the final steps are possibly localized to both mitochondria and plastids (Mochizuki et al. 2010; Tanaka and Tanaka 2007).

Due to its hydrophobicity, free heme will intercalate into biological membranes, potentially leading to membrane rupture. Heme iron might also generate reactive oxygen species and free heme exhibits peroxidase activity. Thus, the cellular accumulation of free heme is highly unfavorable for cells and newly synthesized heme is immediately complexed by heme-binding proteins. However, the contribution of these proteins to heme transport and assembly of TM b-type cytochromes, is presently elusive (Hamza and Dailey 2012).

In all heme species, the central heme iron ion is ligated by four N-atoms of the heme tetrapyrrole ring system. In most cases the iron center is additionally ligated via two histidine side-chains of a polypeptide chain, which serve as fifth and sixth ligands. However, the side-chains of Met, Cys and Tyr can also act as sixth ligand (Smith et al. 2010). When involved in e^--transfer reactions, the redox state of the central heme iron switches between Fe^{3+} (ferri-heme) and Fe^{2+} (ferro-heme), allowing reversible reduction and oxidation.

Five major cytochrome classes can be differentiated (cytochrome *a, b, c, d, o*), and these differ in the nature of the bound heme (derivative) and/or in the mode of heme binding (Koch and Schneider 2007; Thöny-Meyer 1997). The structures of the heme cofactors bound in the respective cytochrome groups are shown in Fig. 28.2. Protoporphyrin IX (heme *b*) is the first heme product in the tetrapyrrole/heme biosynthesis pathway, and protoporphyrin IX derivatives are subsequently generated by heme *b* ring modifications. While in both *b*- and *c*-type cytochromes, protoporphyrin IX is bound
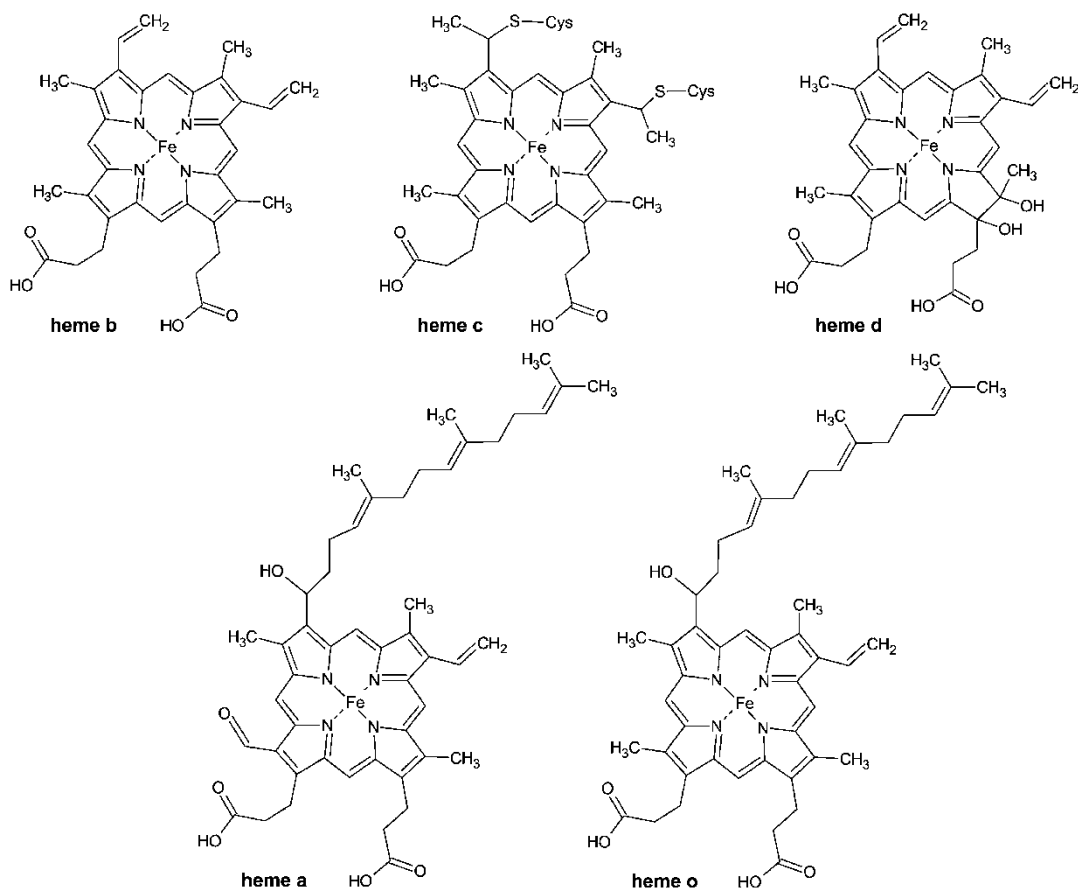
Fig. 28.2. Chemical Structures of naturally occurring hemes *b*, *c*, *d*, *a*, *o*. All hemes are derived from protoporphyrin IX (heme *b*) and thus have the same basic structure. The central iron is Fe^{2+} in the reduced and Fe^{3+} in the oxidized state. While most hemes are non-covalently bound by proteins, in *c*-type cytochromes heme *c* is bound covalently to a protein, typically via two thioether bounds to cysteine (Cys) residues.

to the protein, *c*-type cytochromes have the heme covalently attached (typically via two thioether-linkages), whereas in *b*-type cytochromes the heme is non-covalently bound and bound only by two axial histidine residues. Similarly, in *a*-, *d*- and *o*-type cytochromes the heme molecules are non-covalently attached to proteins. In contrast to *c*-type cytochromes, there is no need for a specialized machinery catalyzing covalent attachment of the heme molecule to a *b*-type apo-cytochrome, nor is the cofactor further modified, as in *a*-, *d*- and *o*-type cytochromes. Thus, formation of a holo-cytochrome is relatively simple for *b*-type cytochromes, and they probably represent the

most ancient members of TM cytochromes. However, during evolution, some proteins have changed their heme cofactors. This is illustrated by the terminal cytochrome *c*-oxidases, which contain a variety of different heme species associated with subunit I. So far, *aa*₃-, *ba*₃-, *cbb*₃- or *bb*₃-type cytochrome oxidases have been characterized (Garcia-Horsman et al. 1994; Thöny-Meyer 1997). The *cbb*₃-type cytochrome c oxidase contains two heme *b* cofactors and closely resembles the NO-reductases (Ekici et al. 2012). This led to the hypothesis that subunit I of cytochrome oxidases evolved from subunit I of the NO-reductase and that the *b*-type heme-containing oxidases appear to

be the primordial enzyme (Pereira et al. 2001; Saraste and Castresana 1994).

II. Transmembrane *b*-Type Cytochromes in Respiratory and Photosynthetic e⁻-Transfer Chains

Several cofactor-containing TM protein complexes are involved in respiratory and photosynthetic electron transfer reactions. Cofactors involved in e⁻-transfer include not only hemes but also iron-sulfur clusters and other metal centers, such as Fe, Mo, Cu or Ni. However, utilizing membrane-integrated cytochromes, and in particular *b*-type cytochromes, has evolved as an efficient principle to catalyze charge transfer across membranes. This is reflected by the fact that despite the great diversity of e⁻-transfer chains, TM *b*-type cytochromes are key subunits of most multi-subunit TM complexes involved in aerobic and anaerobic respiratory electron transfer chains in eu- and prokaryotes. They are present in dehydrogenases, such as succinate or formate dehydrogenases, as well as in cytochrome bc_1 and b_6f complexes, which transfer electrons from a quinol to the soluble e⁻-carrier cytochrome c_6 or to plastocyanin. Furthermore, the bacterial quinol as well as cytochrome c oxidases mostly contain TM *b*-type cytochrome subunits. In addition, also photosystem II of cyanobacteria and higher plants contains a conserved TM *b*-type cytochrome subunit (cytochrome b_{559}).

Despite the wealth of structural, biophysical and biochemical data on TM cytochromes, surprisingly little is known about their biogenesis. In case of *c*-type cytochromes, four defined systems have been identified and described, which are involved in covalent attachment of the heme cofactor to the protein moiety, and thus in the conversion of an apo- to a holo-cytochrome (Ferguson et al. 2008). In contrast, no specialized biogenesis system and/or heme chaperone system for delivery of heme to *b*-type cytochromes and/or for biogenesis of TM b-type cytochromes has been identified so far. Instead, it appears to be likely that *b*-type cytochromes assemble spontaneously from the apo-protein and free heme, as further discussed below.

III. Targeting and Membrane Insertion of *b*-Type Apo-Cytochromes

b-type cytochromes execute their functions in different membrane environments, and correct targeting and membrane insertion is crucial for their function in electron transport processes. Prokaryotic and eukaryotic cells engage multiple targeting and insertion pathways to ensure the correct cellular localization of membrane-bound *b*-type cytochromes (Kudva et al. 2013). In this chapter we will mainly focus on their insertion into the bacterial cytoplasmic membrane and only briefly outline other membrane systems (Fig. 28.3).

A. Co-Translational Membrane Targeting by the Signal Recognition Particle (SRP) Pathway

Lipid-anchoring of membrane proteins, such as *b*-type cytochromes, is usually achieved via a single or via multiple hydrophobic TM α-helices. These α-helices are not only involved in membrane insertion, but the N-terminal helix usually also serves as a recognition signal during the targeting process and is hence referred to as signal anchor sequence (SA) (Kudva et al. 2013). Upon synthesis on cytosolic ribosomes, hydrophobic α-helices tend to aggregate in the aqueous cytosolic environment and this would impair subsequent targeting and insertion. Co-translational protein targeting overcomes this problem by coupling protein synthesis with protein targeting. Co-translational membrane targeting is facilitated by the signal recognition particle (SRP) and its membrane-bound receptor (SRP-receptor,
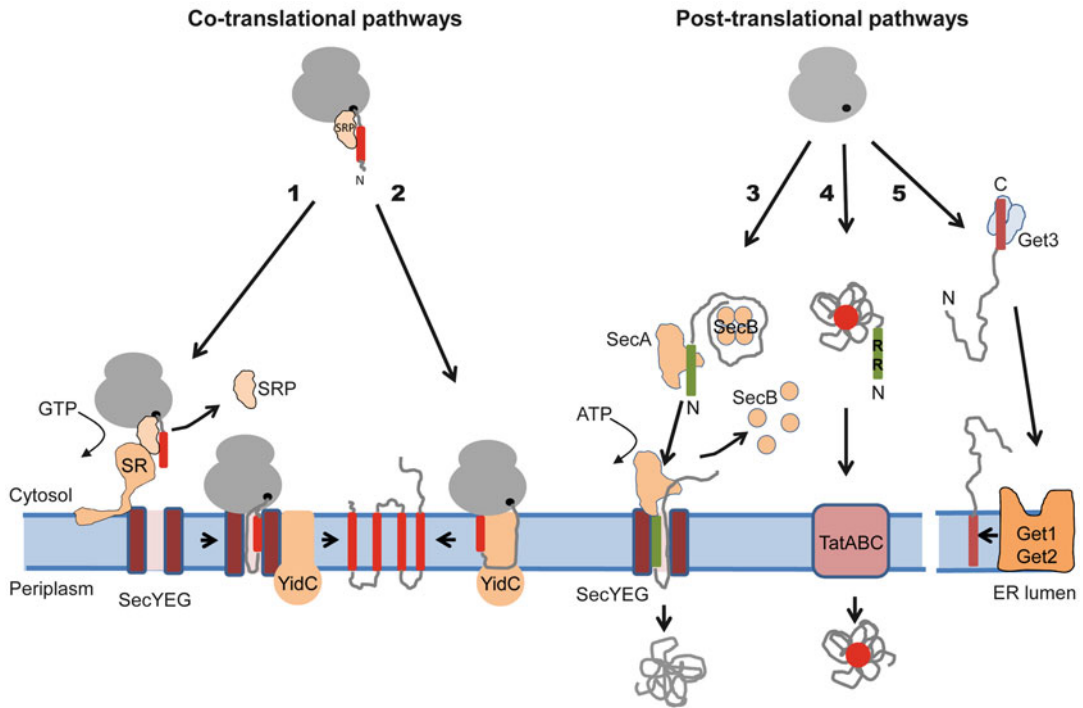
Fig. 28.3. Co- and post-translational protein-targeting pathways. Co-translational protein transport is initiated by binding of the signal recognition particle (SRP) to a signal anchor sequence (*red*) emerging from the ribosomal tunnel: (1) SRP then delivers the SRP-ribosome nascent chain complex (SRP-RNC) to the membrane-bound SRP receptor (SR) and the RNC is transferred to the SecYEG translocon. GTP hydrolysis by SRP and SR induce their dissociation allowing another round of targeting. The SecYEG translocon cooperates with YidC during membrane protein insertion and YidC is thought to facilitate folding and assembly processes. (2) YidC can also function independently of the SecYEG translocon and insert membrane proteins on its own. Targeting of most proteins to YidC also appears to be SRP-dependent. (3) The best studied example of a post-translational protein-targeting pathway is the bacterial SecA-dependent transport. Secretory proteins, i.e. periplasmic and outer membrane proteins, are recognized by the tetrameric chaperone SecB after their release from the ribosome. SecA binds to the signal sequence (*green*) and targets the protein to the SecYEG translocon. SecB is released and SecA translocates the protein in ATP-dependent steps across the SecYEG channel. The signal sequence is cleaved off by a signal peptidase and further degraded. Sec-independent post-translational protein transport pathways also exist in prokaryotes and eukaryotes. (4) The twin-arginine translocation (Tat) is able to transport cofactor-containing folded proteins across the bacterial cytoplasmic membrane or the chloroplast thylakoid membrane. The TatBC proteins serve as a signal sequence receptor, recognizing the canonical RR-motif. TatA oligomers are probably required for TM translocation, but the exact mechanism of transport is still unknown. (5) Proteins containing a C-terminal TM domain (tail-anchored (TA) proteins) are inserted into the endoplasmic reticulum (ER) membrane by the GET pathway. The homo-dimeric ATPase GET3 functions as an ER targeting factor. The minimal membrane insertion machinery for TA proteins in yeast appears to be provided by the ER-integral GET1 and GET2 proteins. The insertion mode of bacterial TA proteins is largely unknown, but might involve YidC (Aschtgen et al. 2012).

SR) (Koch et al. 1999). The SRP pathway is the only protein-targeting pathway that is universally conserved and essential in almost all domains of life. Notable known exceptions are *Saccharomyces cerevisiae* and the Gram-positive bacterium *Streptococcus*

mutans, which tolerate SRP inactivation (Koch et al. 2003a, 2013).

SRP is a ribonucleoprotein complex that consists of 6 protein subunits in mammals, which are bound to the 300-nucleotide-long 7S RNA. The bacterial SRP is reduced

to a functional core comprising the signal sequence binding subunit Ffh (homologous to the eukaryotic SRP54 subunit) and the 4.5S SRP-RNA, which in *E. coli* is 114 nucleotides long. This minimal core is present in all organisms, with the intriguing exception of higher plant chloroplasts, where the SRP lacks the RNA subunit (Trager et al. 2012).

Ffh/SRP54 and the bacterial SRP receptor FtsY belong to the SIMIBI family (**Si**gnal recognition particle – **Mi**nD – **Bi**oD) of nucleotide-hydrolyzing enzymes (Grudnik et al. 2009) and display a modular organization (Fig. 28.4). Both, Ffh/SRP54 and FtsY, contain a highly similar four-helix bundle domain (N-Domain) that is connected to a Ras-like GTPase domain (G-domain). In contrast to classical GTP-hydrolyzing enzymes, such as Ras, EF-Tu or Ran, neither Ffh nor FtsY require external guanine nucleotide exchange factors (GEFs) or GTPase-activating enzymes (GAPS). Instead, the GTPase activities of Ffh and FtsY are stimulated upon Ffh-FtsY heterodimer formation, which occurs via their respective NG-domains (Bange and Sinning 2013). Both, Ffh and FtsY, contain an additional functional domain that is fused to their respective NG domains;
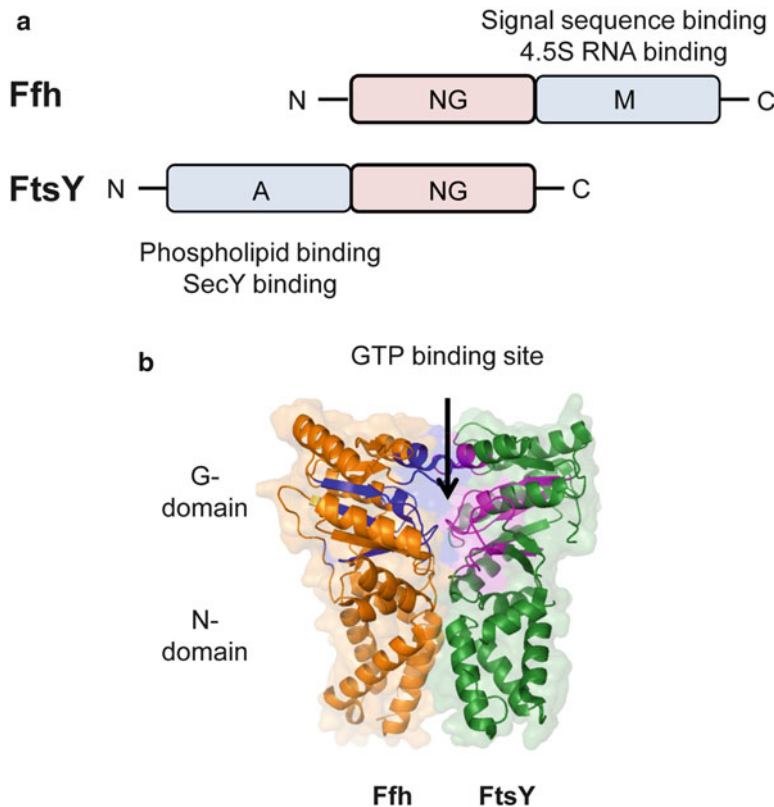
Fig. 28.4. Domain structure of the bacterial Signal Recognition Particle pathway (SRP) components. (**a**) Ffh is the protein component of the bacterial SRP and forms together with the 4.5S RNA the bacterial SRP. The conserved NG-domain is required for GTP hydrolysis and regulation, while the C-terminal M-domain harbors the signal sequence-binding site and the contact site for the 4.5S RNA. The NG-domain of FtsY is very similar to the NG domain of Ffh. The N-terminal A-domain contains in *E. coli* binding sites for SecY and for negatively charged phospholipids. The A-domain exhibits only low sequence conservation and has a variable size in different bacterial species (Kudva et al. 2013; Weiche et al. 2008). (**b**) The heterodimer of the NG-domains of Ffh and FtsY from *T. aquaticus* (PDB: 1OKK). The structure shows a large common surface area, which also includes a composite GTPase site.

the M-domain in Ffh and the A-domain in FtsY (Kuhn et al. 2014). The C-terminal M-domain in Ffh is the signal sequence binding region, which is characterized by an over-representation of methionine residues in mesophilic organisms. This flexible methionine-rich domain probably allows the accommodation of a large variety of signal anchor sequences. The N-terminal A-domain of FtsY is involved in membrane binding and contains two amphipathic lipid-binding helices with high affinity to anionic phospholipids, such as phosphatidylglycerol and cardiolipin (Braig et al. 2009; Weiche et al. 2008). The A-domain varies significantly in length and amino acid composition between different bacterial species (Weiche et al. 2008), and only the second lipid-binding helix, located close to the NG-domain, appears to be essential for function (Braig et al. 2011; Eitan and Bibi 2004; Parlitz et al. 2007). Lipid binding of FtsY triggers a conformational switch that strongly enhances its subsequent interaction with SRP (Braig et al. 2009, 2011; Klostermann et al. 2002; Lam et al. 2010; Stjepanovic et al. 2011).

SRP binds to ribosomes with high affinity ($K_d \cong$ 50–70 nM) (Bornemann et al. 2008). Its binding site is located close to the ribosomal tunnel exit, which allows SRP to scan emerging polypeptides for signal anchor sequences and to initiate membrane targeting of the translating ribosome (ribosome-nascent-chain complex (RNC)) (Gu et al. 2003). If the emerging protein does not contain a suitable signal sequence, the affinity of SRP for ribosomes drops significantly (>200 nM). This probably explains why a rather small number of SRP molecules (approx. 200–300 in *E. coli*) can scan a vast number of ribosomes (approx. 20,000–30,000 in *E. coli*). Although SRP binding to emerging polypeptides is mainly determined by their hydrophobicity, other factors, such as the presence of helix-breaking amino acids (Beha et al. 2003) or the translation speed, also influence SRP binding (Zhang and Shan 2012).

After binding to the polypeptide, SRP targets RNCs to the membrane-bound FtsY.

FtsY not only binds to phospholipids, but also directly to the surface-exposed loops of the membrane-bound SecYEG translocon (Angelini et al. 2006, 2005; Kuhn et al. 2011), the main protein transport channel in bacterial membranes (Kudva et al. 2013). Thus, FtsY recruits the SRP-RNC complex directly to the protein transport channel and is probably displaced by the SRP-RNC, because FtsY and ribosomes use partially overlapping binding sites on SecY (Kuhn et al. 2011). GTP hydrolysis by FtsY and SRP then induces their complete dissociation and allows for the next round of targeting. FtsY was also found to contact YidC, which can constitute a SecYEG-independent insertion site for some membrane proteins (Welte et al. 2012).

B. The Integration Channels: The SecYEG Translocon and the YidC Integrase

In *E. coli* and probably most bacteria, SRP-dependent membrane proteins are preferentially targeted to the SecYEG translocon, which is homologous to the eukaryotic Sec61 complex in the endoplasmic reticulum (ER) membrane (Kudva et al. 2013; Zimmermann et al. 2011). The SecYEG translocon is a trimeric membrane protein complex that consists of the three integral membrane proteins SecY, SecE and SecG (du Plessis et al. 2011). Additional subunits associate at least transiently with the SecYEG/Sec61 core complexes and are required for substrate folding and processing (Fig. 28.5).

The protein conducting channel of the SecYEG translocon is formed by the SecY subunit, an hour-glass-like structure with a hydrophobic constriction in the center that is thought to seal the channel during protein transport (Van den Berg et al. 2004). In its inactive state, the channel is further blocked by a short helix on the periplasmic side of the constriction, which is believed to serve as a plug (Li et al. 2007). The SecYEG translocon is activated when RNCs bind to surface-exposed SecY loops (Park et al. 2013). The signal anchor sequence is threaded into the
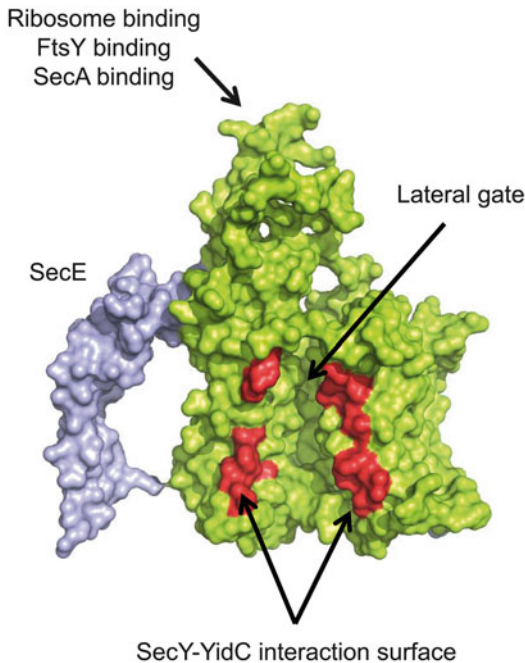
Fig. 28.5. The SecYE translocon in *E. coli*. SecY and SecE form the core components of the bacterial Sec translocon and are essential for function. The structure is adapted from Frauenfeld et al. (2011, pdb:3J01). SecE is shown in *blue* and SecY in *green*. The cytosolically exposed loops of SecY provide overlapping binding sites for the ribosome, the SRP receptor FtsY and the ATPase SecA. Transmembrane domains are thought to exit the SecYE translocon via the lateral gate. Residues of the lateral gate helices that were shown to be in contact with YidC are shown in *red*.

channel, where it contacts TM helices 2 and 7 of SecY, relocating the plug slightly to the periphery of the channel. This probably results in the opening of a lateral gate within SecY that is formed by TM helices 2 and 3 on one side and TM helices 7 and 8 on the other side (Fig. 28.5) (Park et al. 2013). Opening of the SecY channel laterally towards the membrane probably allows membrane proteins to exit the channel and to partition into the lipid phase (du Plessis et al. 2009; Hizlan et al. 2012). The essential SecE subunit of the SecYEG translocon is located opposite to the lateral gate at the back of the SecY channel (Van den Berg et al. 2004). SecE probably stabilizes SecY, because in its absence, SecY is rapidly degraded by the protease FtsH

(Kihara et al. 1995). SecG, the third subunit of the eubacterial SecYEG translocon is not essential for protein transport. It also shows no sequence homology to Sec61β, the third subunit of the archaeal and eukaryotic Sec translocon (Park and Rapoport 2012). Available data indicate that SecG is required for SecA-dependent post-translational transport across the SecYEG channel (see below). This is consistent with the observation that both, SecA and SecG, are exclusively found in bacteria (Kudva et al. 2013).

TM helices exiting the SecY channel via the lateral gate have been shown to contact YidC before they encounter lipids. YidC and its homologues in mitochondria (Oxa1 & Oxa2) and chloroplasts (Alb3 & Alb4) are multi-spanning membrane proteins, containing a conserved core of five TM helices (Funes et al. 2011). In most Gram-negative bacteria, the N-terminus of the conserved core is connected via a large periplasmic loop to an additional TM helix (Saaf et al. 1998). It is believed that YidC assists in the transfer of TM helices from the SecY channel to lipids (Beck et al. 2001). In addition, YidC also facilitates the folding and assembly of membrane proteins (Chen et al. 2002). As YidC is located in immediate proximity to the lateral gate of SecY, it is perfectly positioned to interact with TM helices emerging from the SecY translocon (Sachelaru et al. 2013). Eukaryotic YidC homologues are absent in the ER, but instead located in the chloroplast thylakoid and inner mitochondrial membrane. A SecY-Alb3 interaction was shown in chloroplasts (Klostermann et al. 2002), but does not occur in mitochondria, since the inner mitochondrial membrane usually lacks a Sec machinery (Becker et al. 2012).

YidC not only functions in concert with SecYEG but it can also insert membrane proteins independently of SecYEG (Fig. 28.3). The SecYEG-independent insertion function of YidC was initially observed for small phage proteins (Samuelson et al. 2000), but later it was demonstrated that YidC can also insert small native membrane proteins, such as the c-subunit of the F_1F_0 ATPase (Yi et al. 2003) and the mechano-sensitive

channel of large conductance MscL in *E. coli* (Facey et al. 2007). Further in vitro studies revealed that YidC is also able to insert multi-spanning membrane proteins that were previously considered to be exclusively SecYEG-dependent (Welte et al. 2012). It remains to be determined how YidC inserts single- and multi-spanning membrane proteins. Electrophysiological measurements indicate the presence of a cation-selective channel in the mitochondrial Oxa1, but whether YidC also forms a protein conducting channel is currently unknown. Targeting of most substrates to YidC is co-translational and mediated by the SRP pathway. Some Gram-positive bacteria, such as *Streptococcus mutans*, express YidC derivatives with an extended C-terminus, which serves as a ribosome-binding site (Palmer et al. 2012). In these bacteria sufficient membrane protein targeting occurs even when the SRP pathway is inactivated. This probably explains why *S. mutans* SRP mutants are viable.

It is currently unknown why bacteria contain two integration sites for membrane proteins and to which extent they are engaged in vivo. In *E. coli*, YidC is present in approx. 5-fold excess over SecYEG (Kudva et al. 2013). In addition, the limited number of SecYEG translocons is also required for the post-translational translocation of periplasmic and outer membrane proteins (see below; Fig. 28.3). This could suggest that YidC is the preferred integration site. However, YidC is unable to insert membrane proteins with large periplasmic loops (Welte et al. 2012). Membrane proteins, which harbor periplasmic loops of more than approx. 30 amino acids, are still targeted to the membrane by the SRP pathway, but the translocation of the periplasmic loop requires the SecA motor protein ATPase (Deitermann et al. 2005). SecA can interact with SecYEG but probably not with YidC (Kuhn et al. 2011; Welte et al. 2012). Thus, membrane proteins which harbor large periplasmic domains are probably strictly dependent on SecYEG for insertion, while closely spaced membrane proteins can probably be inserted by either SecYEG or YidC.

C. The Insertion Mode of Membrane-Bound b-Type Cytochromes

The exact insertion mode has been experimentally verified only for a few membrane-bound *b*-type cytochromes and in most cases it can only be predicted based on the topological features of *b*-type cytochromes. In *E. coli*, the heme *b*-containing subunits CyoB of the cytochrome bo_3 oxidase and HybB of the Ni/Fe hydrogenase 2 both contain only small periplasmic loops and can probably be inserted into the *E. coli* membrane by either, SecYEG or YidC. On the other hand, larger periplasmic loops are present in the CydA and CydB subunits of cytochrome *bd* oxidase (146 amino acids between TM helices 6 and 7; 51 amino acids between TM helices 1 and 2, respectively) (Borisov et al. 2011) or in cytochrome b_{561} (36 amino acids between TM3 and TM4), which would probably render both proteins SecYEG-dependent. In addition to the size of periplasmic loops, the charge composition of a membrane protein also influences whether a protein is able to insert via SecYEG or YidC (Price and Driessen 2010; Zhu et al. 2013). A situation, thus far unique, is observed for the CyoA subunit of cytochrome bo_3 oxidase. The N-terminus of CyoA is inserted via YidC, while the large periplasmic loop is translocated via SecYEG (du Plessis et al. 2006). This could indicate that when YidC encounters large periplasmic domains during insertion, SecYEG is recruited for further translocation. However, this needs to be experimentally verified for additional substrates.

The mitochondrial YidC homologue Oxa1 is believed to provide the main integration site for the strongly hydrophobic subunits of the cytochrome bc_1 complex, cytochrome *c* oxidase, and F_1F_o ATP synthase into the mitochondrial membrane (Hell et al. 2001). These proteins are encoded by the mitochondrial genome and assemble with

less hydrophobic nuclear encoded subunits, which are post-translationally imported into mitochondria via the TOM-TIM machinery (Becker et al. 2012). Oxa1 consists of an extended C-terminus that serves as a high affinity binding site for ribosomes. This extended C-terminus causes targeting to Oxa1 SRP-independent, in line with the lack of an SRP-dependent targeting system in mitochondria (Funes et al. 2013; Ott and Herrmann 2010). Importantly, bacterial Ffh can partially rescue the co-translational insertion defect in Oxa1 mutants lacking the ribosome-binding site (Funes et al. 2013). Ribosome binding to the mitochondrial membrane involves at least two additional proteins, Mba1 and Mdm38 (Bauerschmitt et al. 2010; Ott et al. 2006). Both bind to the large ribosomal subunit and *mba1-mdm38* double mutants lack a functional respiratory chain (Bauerschmitt et al. 2010).

D. Post-Translational Transport across the Sec Translocon

The co-translational SRP pathway is highly advantageous because it minimizes the risk of misfolding/aggregation of nascent membrane proteins before they encounter their target membrane. Still, several post-translational protein-targeting pathways are also utilized and both the bacterial and the eukaryotic Sec channels can operate in a post-translational mode (Rapoport et al. 1996). In eukaryotes, this mode depends on the Sec62/63 complex, which associates with the Sec61 complex. Substrate recognition requires the Hsp70 and Hsp90 chaperone systems. So far, mainly short proteins/peptides and GPI-anchored proteins appear to be transported post-translationally in eukaryotes (Zimmermann et al. 2011).

The post-translational mode of the bacterial SecYEG translocon depends on the interaction with the cytosolic ATPase SecA and is required for the ATP-dependent translocation of secretory, *i.e.* periplasmic and outer membrane proteins across the SecYEG translocon (du Plessis et al. 2011). After the release of a fully synthesized

protein from the ribosome, the homo-tetrameric chaperone SecB binds to the protein and keeps it in a transport-competent conformation. The substrate-SecB complex is then recognized by the ATPase SecA that binds with high affinity to the N-terminal signal sequences of secretory proteins. In comparison to the signal anchor sequences mentioned above, these signal sequences display lower hydrophobicity and contain a signal peptidase cleavage site that is characterized by an Ala-x-Ala motif (Kudva et al. 2013). SecA binds, like FtsY, directly to the cytosolic loops of the SecYEG translocon and to anionic phospholipids, with recent data suggesting that FtsY and SecA compete for binding to the limited number of SecYEG translocons (Kuhn et al. 2011). SecA-dependent translocation of secretory proteins across the SecYEG translocon is a stepwise process that is coupled with multiple rounds of ATP hydrolysis. During translocation, the signal sequence is cleaved off and further degraded by peptidases; the mature protein is then released into the periplasmic space. The SecA/SecY pathway also operates at the thylakoid membrane in plants, but SecB homologues have so far not been identified (Koch et al. 2003b; Muller et al. 2001).

Cytochrome b_{562} (CybC) is the only periplasmic *b*-type cytochrome in *E. coli* and it contains a typical signal sequence (Nikkila et al. 1991) that routes it very likely into the post-translational SecB/SecA targeting pathway. The periplasmic protein NikA also contains a cleavable Sec signal sequence and was shown to bind heme (Shepherd et al. 2007). However, whether heme binding is required for the function of NikA in Ni uptake remains to be determined.

E. Alternative Protein Targeting and Transport Pathways

1. The Twin-Arginine Translocation Pathway

The twin-arginine translocation (Tat) pathway is a protein transport system that exists in bacteria, archaea and chloroplasts (Palmer

and Berks 2012). Its ability to export proteins in a fully folded conformation makes it distinct from most other protein transport systems. The Tat system in *E. coli* consists of three membrane proteins TatA, TatB and TatC. TatC and TatB form a receptor complex that is required for the initial recognition of the N-terminal S-R-R-x-F-L-K consensus sequence of Tat substrates (Alami et al. 2003). The name-giving arginine pair within the signal sequence is almost invariant and required for transport. However, a few naturally occurring exceptions have been described, in which one Arg is replaced by Lys, Asn or Gln (Kudva et al. 2013). Translocation of Tat substrates across the membrane is mediated by TatA oligomers (Kudva et al. 2013). It is however being debated whether TatA oligomers form a membrane pore, like the SecYEG translocon, or whether they destabilize the membrane, thereby weakening the permeability barrier (Kudva et al. 2013). The number of Tat substrates in different bacterial species varies significantly. 27 substrates have been identified so far in *E. coli*, while almost 190 predicted Tat substrates exist in *Streptomyces coelicolor* (Palmer and Berks 2012). Several reasons have been established for explaining why proteins engage the Tat pathway instead of the Sec pathway: (1) The dependence on cytosolic cofactor insertion machineries that induces a partial or complete folding of the protein. This applies to *E. coli* proteins containing [Fe-S] clusters (e.g. HyaA, HybO, NapG or NrfC), Cu-cofactors (CueO or PcoA), or molybdenum cofactors (TorA, DmsA); (2) Avoidance of uncontrolled cofactor insertion in the periplasm. This has been established for the Mn^{2+} binding protein MncA of *Synechocystis* sp. PCC 6803 (Tottey et al. 2008), which would incorporate Cu or Zn if exported into the periplasm in an unfolded conformation; (3) the association with partner proteins in the cytosol before transport. This was shown for Hydrogenase-1 and -2 in *E. coli*, where only the small subunits (HyaA, HybO) contain a Tat signal sequence. By association with the small subunits, the large subunits (HyaB, HybC) can be translocated across

the membrane by a hitchhiker mechanism (Palmer and Berks 2012); (4) the requirement for cytosolic folding catalysts or unfavorable extracellular conditions for protein folding. The latter argument might explain why many halophilic archaea employ the Tat pathway (Pohlschroder et al. 2005).

So far, the DyP-like periplasmic peroxidase EfeB (YcdB) is the only Tat-dependent heme-containing protein identified in *E. coli* (Sturm et al. 2006). The Tat pathway is nevertheless important for the assembly of several cytochrome *b*-containing membrane protein complexes, as they contain Tat-dependent subunits. HybB is a membrane integral cytochrome *b* subunit of the *E. coli* hydrogenase. It contains 10 TM domains and is likely a substrate for the SRP pathway, while HybO and HybC are translocated together via the Tat pathway. A similar situation is encountered for formate dehydrogenases in *E. coli*, which contain a membrane integral *b*-type cytochrome subunit (γ-subunit) that assembles with the G- and H-subunits. The G- and H- subunits are translocated together as a heterodimer via the Tat pathway due to a Tat signal sequence present in the G-subunit.

2. The Insertion of Tail-Anchored Membrane Proteins: The GET Pathway

Several membrane proteins are membrane-anchored via a C-terminal TM domain (Tail-anchored (TA) proteins), which escape co-translational SRP binding and targeting. Bioinformatics screens have identified 411 TA proteins in humans (Kalbfleisch et al. 2007), 55 TA proteins in *S. cerevisiae* (Beilharz et al. 2003) and 11–14 TA proteins in *E. coli* (Borgese and Righi 2010; Renthal 2010). Examples are cytochrome b_5 of mammalian and plant cells or the β-subunit of *E. coli* formate dehydrogenase-N (Borgese and Righi 2010). In eukaryotic cells, TA proteins of the ER employ a post-translational insertion pathway, the **G**uided **E**ntry of **T**ail-anchored proteins (GET) system (Sinning et al. 2011). The central component of the GET pathway is the homo-dimeric ATPase GET3, which functions as an

ER targeting factor. GET3 shows homology to the bacterial ATP-dependent arsenite transporter ArsA, and belongs, together with Ffh/FtsY, to the SIMIBI family of NTPases. The minimal membrane insertion machinery for TA proteins in yeast appears to be provided by the GET1 and GET2 proteins, which are integral ER membrane proteins that interact with GET3. For a subset of eukaryotic TA proteins an involvement of the SRP-Sec pathway has also been suggested (Rabu et al. 2009).

How TA proteins are inserted into bacterial membranes is largely unknown. YidC has been implicated in the insertion of the *E. coli* TA protein TssL (Aschtgen et al. 2012) and some *Streptomyces coelicolor* TA proteins (Craney et al. 2011). The osmosensor ProW contains a 100-amino-acid-long periplasmic N-tail followed by seven closely spaced TM helices. Biochemical studies indicate that ProW follows an SRP-dependent but SecYEG-independent insertion pathway (Cristobal et al. 1999), which could involve YidC. The involvement of chaperones of the Hsp70/Hsp40 families in recognition and targeting of TA proteins has also been suggested (Borgese and Righi 2010).

3. The Insertion of Small Membrane Proteins

Small, single-spanning membrane proteins are frequently found as accessory subunits of respiratory complexes, and it has been suggested that they are involved in organizing the large hydrophobic subunits within the membrane (Pawlik et al. 2010; Zickermann et al. 2010). *E. coli* synthesizes more than 60 proteins less than 50 amino acids long that are predicted to be membrane-inserted (Fontaine et al. 2011). Co-translational recognition and insertion of these proteins by the SRP-Sec or SRP-YidC pathway is conceptually difficult because approximately 30–40 amino acids are shielded by the ribosomal peptide tunnel during synthesis. However, data from conditional depletion strains suggest that the insertion of some of these proteins appears to be impaired upon SecYEG or YidC depletion (Fontaine et al. 2011).

4. Translation-Independent Targeting Pathways

The general concept of protein targeting and transport relies on the presence of a transport signal within the protein itself. However, recent data have indicated that mRNAs encoding membrane proteins localize to the inner membrane in *E. coli* by a translation-independent mechanism (Nevo-Dinur et al. 2011). Although details on the recognition and membrane targeting of these mRNAs are still missing, it has been shown that mRNAs coding for membrane proteins have a significantly higher uracil content than mRNAs encoding cytoplasmic proteins (Bibi 2011).

IV. Structural Features Determining Heme Binding to Membrane-Bound Apo-Cytochromes

It still is not entirely clear why the heme cofactors are covalently attached in c-type cytochromes (Barker and Ferguson 1999). Covalent attachment reduces the need for stabilizing heme-protein interactions via additional forces, such as Van der Waals or hydrophobic interactions, which are involved in stabilizing heme-protein contacts in *a*-, *b*-, *d*- or *o*-type cytochromes. At least four different systems exist, which are involved in covalent attachment of hemes to the protein moiety of *c*-type cytochromes. Thus, in case of soluble c-type cytochromes, the three-dimensional protein structure might fold after covalent heme attachment to small polypeptide stretches, and the heme might be involved in formation of a folding core. However, in case of TM c-type cytochromes, where the heme is bound in the TM region, it appears to be rather unlikely that major parts of a polypeptide chain fold after heme attachment, as unfolded polypeptide

chains are energetically unfavorable in the hydrophobic membrane environment (Popot and Engelman 1990). As all TM cytochromes belong to the class of α-helical TM proteins, which span the lipid bilayer in an α-helical conformation and form TM helix bundles (in contrast to β-barrel TM proteins), it appears to be likely that at least the membrane-integrated TM α-helices are pre-structured. In case of TM *c*-type cytochromes, it still is possible that individual TM helices assemble around the cofactor after covalent attachment of the heme to a single TM helix and thereafter form the final three-dimensional structure. However, as in case of all other cytochrome types, the heme cofactors are non-covalently bound, such folding pathways appear to be rather unlikely. More likely, heme molecules will bind to predefined, small hydrophobic pockets, which have a shape complementary to the shape of the heme cofactor. Such steric complementation results in a close fit between the heme cofactor and the protein moiety. Existence of a predefined heme-binding pocket and specific packing interactions maximizes non-polar contacts of the heme cofactor with the protein, resulting in tight heme binding. Initially, it was suggested that ligation of the heme central iron via the two ligating amino acid side chains (mostly His residues) is the major force driving formation of b-type cytochromes and mainly stabilizes the final cytochrome structures (Schneider et al. 2007). In line with this suggestion, in synthetic heme proteins, resembling TM cytochrome structures, essentially only the crucial His residues were incorporated and the resulting *b*-type cytochrome structures were competent to bind heme (Robertson et al. 1994). However, after mutating the His ligands in soluble *b*-type cytochromes, binding of the heme cofactor was still observed, indicating that additional forces guide specific heme recruitment (Barrick 1994; McRee et al. 1994). Also, after mutation of a conserved heme ligand in the TM protein cytochrome b_6 of spinach, which binds two heme cofactors, binding of the hemes was still observed (Dreher

et al. 2008), and a naturally occurring cytochrome b_6 variant, in which a (otherwise conserved) His ligand is replaced by an Ala, has been described in the cyanobacterium *Gloeobacter violaceus* (Dreher et al. 2010). Thus, while binding of heme via the fifth and sixth His ligand is important, it appears to be dispensable in some cases, and other forces must be involved in binding of heme cofactors to proteins.

Structural analyses of multiple (soluble as well as membrane-integrated) cytochromes has indicated that the heme cofactor is typically deeply buried within a protein structure without significant surface exposure (Smith et al. 2010). However, it must be noted that solvent accessibility might be especially problematic for soluble cytochromes, where the hydrophobic heme group is exposed to an aqueous environment, whereas in TM cytochromes the solvent is mainly represented by the hydrophobic acyl side chains of the membrane lipids. Thus, in a membranous environment an increased solvent exposure might be less problematic than in aqueous environments. In line with this, in the simple TM *b*-type cytochrome b_{559}, which is formed by only two TM α-helices that sandwich a single heme (Herrmann et al. 1984), the heme cofactor is rather exposed to the lipid environment (Prodöhl et al. 2005). However, in naturally occurring TM di-heme proteins the heme molecules are almost completely buried within the protein (Smith et al. 2010). Maximizing heme-protein contacts will stabilize the holo-cytochrome structure, and additionally, due to an involvement in charge transfer reactions the heme cofactors need to be bound in a hydrophobic pocket with little solvent exposure. Within the rather non-polar heme-binding pockets the character of individual amino acids, rather than the exact identity, appears to be conserved (Smith et al. 2010). The binding pocket typically contains a large number of amino acids with non-polar side chains, and especially aromatic residues are over-represented (Smith et al. 2010). The three aromatic amino acids Phe, Tyr and Trp are involved in contacts with the

heme face, and they predominantly stabilize the heme-protein contact via aromatic-aromatic π-stacking interactions (Schneider et al. 2007). Furthermore, aromatic residues are frequently involved in Van der Waals contacts with the non-polar "edges" of the heme molecules. Similarly, also Leu, Ala and Val residues have been observed to frequently pack at the "edges" of heme molecules (Schneider et al. 2007). These residues are additionally involved in building a fairly rigid heme-binding surface, which keeps the heme cofactor in a rather fixed position (Schneider et al. 2007).

It was previously recognized that in many cytochrome structures the heme propionyl side chains interact with the positively charged amino acids Arg and Lys via the formation of salt bridges. Such interaction anchor and orient a heme molecule within a protein structure (Schneider et al. 2007; Smith et al. 2010). A structural survey has further revealed that also His side chains (other than the Fe-ligands), as well as tyrosine residues, might be involved in specific interactions with a heme propionyl chain, and thus in orienting and anchoring of the cofactor (Schneider et al. 2007; Smith et al. 2010).

Besides those defined interactions with the heme cofactors, other residues are indirectly involved in heme binding, but still crucial for its correct positioning. Some amino acids interact with His side chains and thereby orient the His imidazole ring to a position, which allows optimal interactions with heme (Schneider et al. 2007). Contacts between the heme and the protein moiety strikingly depend on the His rotamer (i.e. low energy side chain conformations), which has a severe impact on the energetics of heme binding and on positioning of the heme cofactor (Huang et al. 2004; Negron et al. 2009). Thus, a defined heme-binding cavity enables selective and high-affinity binding of heme, and such a cavity would have coevolved with the integration of heme *b* derivatives, such as heme *a*, *d* or *o*. Binding of different heme species to a TM *b*-type cytochrome might be possible, although with rather low

affinities, to circumvent nonspecific binding of undesired heme species in vivo. For example, in plant chloroplasts, the concentration of chlorophyll, which is formed from protoporphyrin IX in various steps, is up to 100 times higher than the concentration of heme *b* (Moulin and Smith 2005), and thus, binding of chlorophyll or chlorophyll precursors needs to be excluded. While the mechanisms leading to specific binding are not yet completely resolved, the geometry of the heme-binding pocket, as well as the tetrapyrrole ring substituents and the central metal ion ($Fe^{2+/3+}$ in heme; Mg^{2+} in chlorophyll) might control selective binding of heme *b* (Tome et al. 2013).

V. Assembly of Individual Transmembrane Holo-Cytochromes

Following membrane integration of the apo-cytochrome, individual apo-proteins interact with heme, resulting in formation of the homo-cytochrome. However, in contrast to *c*-type cytochromes, the assembly of *b*-type cytochromes is largely unclear. Originally, based on studies with designed four-helix-bundle heme proteins it has been concluded that heme integrates spontaneously into a protein moiety, resulting in formation of *b*-type cytochrome structures (Robertson et al. 1994). However, these studies were conducted using water-soluble proteins. Thus, the hydrophobic effect might promote intercalation of the hydrophobic heme cofactor into the hydrophobic protein interior of the designed proteins in an aqueous environment. Therefore, only minimal additional forces are needed to generate some specificity in heme-binding, e.g., provided by the rational placement of heme-ligating His residues. In recent years, such designed heme-binding proteins have been characterized in the more hydrophobic environment of detergent micelles or membranes, and hydrophobic heme-binding proteins have been designed, which mimic TM *b*-type cytochromes more closely

(Discher et al. 2003, 2005; Korendovych et al. 2010). Nevertheless, in these cases the heme-binding affinities were quite low, indicating weak interactions and low binding energies.

Folding and assembly of naturally occurring TM b-type cytochromes, which bind heme with high affinities, has been studied in only a few cases. In the following, we discuss recent results obtained by analyzing assembly of the simple mono-heme TM b-type cytochrome b_{559}' as well as of the more complex di-heme TM cytochrome b_6.

A. Cytochrome b$_{559}$′: A Mono-Heme TM b-Type Cytochrome

Cytochrome b_{559} is part of the large TM protein complex photosystem II, where it is the only TM cytochrome subunit. The physiological function of cytochrome b_{559} is still under discussion but its involvement in secondary electron transfer processes and protection of photosystem II have been discussed (Shinopoulos and Brudvig 2012). In vivo, this protein is composed of two subunits, PsbE and PsbF (Babcock et al. 1985; Herrmann et al. 1984; Widger et al. 1985), which both span the membrane with a single TM α-helix (Shinopoulos and Brudvig 2012). As PsbF is only around 40–50 amino acids long, a large portion of this protein is membrane-embedded. In contrast, the PsbE subunit is >100 amino acids long, and thus this protein contains more extended extra-membrane domains. The two TM helices of PsbE and PsbF form a TM helix dimer, and a single heme molecule is sandwiched and non-covalently bound in between the two TM helices. Interestingly, the amino acid sequence of the TM regions of both proteins is highly similar, and amino acids, which are involved in heme binding, are strictly conserved in both sequences (Prodöhl et al. 2005). Consequently, it has been shown some time ago that synthetic PsbF peptides spontaneously form a dimeric, heme-binding TM protein structure (Francke et al. 1999). In the following years this cytochrome b_{559}-like protein (cytochrome b_{559}') has been used as a

simple cytochrome b_{559} model to rationalize individual steps of cytochrome b$_{559}$ assembly (Prodöhl et al. 2005, 2007; Volkmer et al. 2006; Weber et al. 2011).

Understanding assembly of this simple TM b-type cytochrome can be helpful for understanding folding pathways of more complex TM cytochromes and may provide hints for an improved rational design of TM b-type cytochromes. Based on mutational analyses it has been concluded that the apo-cytochrome b_{559}' assembles without heme, and thus TM helix dimerization is not heme-dependent (Prodöhl et al. 2005). In fact, presence or absence of heme has no impact on the interaction propensity of the TM helices (Prodöhl et al. 2005; Volkmer et al. 2006). Addition of heme after TM helix dimerization resulted in the formation of the holo-cytochrome, and thus the heme cofactor integrates into the dimeric apo-cytochrome. Consequently, when dimerization of the PsbF TM helix was disturbed, heme could not bind and formation of the holo-cytochrome was abolished (Prodöhl et al. 2005). This indicates that the heme cofactor inserts into a predefined heme-binding niche. In the apo-cytochrome, the heme-binding domain is still flexible and only after heme binding, the cytochrome structure tightens up like a clam-shell, resulting in formation of the final holo-cytochrome structure (Akdogan et al. 2012) (Fig. 28.6). The PsbF TM region can be divided into a dimerization domain and a heme-binding domain, which are separated by a conserved kink-inducing Pro-residue (Weber et al. 2012). While the TM helices in the dimerization domain have a crossing angle of about 20°, the Pro residue induces a kink, which results in an almost parallel orientation of the two TM helices in the heme-binding domain. Thus, the heme-binding niche is predefined and heme binds to an open state resulting in structural alterations and clam-shell-like closing of the structure (Fig. 28.6). Inspection of the PsbF heme-binding region nicely illustrates that a heme-binding niche is predefined on each monomeric TM helix and many of the aspects derived from in silico
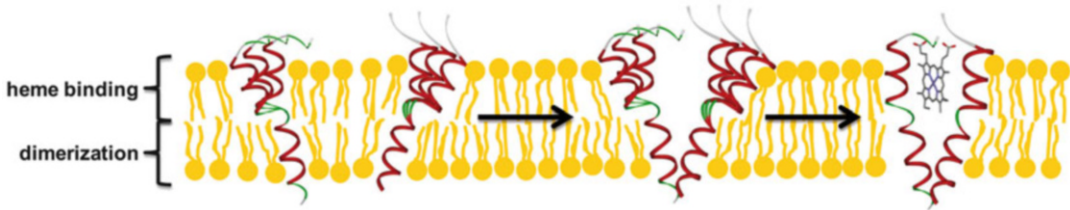
Fig. 28.6. The cytochrome b_{559}' folding pathway. For assembly of a heme-binding TM helix dimer, two monomeric PsbF TM peptides first interact via their dimerization domain and thereby form a homodimer with a flexible heme-binding domain. Heme binding constricts the flexibility of the heme-binding domain, resulting in formation of the final holo-cytochrome structure.
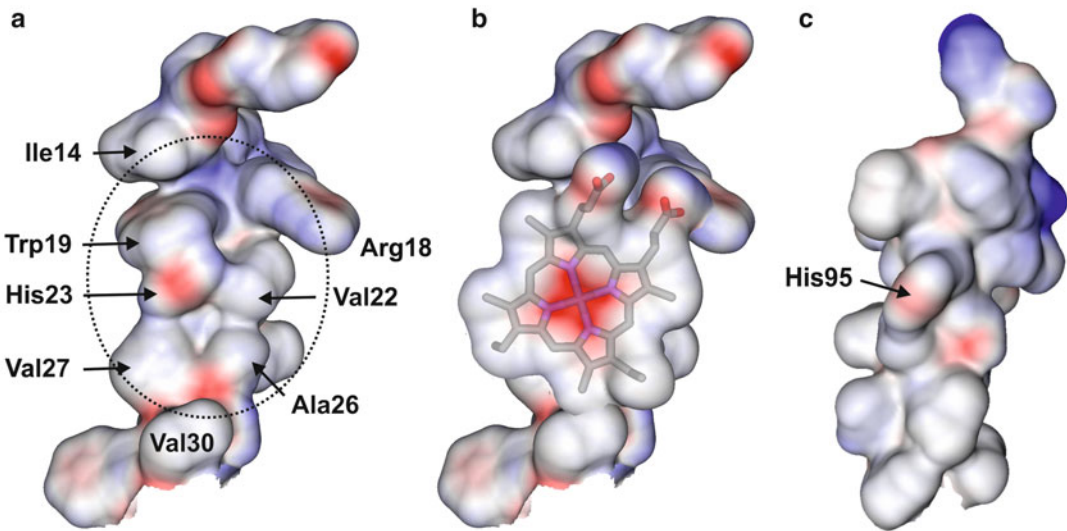

Fig. 28.7. Structure of PsbF in the apo- and holo-cytochrome b_{559}' protein. (**a**) Residues involved in heme binding at the heme-binding cavity formed by a single PsbF protein are shown. The protein surface is visualized with *red* and *blue* colors, indicating positive and negatively charged regions, respectively. Hydrophobic surfaces are colored in *gray*. The circle marks the heme-binding cavity. (**b**) The structure shown in (**a**) with heme bound. The structures shown in (**a**) and (**b**) are derived from the pdb-file 1S5L. (**c**) Modeled surface structure of the designed, heme-binding GpA peptide. The His residue introduced for heme binding (His95) is indicated. The structural model was generated using pdb-file 1AFO as a template.

analyses described above are confirmed (Fig. 28.7a).

On a single PsbF TM helix a cavity is formed, which is confined by Ile14 and Arg18 on one side and Val30 on the other side of the helix. This cavity has a rather flat and hydrophobic surface, composed of Val22, Ala26 and Val27, and the heme-ligating His23 is part of the surface without protruding out of the heme-binding plane. Additionally, Trp19 is in an almost parallel orientation to His23 and positions the His

imidazole ring, which most likely improves the stability of the His-iron bond. As can be seen in Fig. 28.7b, the heme molecule nicely fits into the predefined heme-binding cavity, and hydrophobic contacts are maximized. Furthermore, the heme propionyl side chains are positioned in a way that one side chain interacts with Arg18 of one TM helix (shown) and the other will interact with the corresponding Arg residue on the adjacent TM helix (removed in Fig. 28.6a, b). Such a perfect fit results in tight heme binding.

Nevertheless, deletion of the heme-ligating His residue completely abolished heme binding (Prodöhl et al. 2005). In contrast, when a heme-ligating His residue together with an Arg residue, which potentially salt-bridges the propionyl side-chain, are placed into the sequence context of the simple TM helix dimer glycophorin A (GpA), the protein was shown to bind heme (Cordova et al. 2007; Shinde et al. 2012). While this illustrates the predominant role of the His and Arg/Lys residues in heme binding, the heme-binding affinity of the designed protein was low. Inspection of the modeled surface structure of the designed TM b-type cytochrome nicely illustrates likely reasons for the low heme-binding affinity: In contrast to PsbF, in the designed protein no heme-binding cavity is preformed, the heme-ligating His side-chain protrudes out of the heme-binding surface, and besides interactions with the heme-ligating His and the Arg residues, the heme cofactor will not establish additional stabilizing Van der Waals interactions, as observed for cytochrome b_{559}' (Fig. 28.7c). Furthermore, the kink introduced in the cytochrome b_{559}' structure by Pro28 establishes formation of a parallel helix dimer in the heme-binding region, and thus the distance of the two adjacent TM helices to the heme cofactor is almost identical throughout the length of the heme molecules. In contrast, in the case of the designed heme-binding TM helix dimer, the heme-binding cavity narrows down, and thus the distance of the individual TM helices to the heme molecules differs throughout the length of the heme-protein contacts. Therefore, it appears to be rather difficult to establish stabilizing Van der Waals interactions at major heme parts. Consequently, future design of dimeric mono-heme TM cytochromes may include optimization of hydrophobic contacts in the heme-binding region. Furthermore, in case of cytochrome b_{559}', an induced fit has been observed upon heme binding (Akdogan et al. 2012), and thus the apo-cytochrome structure was flexible to some extent in the heme-binding region. Such flexibility appears to

be a prerequisite if the heme-binding cavity will enclose the heme molecules perfectly to establish maximal Van der Waals contacts. The designed TM helix cytochrome dimer is based on the rather rigid structure of the TM helix dimer GpA, and thus engineering some flexibility might further improve heme-binding affinities.

B. Cytochrome b_6: A Di-Heme Transmembrane Four-Helix Bundle

The role of individual amino acids in formation of the TM cytochrome b subunit of cytochrome bc_1 complexes has been analyzed by site-directed mutagenesis years ago [summarized in (Brasseur and Brivet-Chevillotte 1995)]. In earlier studies it has been observed that mutation of the heme-ligating His residues results in loss of heme binding and thus in disturbed assembly of the cytochrome b subunit (Gennis et al. 1993). Furthermore, a crucial importance of positively charged Lys or Arg residues, which interact with the heme propionyl side chains, has also been described early on. In addition, small residues located in the TM helices, which are not directly involved in heme binding but are part of the four-helix bundle, sandwiching the two b-type hemes, have been identified to be crucial for assembly of the proteins (Esposti et al. 1993; Gennis et al. 1993; Rau and Haehnel 1998). Thus, in case of the four-helix bundle proteins of the cytochrome b-type class of proteins, heme-ligating His residues are conserved as well as positively charged residues, which salt-bridge the heme propionyl side chains. Furthermore, the heme-binding niches are pre-defined and involve conserved Gly residues, since residues with small side chains are crucial to exclude steric clashes in the structure caused by heme incorporation. Thus, cytochrome b proteins contain symmetric motifs of conserved Gly and His residues in their TM regions and positively charged amino acids in the juxtamembrane regions of the TM helices (Esposti et al. 1993). This has been recognized and used in the improved design of (soluble) four-helix bundle proteins,
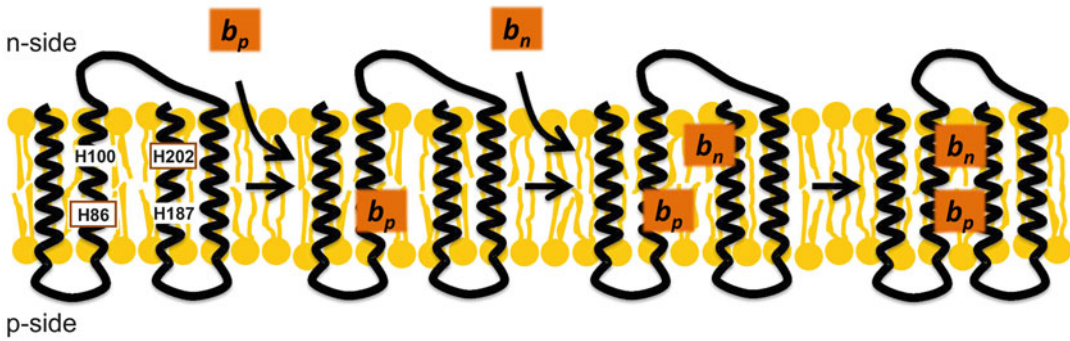
Fig. 28.8. The cytochrome b_6 assembly pathway. Conversion of the apo- to the holo-cytochrome b_6 involves multiple steps. Following membrane integration of the apo-cytochrome, the heme cofactor on the electrochemically negative side (n-side), heme b_p, binds predominantly via His86 to the protein moiety. Subsequently, the heme on the electrochemically positive side of the membrane (p-side), heme b_n, binds primarily via His202 to an assembly intermediate, finally resulting in a structural rearrangement and formation of the holo-cytochrome. The two residues His187 and His100 are involved in stabilization of bound heme b_p and heme b_n, respectively. In the case of chloroplast thylakoid membranes, the n- and p-side of the membrane correspond to stroma and lumen, respectively.

which bind two heme molecules, as further discussed below. The first four TM helices of cytochrome *b*, found in some prokaryotes and in eukaryotic mitochondria, are homologous to the four-TM protein cytochrome b_6 (Widger et al. 1984), which also binds two heme cofactors non-covalently. The two hemes located on the n- and p-sides of the membrane, are designated hemes b_n and b_p, respectively (Fig. 28.8).

As individual assembly steps are analyzed in greater detail in case of the cytochrome b_6 protein, in the following we use the amino acid numbering of the spinach cytochrome b_6 protein (See Chap. 9 by Cramer and Hasan).

Deletion of the His residues, ligating the high-potential heme b_n in the cytochrome *b* protein of *Rhodobacter* species, has resulted in loss of heme b_n as expected (Gennis et al. 1993). However, mutation of the His residue, ligating the low-potential heme b_p to large polar or charged residues has resulted in completely abolished heme binding (Gennis et al. 1993). Similarly, when both His residues of the spinach cytochrome b_6 protein, which are involved in heme b_n binding, are mutated to Ala, binding of heme b_p was abolished, and when the two heme b_n ligating His residues were mutated to Ala, both, heme b_p and heme b_n, were absent

(Dreher et al. 2008). These observations indicate that binding of heme b_p is a prerequisite for binding of heme b_n and thus, heme b_n binds after heme b_p. However, while in the originally described mutational study replacement of individual His residues by large polar or even charged residues resulted in complete loss of heme binding, the effects observed after replacement of the individual His residues by the small Ala residues were slightly different. In case of the spinach cytochrome b_6 protein, replacement of His100 still resulted in binding of heme b_p, indicating that a single heme-ligating His residue is sufficient for heme binding (Dreher et al. 2008). This observation is not completely unexpected as such heme coordination mimics binding of heme in catalytically active heme proteins or e.g. in oxygen-transporting myoglobins and hemoglobins, where the sixth ligand position is free. However, mutation of solely the second heme b_n ligating His residue (His 202) resulted in complete loss of heme b_n binding, indicating that the importance of the two heme-binding half-niches located on the helix hairpins differs (Dreher et al. 2008). Most likely, dominant interactions between the heme molecule and defined amino acids within the heme b_p binding niche of TM helices C and D promote

heme binding even in the absence of His100. In fact, a cytochrome b_6 protein has been identified in the cyanobacterium *Gloeobacter violaceus*, which has a naturally occurring mutation at the His100 equivalent position but binds two heme molecules with high affinity (Dreher et al. 2010). In contrast to the heme b_n ligating residues, replacement of the heme b_p ligating residues His 86 and His187 in combination resulted in absence of both, heme b_p and heme b_n. However, mutation of solely His187 did not impair binding of the two heme molecules, whereas mutation of solely His86 completely abolished binding of any *b*-type heme (Dreher et al. 2008). Based on these observations a likely folding pathway involves initial formation of two helical-hairpins (helix pairs AB and CD) after integration of the TM helices into the membrane. Subsequently, heme b_p binds, and His86 appears to have a predominate function in retaining this heme cofactor bound. Most likely, the structure of the four-helix bundle has not closed, as the second heme has to bind and the heme b_n binding niche has to be accessible. Thus, it appears to be likely that molecular interactions stabilize binding of heme b_n primarily at the helical hairpin formed by the TM helices A and B. After initial binding of heme b_p, heme b_n binds and here interactions mediated by His202 and the heme-binding half-cavity formed by the helical hairpin CD predominantly stabilize the bound heme cofactor. Binding of heme b_p obviously triggers formation of the final two-heme-binding four TM helix bundle structure, which appears to be a prerequisite for subsequent assembly of the entire cytochrome b_6f complex (Hasan et al. 2013; Saint-Marcoux et al. 2009).

Thus, on each helical hairpin of the heme-binding four helix bundle, two His residues are conserved in one helix, and one of these His residues has a predominant function. Furthermore, Gly residues are conserved in the other TM helix of the helical TM hairpin, as discussed above. The two helical hairpins (AB and CD) have an inverse TM topology, and thus the di-heme cytochrome b_6 protein has an internal quasi-two-fold symmetry

with the two helical hairpins oriented at 180° to each other. Such organization has been mimicked in some approaches aiming to design a four-helix bundle *b*-type cytochrome, as further discussed below.

VI. Naturally Occurring and Designed Transmembrane Four-Helix Bundle *b*-Type Cytochromes

Noteworthy, TM four-helix bundle structures, which bind two *b*-type heme molecules, are also found in other TM proteins, the structure of which has been solved, such as the γ subunit of the formate dehydrogenase, and subunit C of the fumarate reductase (Jormakka et al. 2003; Lancaster 2002). In all three cases, two heme molecules are non-covalently bound by a TM four-helix bundle and the heme residues are always ligated via two His residues. Nevertheless, the occurrence of two related helical-hairpin structures in cytochrome b and b_6 proteins and the symmetric motifs of conserved His and Gly residues found in these two helical-hairpin structures is not seen in the other di-heme TM proteins. In fact, the three structures have very diverse heme coordination patterns (Fig. 28.9). In the formate dehydrogenase γ-subunit, the heme-ligating His residues are provided by three TM helices, and in case of the fumarate reductase C subunit all four heme-ligating His residues belong to different TM helices. Consequently, the three TM four-helix bundle proteins have no sequence homology, and thus may have evolved independently (Jormakka et al. 2003; Lancaster 2002).

As the structural organization of the first four TM helices of cytochrome b proteins, which correspond to the cytochrome b_6 protein, is the least complex organization, these structures have served in the past as models for the design of water-soluble, di-heme-binding TM cytochrome models. In the initial design, a potential role of conserved cytochrome b amino acids has
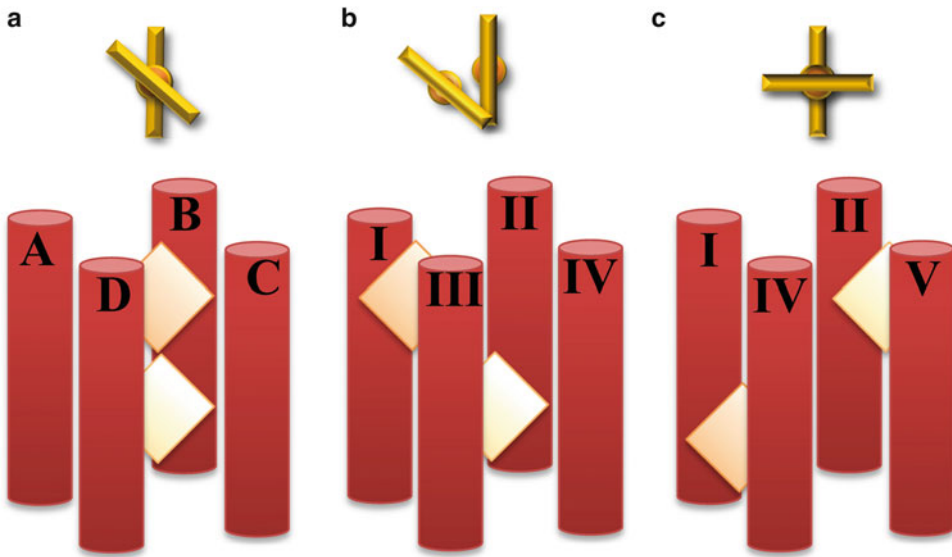
Fig. 28.9. Structures of transmembrane four-helix bundle *b*-type cytochromes. Three types of TM four-helix bundles, which bind two *b*-type hemes non-covalently, have been identified in TM protein structures, represented by the cytochrome b/b_6 subunit of cytochrome bc_1/b_6f complexes (illustrated in **a**), the γ subunit of the formate dehydrogenase (**b**), and the subunit c of the fumarate reductase (**c**) (Jormakka et al. 2003; Lancaster 2002). Two heme molecules are always non-covalently bound by a TM four-helix bundle and the heme residues are always ligated via two His residues. However, different TM helices are involved in heme binding, as discussed in detail in the text. The orientation of the two heme molecules with respect to each other is indicated above each four-helix bundle.

been recognized, though the identified residues were not completely included in the sequence of a synthetic peptide, which assembled to a water-soluble, heme-binding four-helix bundle (Robertson et al. 1994). Here, only the heme-ligating His residues, together with Arg residues involved in interaction with the heme propionyl groups, have been retained in the synthetic peptide. This initial design of a so-called molecular "Maquette" structure was a key step, as it was shown for the first time that heme-binding four helix bundles spontaneously assemble in aqueous solutions. However, in this design more specific heme-binding cavities or caves, as seen in naturally occurring *b*-type cytochromes, were not considered, and four identical helices assembled to form the four-helix bundle structure. Two identical helices were dimerized by disulfide linkage and these helix dimers finally assembled into a four-helix bundle with parallel topology. All four helices of the four-helix bundle

protein contained His residue potentially involved in heme binding. For enhancing Van der Waals packing of the heme to the protein moiety, topologically defined four-helix bundles were subsequently designed and synthesized, which mimic the heme-binding core of cytochrome *b* proteins more closely (Rau and Haehnel 1998). Two different helices were synthesized: a heme-binding helix, which contains the conserved His and Arg residues involved in heme binding, as well as a heme-shielding helix, which contains the conserved Gly residues crucial for heme packing as they provide space for tight packing and an optimal fit for the heme. Later on it has been shown that all residues needed for heme binding (His & Arg) and for heme space organization (Gly) might be included in a single helix (Ghirlanda et al. 2004). Furthermore, in the latter study a Thr residue was included, which was involved in correctly positioning the His residues, as the His residue position has been

shown to be crucial for high-affinity binding of heme molecules (Huang et al. 2004; Negron et al. 2009). In subsequent studies, heme-binding four-helix bundles have been designed, which reside in the hydrophobic environment of micelles or lipid bilayers (Discher et al. 2005; Korendovych et al. 2010; Ye et al. 2005). In these cases, heme binding occurred with rather low affinity, indicating that the forces driving assembly of *b*-type cytochromes severely differ in a polar aqueous *vs.* non-polar membranous environment. In contrast to a membrane environment, formation of a water-soluble four-helix bundle is mainly driven by formation of the hydrophobic bundle core. Consequently, further optimization of heme-binding properties within the hydrophobic membrane environment is needed.

VII. Assembly of Cytochrome *b*-Containing Membrane Protein Complexes

The vast majority of the membrane-bound *b*-type cytochromes function as part of larger protein assemblies, but there is surprisingly little information available on how these complexes assemble within the membrane. The best studied example in the *E. coli* inner membrane is cytochrome bo_3 oxidase, the major terminal oxidase under high oxygen conditions. Another well studied example is the assembly of cytochrome cbb_3 oxidase of *Rhodobacter capsulatus*, which is described in more detail in the chapter in this volume by Daldal and Koch. Finally, recent data have also shed light on the assembly of the cytochrome bc_1 complex in the bacterial and mitochondrial membrane (Gruschke et al. 2011, 2012) and the cytochrome b_6f complex in the thylakoid membrane (Hasan et al. 2013).

A. Cytochrome bo_3 Oxidase Assembly

Cytochrome bo_3 oxidase is a four-subunit membrane complex containing heme *b*, heme *o* and a Cu_B center within the large subunit I (CyoB), which spans the membrane 15 times (Pereira et al. 2001; Stenberg et al. 2007). Subunit II (CyoA) contains two TM helices and a large periplasmic loop, while subunits III (CyoC) and IV (CyoD) are closely spaced membrane proteins, containing five and three TM helices, respectively. An elegant study by the Daley group (Stenberg et al. 2007) has revealed a sequential assembly process in which subunits III and IV assemble first within the membrane, forming an intermediate migrating at approx. 63 kDa during Blue-native polyacrylamide gel electrophoresis (BN-PAGE). CyoB is then recruited to this intermediate, resulting in a 123 kDa complex when visualized on BN-PAGE. Finally, CyoA binds to the CyoB-CyoC-CyoD sub-complex to form the holo-complex. The process and timing of cofactor insertion into respiratory complexes, such as the cytochrome bo_3 oxidase, is still unresolved. The cofactors are deeply buried within CyoB and their insertion is probably important for its folding and stability. It was therefore generally assumed that cofactor insertion occurs early during assembly, and probably even co-translationally (Ekici et al. 2012; Koch and Schneider 2007; Stenberg et al. 2007). However, the only indication for co-translational cofactor insertion so far is the observed interaction between ribosomal proteins and Cox11, a protein required for Cu_B center formation in the cytochrome aa_3 oxidase (Khalimonchuk et al. 2005). Strikingly, a recent study using ^{55}Fe-labeled heme indicates that at least the heme groups are inserted at a late step in cytochrome bo_3 oxidase assembly pathway (Palombo and Daley 2012). It has been suggested that the heme-binding pocket is only formed upon the interaction of CyoB with the CyoDC subcomplex. This would also explain why monomeric CyoB binds heme only with low efficiency (Nakamura et al. 1990). Whether Cu_B insertion also occurs at a late step during assembly, is currently unknown. Multiple accessory proteins assist in the stepwise assembly process of cytochrome aa_3 oxidases (Kim

et al. 2012; Mick et al. 2011) or cytochrome cbb_3 oxidases (Ekici et al. 2012), but similar proteins have not yet been described for cytochrome bo_3 oxidase.

B. Cytochrome bc₁ and Cytochrome b₆f Complex Assembly

The cytochrome bc_1 complex (or its thylakoidal equivalent, the cytochrome b_6f complex) is a central component of respiratory and photosynthetic electron transport chains in bacteria and eukaryotes, although it is absent in some bacteria, such as *E. coli* (Cramer et al. 2005). All bc_1 complexes contain three core subunits with redox prosthetic groups (Daldal et al. 1987). Cytochrome *b* is an integral membrane protein with 8 TM helices that ligates two heme groups of different redox potentials and absorbance maxima, b_p (b_{562}) and b_n (b_{565}), located near the electrochemically positive (p) and negative (n) side of the membrane (compare above). Cytochrome c_1 and the Rieske Fe/S protein both consist of a single TM helix and a larger periplasmic/soluble domain harboring a covalently attached heme group and a 2Fe-2S cluster, respectively. As for the cytochrome bo_3 oxidase, so far no accessory proteins have been identified that are required for the attachment of heme *b* to apo-cytochrome *b*, and it is therefore assumed that heme is spontaneously inserted into the apo-protein, as discussed above. Heme *b* insertion is considered to occur early during assembly and probably concomitantly with folding. Mutating the heme-ligating residues in apo-cytochrome *b* impairs bc_1 complex assembly (Brasseur et al. 1996). Cytochrome *b* and cytochrome c_1 probably assemble first and form a protease-resistant intermediate to which the Rieske Fe/S protein is later recruited (Valkova-Valchanova et al. 1998). Mutations that interfere with cofactor insertion into the Rieske Fe/S protein have no impact on cytochrome *b* or cytochrome c_1 maturation, suggesting that these processes occur independently (Davidson et al. 1992).

The eukaryotic bc_1 complex contains additional subunits and its assembly pathway is far more complex compared to its prokaryotic homologue. The *S. cerevisiae* cytochrome bc_1 complex consists of 10 subunits and forms a dimer in the mitochondrial membrane. Translation of the cytochrome *b*-encoding mRNA requires processing by the matrix protein Cbp2 and the translational activators Cbs1 and Cbs2. In addition, Cbp3-Cbp6 has to bind to the ribosomal tunnel exit to allow efficient cytochrome *b* synthesis (Gruschke et al. 2011). After synthesis is completed, the Cbp3-Cbp6 complex is released from the ribosome but maintains contact to cytochrome *b*. Sequential recruitment of the assembly factor Cbp4 and the Qcr7 and Qcr8 subunits induces the release of Cbp3-Cbp6 and allows subsequent binding of the two additional subunits Cor1 and Cor2, which do not contain cofactors. During this step Cbp4 is also released. Stepwise binding of Qcr6, cytochrome c_1 and of Qcr9, Qcr10 and the Rieske Fe/S protein then completes the assembly process (Gruschke et al. 2012). Blocking later stages of assembly sequesters Cbp3-Cbp6 in the early intermediates and prevents further translational activation of the cytochrome *b* synthesis. This feed-back loop regulates cytochrome *b* synthesis in response to the efficiency of cytochrome bc_1 complex assembly.

The cytochrome b_6f complex is a symmetric dimer of eight TM subunits. The b_6f monomer contains two multi-spanning membrane subunits (Cramer et al. 2005). Cytochrome b_6 has four TM helices and a short N-terminal helix, which is surface-exposed. Subunit IV contains three TM helices, which surround the four-helix bundle of cytochrome b_6. This core assembles with additional six TM helices, which are provided by the additional single-spanning subunits cytochrome *f*, the Rieske protein and the four small single TM proteins, PetG, PetL PetM and PetN at the periphery of each monomer of the complex (Hasan et al. 2013).

Seven prosthetic groups are present in the cytochrome b_6f complex monomer (Kurisu et al. 2003; Stroebel et al. 2003). The cytochrome b_6 subunit harbors two bis-His ligated b-hemes (b_p and b_n) and a covalently linked heme c_n, which is unique in not having any amino acid as an axial ligand (Kurisu et al. 2003; Stroebel et al. 2003). In addition, a chlorophyll-a (chl-a) and a β-carotene (β-car) molecule are intercalated within the four TM domains of cytochrome b. An additional heme molecule is covalently attached to the cytochrome f subunit and one [2Fe–2S] cluster is bound to the Rieske subunit.

The current model suggests that the cytochrome b_6 and subunit IV assemble first to form the core of the monomeric cytochrome b_6f complex, which subsequently undergoes dimerization (Hasan et al. 2013). At this stage, lipids are thought to stabilize the monomer–monomer interactions (Breyton et al. 1997) (See Chap. 20 by Baniulis et al.). The Rieske FeS protein and the cytochrome f subunit also form a subcomplex that is stabilized by a native acidic sulfolipid interacting with basic residues of both TM helices. This sub-complex then interacts with the cytochrome b_6-SU IV polytopic core nucleus to form a cytochrome b_6-SU IV-ISP-cytochrome f sub-complex. The four small subunits are thought to insert spontaneously into the membrane and to assemble as a four-helix bundle around the β-carotene molecule at the periphery of the cytochrome b_6f complex core. Mutagenesis studies suggest that the presence of the PetG, L, M, N subunits is required for a stable cytochrome b_6f complex dimer (Hasan et al. 2013).

Heme binding is probably an important step in the stabilization of the assembly intermediates. In the absence of heme, the cytochrome b_6 and cytochrome f apo-proteins are highly susceptible to protease cleavage (Kuras et al. 1995). Heterologous expression and stable membrane insertion of cytochrome b_6f subunits in *E. coli* is possible, when heme is added to the medium, suggesting that no dedicated assembly factors/chaperones are required for heme insertion (Dreher et al. 2007). However, the exact timing of subunit assembly and cofactor insertion still needs to be determined.

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Chapter 29

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Respiratory Cytochrome Supercomplexes

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Summary

Evidence from several investigations demonstrates the existence of supramolecular units of Complex I, Complex III, and multiple copies of Complex IV in mitochondria and indicates that specific respiratory complexes may preferentially associate to form cytochrome-containing supercomplexes in the native membrane. There are now indications that

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cardiolipin, a distinctive mitochondrial lipid, stabilizes the respiratory assemblies. The isolated supercomplexes are active with respect to both their component individual complexes and the entire respiratory function that relies on Coenzyme Q and cytochrome c as intermediate substrates. The latter finding argues against previous models of a random distribution of the respiratory complexes in mitochondria. The supercomplex organization is compatible with electron transfer, but experimental evidence is scant for an effective mechanism via substrate channeling compared to free diffusion of substrates in accordance with the random collision model. The finding that Complex I is almost totally associated in a supercomplex with Complex III seems to exclude a role for the ubiquinone pool in physiological electron transfer between these two complexes, whereas it is certainly required for electron transfer from Complex II or from other dehydrogenases to Complex III; likely, only a small fraction of Complex IV forms a functional supercomplex with channeling of cytochrome c. Nevertheless, the supercomplexes may physiologically exist in equilibrium with free complexes (plasticity model). The supercomplex organization appears to prevent excessive generation of reactive oxygen species from the respiratory chain; accordingly, many pathological conditions and the mitochondrial aging phenotype characterized by a loss of supercomplex assembly correlate with mitochondrial dysfunction and increased oxidative stress. Specific metabolic signals may also arise in the cell in response to a tuned production of reactive oxygen species as a consequence of the controlled dynamics of supercomplex assembling/disassembling at different physio-pathological conditions. The present review paper provides an updated and extensive discussion on the subject.

I. Introduction

A. The Respiratory Chain of Mitochondria

The *electron transfer chain* or *respiratory chain* is a multi-enzyme system that collects reducing equivalents (hydrogen atoms)

Abbreviations: AOX – Alternative oxidase; BHM – Bovine heart mitochondria; BN-PAGE – Blue-native polyacrylamide gel electrophoresis; C_I – Complex I, NADH-coenzyme Q reductase; C_{II} – Complex II, succinate-CoQ reductase; C_{III} – Complex III, ubiquinol-cytochrome c reductase; C_{IV}, COX – Complex IV, cytochrome c oxidase; CJ – Cristae junction; CL – Cardiolipin; CN-PAGE – Colorless-native polyacrylamide gel electrophoresis; Complex V – ATP synthase; CoQ – Coenzyme Q, ubiquinone; cyt. c – Cytochrome c; $\Delta\tilde{\mu}_H{}^+$ – Electrochemical proton gradient; ETF – Electron transfer flavoprotein; FAO – Fatty acid β-oxidation; IM – Inner mitochondrial membrane; OM – Outer mitochondrial membrane; OXPHOS – Oxidative phosphorylation; PC – Phosphatidyl choline; PE – Phosphatidyl ethanolamine; R4B – Crude mitochondrial preparation enriched in complexes I and III; ROS – Reactive oxygen species

released from oxidations of intermediary metabolism and conveys them to molecular oxygen, reducing it to water (four electron reduction). In eukaryotes the respiratory chain is associated to the inner membrane of mitochondria and collects reducing equivalents mostly from oxidations occurring in the mitochondrial matrix. Most oxidizable substrates have redox potentials close to the midpoint potential of the $NAD^+/NADH$ couple ($E_{m7} = -320$ mV). Therefore, NADH is used to shuttle electrons from these substrates to the respiratory chain; a few oxidizable substrates, however, have midpoint potentials close to zero on the biochemists' scale, and their reducing equivalents must reach the respiratory chain at a site downstream energetically relative to the entry of electrons from NADH.
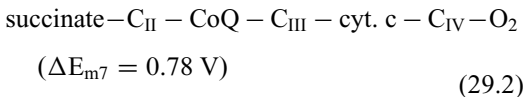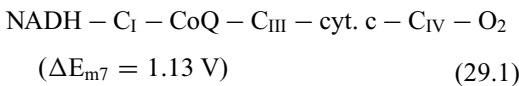
The free energy decrease accompanying electron transfer is exploited to create an electrochemical proton gradient ($\Delta\tilde{\mu}_H{}^+$) by proton translocation from the mitochondrial inner space, the matrix, to the space existing between the inner and outer mitochondrial membranes (Nicholls and Ferguson 2013). The proton gradient is then used as a

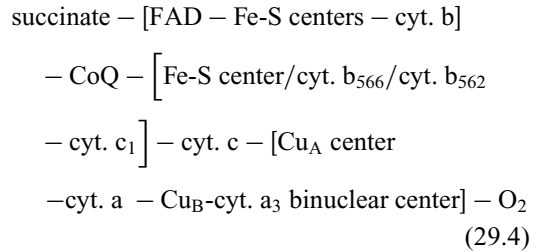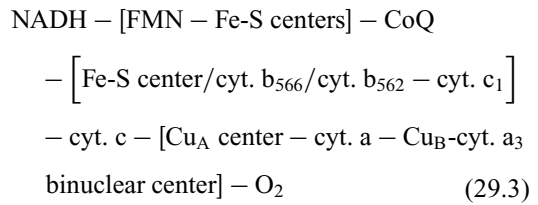source of energy mainly to synthesize ATP from ADP and Pi by the ATP synthase complex: the overall process is called oxidative phosphorylation (OXPHOS). The ATP synthesized is moved to the cytoplasm in exchange with ADP by the ATP/ADP translocase at the expense of the proton gradient. Alternatively, the proton gradient is used to drive other energy-linked reactions such as NADH/NADP$^+$ transhydrogenation and up-hill ion movements across the inner membrane.

The classic electron transfer chain was first described by Hatefi et al. (1962a) in David Green's laboratory (cf. Green and Tzagoloff 1966, for a comprehensive review) as the functional sequence of four major multi-subunit complexes, randomly dispersed in the inner mitochondrial membrane, and designated as NADH-Coenzyme Q reductase (Complex I, C_I), succinate-CoQ reductase (Complex II, C_{II}), ubiquinol-cytochrome c reductase (Complex III, C_{III}) and cytochrome c oxidase (Complex IV, C_{IV}); the enzyme complexes are connected by two mobile redox-active molecules, i.e. a lipophilic quinone, designated Coenzyme Q (CoQ) or ubiquinone, embedded in the membrane lipid bilayer, and a hydrophilic heme protein, cytochrome c (cyt. c), localized on the external surface of the inner membrane (see Sect. I.B for details). The chain functions in the following sequences, where ΔE_{m7} is the change in redox potential between the initial electron donor and the final acceptor,

$$NADH - C_I - CoQ - C_{III} - cyt.\ c - C_{IV} - O_2$$
$$(\Delta E_{m7} = 1.13\ V) \qquad (29.1)$$

$$succinate - C_{II} - CoQ - C_{III} - cyt.\ c - C_{IV} - O_2$$
$$(\Delta E_{m7} = 0.78\ V) \qquad (29.2)$$

and in which several redox centers (Schemes 29.3 and 29.4) that are associated with the enzyme complexes allow electron transfer down the midpoint potential gradient:

$$NADH - [FMN - Fe\text{-}S\ centers] - CoQ$$
$$- \left[Fe\text{-}S\ center/cyt.\ b_{566}/cyt.\ b_{562} - cyt.\ c_1 \right]$$
$$- cyt.\ c - [Cu_A\ center - cyt.\ a - Cu_B\text{-}cyt.\ a_3$$
$$binuclear\ center] - O_2 \qquad (29.3)$$

$$succinate - [FAD - Fe\text{-}S\ centers - cyt.\ b]$$
$$- CoQ - \left[Fe\text{-}S\ center/cyt.\ b_{566}/cyt.\ b_{562} \right.$$
$$- cyt.\ c_1 \left. \right] - cyt.\ c - [Cu_A\ center$$
$$- cyt.\ a - Cu_B\text{-}cyt.\ a_3\ binuclear\ center] - O_2$$
$$(29.4)$$

The best fit unit stoichiometry between complexes in beef heart mitochondria is: 1 Complex I : 1.3 Complex II : 3 Complex III : 6.7 Complex IV (Schägger and Pfeiffer 2001). In addition, there are 0.5 ATP synthase (also called Complex V) and 3–5 units of the ADP/ATP translocase (catalyzing the equimolar exchange of ADP and ATP across the inner membrane) for each cytochrome oxidase, and there is one NADH/NADP$^+$ transhydrogenase per Complex I (Capaldi 1982). Nevertheless, wide differences in cytochrome, Coenzyme Q, and pyridine nucleotide contents of mitochondria from different species, as well as from different organs and tissues of the same species, have been reported (Lenaz and Genova 2010); data in the literature indicate that the molar ratios of the respiratory components vary significantly.

The inner membrane contains other proteins having electron transfer activity in smaller amounts (Lenaz and Genova 2010); among these there are electron transfer flavoproteins capable of feeding electrons to the respiratory chain by pathways not involving Complex I and/or NADH, i.e. glycerol-3-phosphate dehydrogenase, electron transfer flavoprotein (ETF)-ubiquinone oxidoreductase, dihydro-orotate dehydrogenase, choline dehydrogenase, in addition to alternative NADH dehydrogenases in mitochondria from several organisms, especially plants

and fungi. Moreover, alternative or branched pathways of electron transfer also occur, departing from CoQ: these are the alternative ubiquinol oxidases from bacteria and plant and fungi mitochondria.

B. Organization of the Respiratory Chain: Historical Outline

The systematic resolution and reconstitution of four respiratory complexes from mitochondria was accomplished by Hatefi et al. (1962a), leading Green (Green and Tzagoloff 1966) to postulate that the overall respiratory activity is the result of both intra-complex electron transfer in the 'solid' state between redox components having fixed steric relation and, in addition, of inter-complex electron transfer ensured by rapid diffusion of the mobile components acting as co-substrates, i.e. CoQ and cyt. c. This proposal was substantially confirmed over the following years, leading to the postulation by Hackenbrock et al. (1986) of the *Random Collision Model of Electron Transfer*. The organization of the respiratory chain has represented a major research subject in the 1970–1980s, culminating with acceptation of the random collision model by the majority of investigators in the field.

The accumulation of recent experimental evidence obtained with newly developed techniques has replaced the random collision model of electron transfer, based on random interactions between randomly dispersed components, with a model of supramolecular organization based upon specific, though dynamic, interactions between individual components, and leading to the acquisition of new properties (substrate channeling, assembly, morphological organization) that were unpredictable in the previous reductionist approach.

A retrospective analysis of the literature reveals that the idea of supramolecular associations between respiratory enzymes has been present since the early times, dating from the original view derived from the pioneering studies of Chance and Williams (1955), who depicted the respiratory chain

as a solid-state assembly of flavins and cytochromes in a protein matrix.

Evidence against a random distribution of respiratory complexes was also derived from early investigations reporting isolation of Complex I–Complex III (Hatefi et al. 1962b) and Complex II–Complex III units (Yu et al. 1974), indicating that such units may be preferentially associated in the native membrane. The authors of the original studies were well aware that these preferential associations were physiological and indicated the existence of supramolecular units of electron transfer. Hatefi et al. (1962a) in their paper on the reconstitution of the electron transfer system stated that the four primary enzyme systems may be suitably combined to form secondary units capable of catalyzing the sums of the reactions catalyzed by the respective subunits. A careful examination of these pioneering papers allows one to conclude that respiratory supercomplexes, as well as the entire DPNH oxidase (the *respirasome,* see later) had been isolated and reconstituted. The appearance of the fluid mosaic model of membranes (Singer and Nicolson 1972) strongly influenced the researchers involved in the study of mitochondrial membranes, and this is probably the reason that the random collision model of Hackenbrock was so well and uncritically accepted.

There were, however, a few reports before the year 2000 on the possible presence of specific associations between respiratory complexes, either fixed (Ozawa et al. 1987) or dynamic (Hochman et al. 1985).

A breakthrough occurred in 2000 when Schägger applied the previously introduced technique of Blue-Native Polyacrylamide Gel Electrophoresis (BN-PAGE) to digitonin-solubilized yeast and mammalian mitochondria (Schägger and Pfeiffer 2000). The newly discovered associations were considered to represent the physiological state of the respiratory complexes and were called *respirasomes* (see Sect. II). In the same paper the authors also described a dimeric state for the ATP-synthase complex.

Today the evidence of supercomplex association of the respiratory chain is well

consolidated (Wittig and Schägger 2009; Lenaz and Genova 2010), although its possible relation with a random distribution of the individual complexes is not completely clarified (Genova and Lenaz 2013, 2014). This aspect will be widely discussed subsequently in this chapter.

II. Distribution and Composition of Respiratory Supercomplexes

A. Distribution in Different Organisms

BN-PAGE and the closely related though milder colorless native PAGE (CN-PAGE) are the preferred experimental strategy for the detection of the respiratory supercomplexes from various biological samples and for the analysis of their protein composition (Wittig et al. 2006; Krause and Seelert 2008) (Fig. 29.1).

Respiratory supercomplexes have been found in a wide range of living organisms; they were first discovered in yeast and mammalian mitochondria (Schägger and Pfeiffer 2000, 2001). They were subsequently revealed and characterized in several mammalian tissues, such as rat brain (Reifschneider et al. 2006; Dencher et al. 2007; Wernicke et al. 2010), liver (Lenaz et al. 2010; Dani et al. 2009; Reifschneider et al. 2006), kidney (Reifschneider et al. 2006), skeletal muscle (Reifschneider et al. 2006; Lombardi et al. 2009), and heart (Reifschneider et al. 2006; Gómez et al. 2009), in bovine heart (Schägger and Pfeiffer 2001; Schägger 2002; Krause 2006), in human skin fibroblasts (Kovářová et al. 2012; Miyake et al. 2013), human syncytiotrophoblast (De Los Rio Castillo et al. 2011) and human tumor cells (Genova et al. 2008) and in many other organisms, e.g., fish (Schäfer et al. 2007a), fungi (Krause et al. 2004b; Marques et al. 2007; Maas et al. 2009; Nübel et al. 2009; Davies et al. 2011; Cabrera-Orefice et al. 2014), *Caenorhabditis elegans* (Brys et al. 2010), *Drosophila* (Le Pécheur et al. 2009), and also in plants (Eubel et al. 2004; Krause et al. 2004a; Genova

et al. 2008; Peters et al. 2008; Kakizaki et al. 2012), and many bacteria (Schägger 2002; Stroh et al. 2004; Krause 2006; Sousa et al. 2012, 2013).

B. Composition of Respiratory Supercomplexes

All the supercomplexes investigated exhibit highly ordered architectures, thus discarding most doubts on artificial protein-protein interactions, and supporting the idea that such interactions may also be species- or kingdom- specific (Vonck and Schäfer 2009).

Most evidence presently favors the view that supercomplexes are formed by the three "core" complexes of the respiratory chain, i.e. Complexes I, III, and IV, whereas the other respiratory enzymes should be free in the lipid bilayer (Lenaz and Genova 2010). These "core" complexes have the common feature of carrying out proton translocation and of having subunits encoded by mitochondrial DNA.

In bovine heart mitochondria (BHM), only 14–16 % of total Complex I was found in free form in the presence of digitonin (Schägger and Pfeiffer 2001); thus, it seems likely that all Complex I is bound to Complex III in the absence of detergents. According to the stoichiometry of BHM OXPHOS complexes reported by Schägger and Pfeiffer (2001), it is plausible that approximately one-third of total Complex III and >85 % of total Complex IV are not bound in supercomplexes.

The fundamental features of the supramolecular organization of the standard respiratory complexes I, III, and IV as a respirasome are conserved in all higher eukaryotes. The supercomplex I-III$_2$-IV$_{1-4}$ is one of the most intriguing supercomplexes because it contains all the redox enzymes required for the complete pathway of electron transfer from NADH to molecular oxygen, and for this reason was called "*respirasome*". However, supercomplexes containing only two partners are also present in detectable quantities, as the supercomplex I-III$_2$ in which Complex IV is not present, as well as respiratory assemblies comprising only Complex
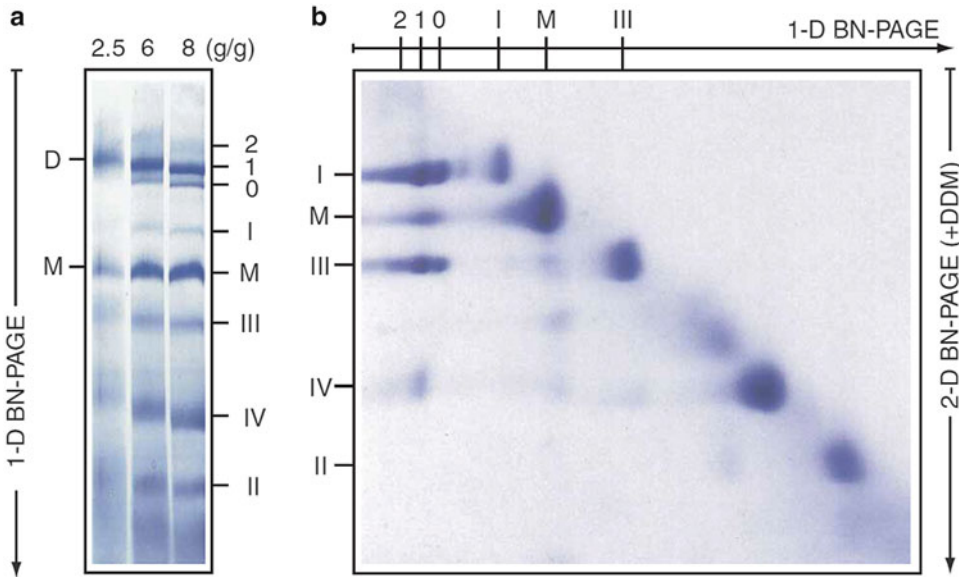
Fig. 29.1. Separation of supramolecular assemblies of oxidative phosphorylation complexes by 1D BN-PAGE and identification of their constituent individual complexes by 2D BN/BN-PAGE. (**a**) Bovine sonic particles were solubilized by digitonin using the indicated digitonin/protein ratios (g/g). The solubilized complexes were separated by 1D BN-PAGE. I, II, III and IV indicate mitochondrial complexes I-IV. Bands 0, 1 and 2 indicate respiratory supercomplexes containing monomeric Complex I, dimeric Complex III and zero (0), one (1) or two (2) copies of Complex IV, respectively. M and D indicate monomeric (M) and dimeric (D) ATP synthase. (**b**) The lane for the digitonin/protein ratio of 8 (g/g) excised from (**a**) was processed by 2D BN/BN-PAGE using 0.02 % dodecylmaltoside added to the cathode buffer. Respiratory supercomplexes were thereby disassembled into the individual constituent complexes I, III and IV (Reprinted from Wittig et al. (2006), with permission).

III and Complex IV. In particular, BN-PAGE in digitonin-solubilized mitochondria of *S. cerevisiae*, which possesses no Complex I, revealed two bands with apparent masses of ∼750 and 1000 kDa containing the subunits of complexes III and IV, as assigned after two dimensional SDS-PAGE followed by N-terminal protein sequencing (Schägger and Pfeiffer 2000). The smaller super complex (III_2IV_1) consists of a Complex III dimer and a Complex IV monomer while the larger super complex (III_2IV_2) is comprised of a Complex III dimer associated with two Complex IV monomers (Stuart 2008; Mileykovskaya et al. 2012).

Although Complex I is usually a monomer, a putative dimeric Complex I has also been described in mitochondria from *Neurospora crassa, Yarrowia lipolytica, Podospora anserina* and *Solanum tuberosum* where it may be involved in forming string-like and patch-like megacomplexes composed of repeated units of respiratory supercomplexes (Krause 2006; Krause et al. 2004b; Bultema et al. 2009; Marques et al. 2007; Nübel et al. 2009) (see Sect. II.C).

A higher level of complexity in the organisation state of supercomplexes is due to the presence of further interaction partners such as the ADP/ATP carrier, some chaperones for cytochrome c oxidase (COX) assembly and the carbonic anhydrase subunits, which co-assemble with the OXPHOS supercomplexes (Stuart 2009; Mick et al. 2007; Sunderhaus et al. 2006). Several additional proteins are probably able to interact with cytochrome c oxidase, and some examples such as monocarboxylate transporters, mitochondrial nitric oxide synthase, and the large-conductance Ca^{2+}-activated K^+ channel have already been identified by immunoprecipitation (Hashimoto et al. 2008; Persichini et al. 2005; Ohya et al. 2005). Recently it was

reported that mitochondrial superoxide dismutases associate with a documented respirasome (Suthammarak et al. 2013).

Wang et al. (2010) provided evidence for a multifunctional fatty acid β-oxidation (FAO) complex within mitochondria that is physically associated with supercomplexes. However, Schönfeld et al. (2010) could not find such an association (see Sect. IV.B.3.b).

It has been observed that several mitochondrial soluble NAD⁺-dependent dehydrogenases such as malate dehydrogenase, β-hydroxyacyl-CoA dehydrogenase, α-ketoglutarate dehydrogenase complex, and the pyruvate dehydrogenase complex specifically associate with Complex I (Sumegi and Srere 1984; Ovàdi et al. 1994; Fukushima et al. 1989). However, their functional role remains controversial (Kotlyar et al. 2004).

The other respiratory enzymes not comprising the "core" of the proton translocation machinery appear not to be associated in supercomplexes (Lenaz and Genova 2010), although only Complex II has been investigated in some detail.

Association of Complex II with other complexes of the respiratory chain has not been identified thus far by BN-PAGE, except in a few cases (Acín-Pérez et al. 2008; Schönfeld et al. 2010). It was recently found that digitonin-solubilised Complex II quantitatively forms high molecular weight structures that can be resolved by CN-PAGE (Kovářová et al. 2013). These structures are enzymatically active and differ in electrophoretic mobility between tissues (500–over 1000 kDa) and cultured cells (400–670 kDa). Complex II has been proposed to be a candidate for physiological association with Complex III (Yu et al. 1974; Gwak et al. 1986).

Mitochondrial glycerol phosphate dehydrogenase has been suggested not to be associated in a supercomplex on the basis of functional studies (Rauchová et al. 1992). This absence has been confirmed in the respirasome, although the enzyme was found to associate in the form of homo-oligomers (Mráček et al. 2014). According to Hildebrandt (2011), sulfide-quinone oxidoreduc-

tase and sulfite oxidase, the two membrane bound enzymes of the pathway catalyzing sulfide oxidation, which transfer electrons into the respiratory chain, might also be associated with a supercomplex in vivo.

C. Megacomplexes or Respiratory Strings

It has been postulated that the assembly of respiratory complexes into supercomplexes is just the first step in the formation of much larger supramolecular structures called *respiratory strings* or *megacomplexes*.

The model of respiratory strings proposed by Wittig et al. (2006) envisions a linear aggregate of alternating Complex III homodimers and Complex IV tetramers, with Complex I bound to some of these units. The existence of these assemblies was demonstrated as the isolation by large pore BN-PAGE of very high mass (35–45 MDa) assemblies of Complex I, III, and IV in bovine heart mitochondria (Strecker et al. 2010). These higher assemblies can be split into individual Complex I, III, and IV complexes under 2D BN/BN-PAGE with dodecylmaltoside, allowing the identification of tetrameric Complex IV and showing the lack of oligomeric Complex I and Complex III. According to Wittig et al. (2006), this evidence suggests a specific and ordered association of these complexes as core pieces of respiratory strings and not random aggregates of respiratory supercomplexes (Fig. 29.2a).

Bultema et al. (2009) postulated a model based on a single-particle electron microscopy of potato mitochondria supercomplex I_2III_2 consisting of two copies of Complex I bound to both sides of a Complex III homodimer (Fig. 29.2b). The model string has a basic unit of Complex III dimer with a Complex I and Complex IV bound to either side, and the contacts are formed by Complex IV dimers.

It is noteworthy that ATP synthase and Complex I occupy different regions of the mitochondrial cristae (Davies et al. 2011). In particular, ATP synthase forms
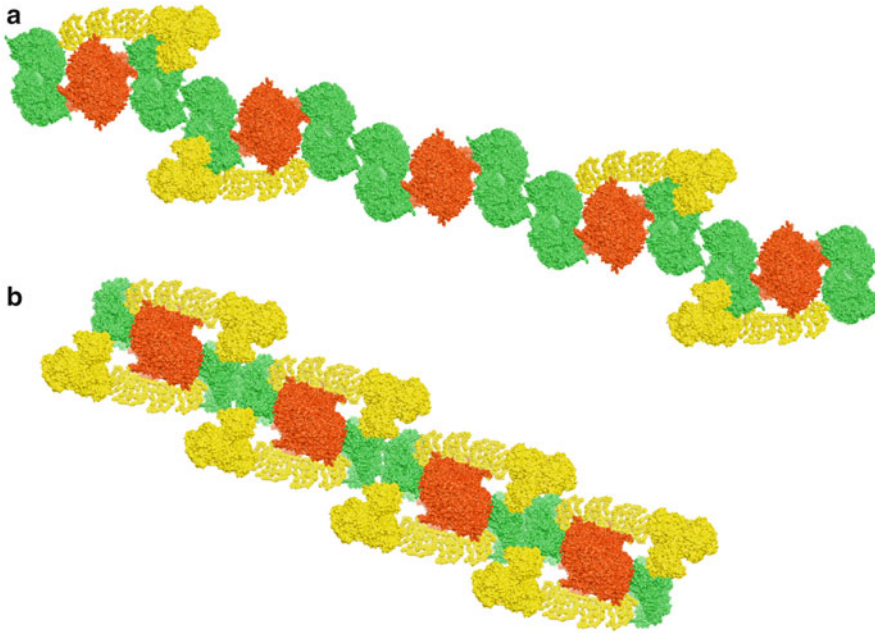
Fig. 29.2. Hypothetical models for a higher organization of respiratory chain complexes (respiratory strings). The model for bovine heart mitochondria in (**a**) is built from a linear combination of $I_1III_2IV_4$ and III_2IV_4 supercomplexes, connected via complex IV tetramers, as described by Wittig and Schägger (2009). The model for potato mitochondria in (**b**) is based on the discussion in Bultema et al. (2009) and consists of $I_2III_2IV_2$ supercomplexes, connected via Complex IV dimers. Complex I is shown in *yellow*, III in *orange*, IV in *green* (Reprinted from Vonck (2012), with permission).

rows of dimers at the cristae tips, whereas the proton pumps of the electron transfer chain reside predominantly on the flat surface of the adjacent membrane regions (Fig. 29.3). The possible correlation between oligomeric rows of ATP synthase and the morphology of the mitochondrial cristae is debated (Habersetzer et al. 2013); electron microscopic studies have shown that the membrane domains of the two partners in the ATP synthase dimer are at an angle to each other, indicating that in vivo the dimers would cause the membrane to bend (Vonck and Schäfer 2009; Chaban et al. 2014). Interestingly, in tomographic reconstructions of mitochondria from some organisms as bovine, yeast and *Arabidopsis,* it is difficult to observe oligomeric chains of dimeric ATP synthases, but the green alga *Polytomella* is an exception to the rule. The tubular cristae of *Polytomella* appear fully covered by oligomeric rows of ATP synthases, suggesting that the other OXPHOS enzymes might be arranged in between, but these can not be easily visualized due to the tight packing of ATP synthases (Dudkina et al. 2010). Few attempts have been made to correlate physiological processes to a potential regulation of the ATP synthase oligomerization. Profound age-dependent changes can be observed in membrane architecture; with increasing age, the typical cristae disappear and the inner membrane vesiculates, the ATP synthase dimers that form rows at the cristae tips dissociate into monomers in inner-membrane vesicles, and the membrane curvature at the ATP synthase inverts (Daum et al. 2013).

III. Structure of Supercomplexes

A. Molecular Structure by Electron Microscopy and Modeling

Supercomplexes can be isolated in their active form after mild detergent solubilization (Stroh et al. 2004; Braun et al. 2009). Partial
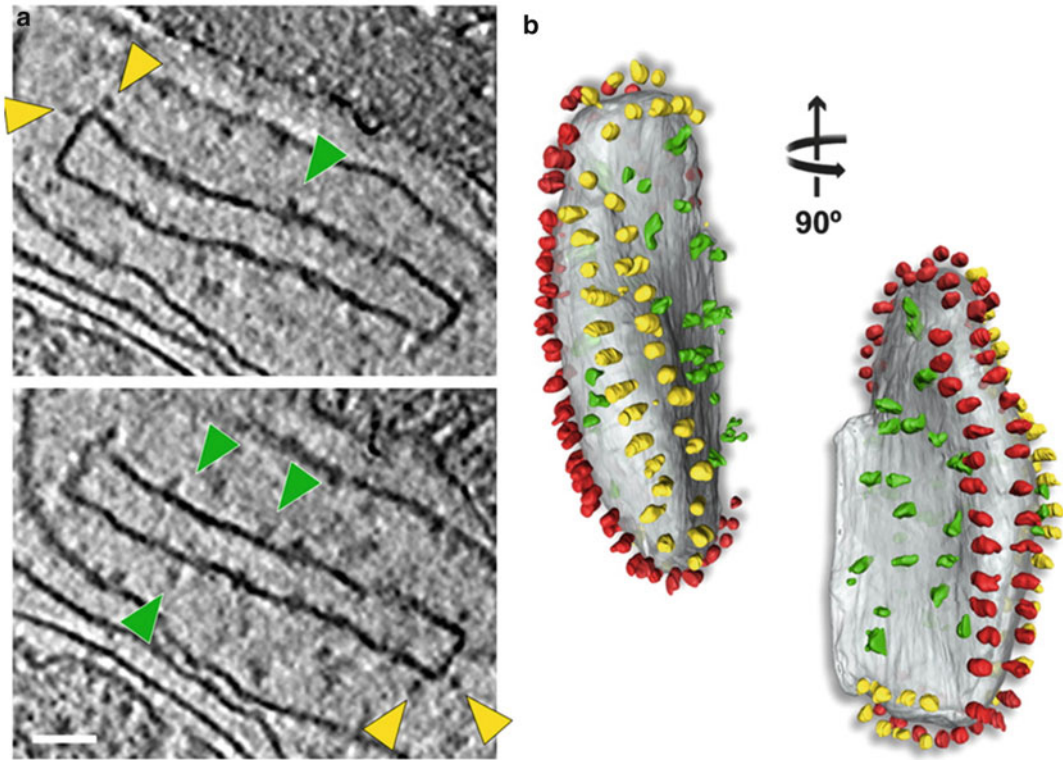
Fig. 29.3. Isolated vesicle from *P. anserina* cristae. (**a**) Two tomographic slices of the same *box-shaped* cristae vesicle showing ATP synthase dimers at approximately 90° bends in the membrane (*yellow arrowheads*) and Complex I densities (*green arrowheads*) in flat membrane regions of the vesicle. (**b**) Segmented surface representation. Two rows of ATP synthase dimers (*yellow* and *red*) in the membrane (*gray*) run along the 90° membrane ridges. Irregularly distributed particles of Complex I or other respiratory chain complexes (*green*) are confined to flat membrane regions. Scale bar, 50 nm (Reprinted from Davies et al. (2011), with permission).

elucidation of the interaction of the individual respiratory complexes within the supercomplex was achieved by single-particle electron microscopy.

Shortly after the first 2D electron projection maps of a supercomplex I_1III_2 from *Arabidopsis* (Dudkina et al. 2005), Schäfer et al. (2006, 2007b) provided initial insights into the shape and size of two respiratory chain supercomplexes isolated from bovine heart mitochondria (I_1III_2 and $I_1III_2IV_1$), whose composition was confirmed by peptide mass fingerprinting.

More recently, new 3D maps at nanoscale resolution allowed interpretation of the architecture of plant and mammalian supercomplexes at the level of secondary structure, confirming the lateral association of dimeric Complex III with the membrane arm of Complex I, while Complex IV, when present, interacts with the distal portion of the same arm. In the new model by Dudkina et al. (2011), the single complexes I, III_2 and IV appear to be at some distance in the bovine respirasome, suggesting that there is little close contact. In particular, the section through the model on the level of the membrane demonstrates gaps between complexes III_2 and IV within the membrane, whereas the same two complexes appear to contact each other in their matrix portions close to the membrane.

Althoff et al. (2011) also proposed that only few points of direct contact are allowed between the three complexes in the mammalian supercomplex $I_1III_2IV_1$ because average distances between the individual complexes exceed 2 nm. Moreover, at 19

Å resolution the membrane-embedded part of the supercomplex shows intermediate values of density between that of the soluble protein and the hydrophobic membrane interior, suggesting that the supercomplex is held together at least partly by lipid-protein interaction. A gap filled with membrane lipid might also facilitate the diffusion of CoQ between Complex I and Complex III. It is interesting to note that the CoQ-binding sites in Althoff's model are conveniently placed for the efficient electron transfer between Complex I and the proximal Complex III monomer over a short distance of about 13 nm to be covered by CoQ. It is proposed that the proximal monomer may be more effective in ubiquinol oxidation, while the distal one may be needed to transfer the electron to cytochrome c via its flexible Rieske domain, in accordance with the half-of-the-sites reactivity model of Complex III (Castellani et al. 2010). Furthermore, a trajectory of 10 nm is envisaged in the respirasome along which cytochrome c may travel to shuttle electrons towards Complex IV (Fig. 29.4).

In agreement with the results of Althoff et al. (2011), three-dimensional density maps of yeast supercomplexes by electron cryo-microscopy show gaps between the transmembrane-localized interfaces of individual complexes consistent with the large excess of cardiolipin in supercomplexes compared to that integrated into the structure of individual respiratory complexes (Mileykovskaya et al. 2012; Mileykovskaya and Dowhan 2014) (cf. Sect. III.B.3).

It has to be borne in mind that, beside the lack of a high resolution crystal structure of the mammalian Complex I, several uncertainties are still present in the interpretation of the 3D maps: for example, there are additional density areas that cannot be filled by the current structural models of complexes III and IV, suggesting that additional proteins may participate in the supercomplexes. This is the case of SCAFI, a recently reported protein required for the super-assembly of complexes III and IV that is not present in the free complexes (Lapuente-Brun et al. 2013), or of the reassignment of NDUF4A as a structural subunit of Complex IV not present in the x-ray structure (Balsa et al. 2012).
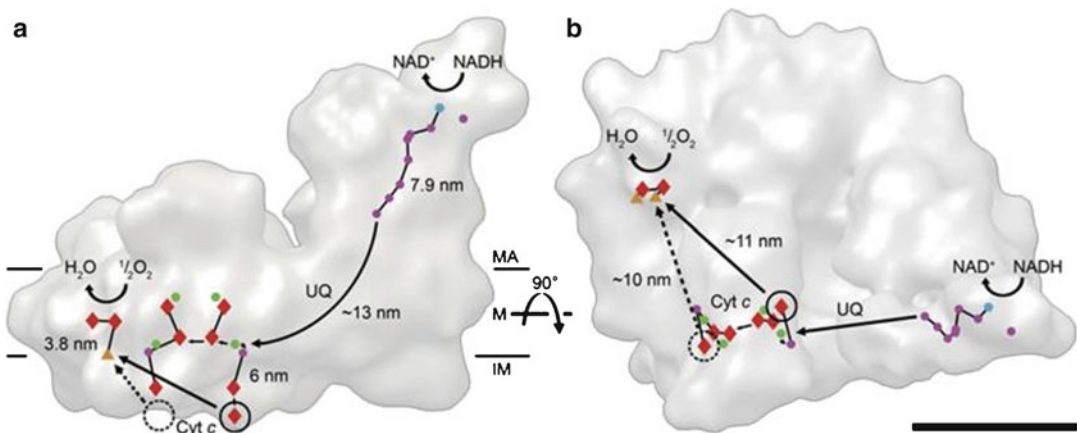
Fig. 29.4. Electron transfer pathways in the supercomplex. Outline of the supercomplex with cofactors active in electron transport marked in *blue* (FMN), *purple* (iron–sulfur-clusters), *green* (quinols/stigmatellins), *red* (hemes) and *orange* (copper atoms), seen from the membrane (**a**) and from the matrix (**b**). Electron trajectories are marked in *black*. The *dashed circle* marks the distal cytochrome c binding site, which is unoccupied in the supercomplex. *Straight arrows* in (**a**) indicate the shortest distances from the cytochrome c binding sites on complex III to the site of cytochrome c oxidation in complex IV. The shorter, proximal branch may be preferred for electron transport. *MA* matrix, *M* membrane, *IM* intermembrane space, *UQ* ubiquinol, *Cyt c* cytochrome c. Scale bar, 10 nm (Image taken from Althoff et al. (2011) and reprinted by permission from Macmillan Publishers Ltd, copyright 2011).

B. Lipid Content and Composition

Lipids are essential determinants of super-complex association (Lenaz and Genova 2007). However, they can modify the nature and extent of the associations only in long time-scales, since their metabolism is affected by the organism species, by the tissue and, within a given tissue and species, by diet and life style.

1. Lipid Composition of Mitochondria

Mitochondria from different cell types have high protein to lipid ratios, as a consequence of the high protein density of the inner membrane (Horvath and Daum 2013), that had been recognized since the early studies (Fleischer et al. 1967). The major phospholipids, accounting for about 80 % of total phospholipids, are phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE). A distinctive feature of mitochondria is the high diphosphatidyl glycerol or cardiolipin (CL) content, in the range of 15–20 %. Phospholipids are not evenly distributed in the inner (IM) and in the outer (OM) mitochondrial membrane nor across their lipid bilayers but rather arranged asymmetrically (Horvath and Daum 2013). Sterols and sphingolipids are only found at low amounts. In different mammalian cells and tissues, the mitochondrial lipid composition is more or less identical. Mitochondria from heart, brain, kidney, adrenal cortex and spleen contain PC and PE plasmalogens in the range of 5–30 % of total phospholipids. Plasmalogens are a class of phospholipids carrying a vinyl ether bond in the *sn-1* and an ester bond in the *sn-2* position of the glycerol backbone. This structure leads to strong lipophilic properties and allows plasmalogens to form inverse hexagonal phase structures. Marine bivalves have considerable amounts of plasmalogens (Kraffe et al. 2004).

Ectotherms tend to have more monounsaturated membranes (lipids with fatty acids with only one C=C) than endotherms of the same size and body temperature (Else et al. 2004).

Decreasing the environment temperature induces fatty acid changes in membrane lipids that have been proposed to help maintaining the fluidity of cellular membranes (*homeoviscous adaptation* (Sinensky 1974)), including mitochondria (Cossins et al. 1980), by producing a generally higher degree of unsaturation (Nozawa 2011).

A unique modification is the presence of non-methylene-interrupted unsaturated fatty acids and branched fatty acids in marine bivalves (Kraffe et al. 2004).

2. Roles of Mitochondrial Lipids

Lipids, and specifically phospholipids, are required for the activity of membrane-bound enzymes (Green and Fleischer 1963; Lenaz 1979). This is achieved in part by a mere dispersive solubilization effect that can be duplicated by appropriate detergents; moreover, lipids are believed to confer on membrane proteins the correct conformation for optimal activity (Lenaz 1979, 1987; Hunte and Richers 2008) and a correct template for membrane protein topogenesis (Dowhan and Bogdanov 2009). In addition, lipids have a specific catalytic effect that is mostly fulfilled by cardiolipin (Vik and Capaldi 1977; Fry and Green 1980, 1981; Robinson et al. 1980; Lee 2004; Wenz et al. 2009). Furthermore, in mitochondria, phospholipids provide a sufficiently lipophilic environment for the interaction of the lipophilic electron carrier, ubiquinone (Lenaz 1988). A further role of lipids is to participate in linking together the protein components of the membrane.

CL differs from all other phospholipids by its dimeric structure, with two headgroups carrying two negative charges and four fatty acyl chains. The fatty acid composition of CL is peculiar (Mileykovskaya et al. 2005; Paradies et al. 2014b): in animals and higher plants, CL almost exclusively contains polyunsaturated linoleic acid (C18:2), while in yeast *S. cerevisiae* there are 16 and 18 monounsaturated chains. CLs in bacteria contain monounsaturated and saturated fatty acids with 14–18 carbons.

It is worth noting that newly synthesized CL contains primarily saturated fatty acids, but CL fatty acyl chains are remodeled in mature CL by phospholipid transacylation in mammalian and yeast cells by a transacylase denominated *tafazzin* (Bogdanov et al. 2008; Baile et al. 2014a, b; Mejia et al. 2014; Horvath and Daum 2013). Although tafazzin *per se* promotes random fatty acyl replacements, it is the physical state of the lipid substrates that provides specificity to the transacylation process (Schlame et al. 2012). Both the precursor phosphatidic acid and mature CL are distributed between the different mitochondrial compartments by the action of protein factors, among which apoprotein O-like (APOOL) (Koob and Reichert 2014) demonstrates a role of CL in determining cristae morphology.

Although CL has two phosphate groups, it is not fully ionized at physiological pH due to intra-molecular hydrogen bonding between the free hydroxyl of the central glycerol and a protonated phosphate (Kates et al. 1993). Due to the four acyl chains and a small headgroup, CL can organize into domains, which have the potential to serve as a proton sink in membranes particularly when in close proximity to OXPHOS complexes that generate and consume $\Delta\tilde{\mu}_H^+$ (Haines and Dencher 2002). Being a dimeric lipid, CL can serve as a "flexible linker", which fills cavities at protein interfaces and thus stabilizes interactions between individual subunits of oligomeric proteins as well as between multi-subunit individual complexes organized into supercomplexes (Hunte 2005; Bogdanov et al. 2008; Mileykovskaya and Dowhan 2009; Schlame et al. 2000; Houtkooper and Vaz 2008) (Sect. III.B.3). The ubiquitous and intimate association between CL and energy-transducing membranes suggests an important role for CL in mitochondrial bioenergetic processes (Mileykovskaya et al. 2005; Paradies et al. 2009, 2014a).

Indeed, CL is required for optimal activity of complexes I, III, IV, and V. Crystallographic studies have shown the presence of a few tightly bound CL molecules in each of the crystal structures of Complex III (Lange et al. 2001; Hunte 2005), Com-

plex IV (Ozawa et al. 1982; Shinzawa-Itoh et al. 2007), and ADP/ATP carrier (Pebay-Peyroula et al. 2003). These results suggest that CL is an integral component of these proteins, the presence of which is critical to protein folding and function. In Complex III, one CL molecule resides at the Qi site (the inner site where CoQ is reduced in the Q-cycle mechanism) suggesting direct involvement of CL in proton uptake at the ubiquinone reduction site. A second CL was found in the hydrophobic cleft close to the homodimer interface of Complex III (Hunte 2005). Two CLs were resolved in Complex IV (Shinzawa-Itoh et al. 2007). A third CL, found by photolabeling with arylazido-containing CL analogues (Sedlák et al. 2006), is located near the entrance to the putative proton pumping channel of Complex IV and thus might facilitate proton entry into the channel.

CL is also observed in the crystal structure of *R. sphaeroides* reaction center, where one CL molecule stabilizes two membrane-spanning α-helices of the M polypeptide (Fyfe and Jones 2005), in *E. coli* succinate dehydrogenase (Yankovskaya et al. 2003), formate dehydrogenase-N (Jormakka et al. 2002) and cytochrome c (Musatov et al. 2013; Muenzner and Pletneva 2014).

3. Lipids and Supercomplex Stabilization

The forces responsible for supercomplex association strongly depend on the lipid content and composition and likely on the shape of the inner mitochondrial membrane (Lenaz and Genova 2007).

At low protein concentrations in lipid bilayers, proteins are usually randomly dispersed (Lee 2004), whereas at high concentrations they tend to aggregate. The forces involved in protein aggregation are both of entropic and enthalpic origin.

Lipid-mediated protein-protein interactions originate by hydrophobic mismatch: the same reasons inducing the formation of a lipid annulus to compensate for the mismatch by attracting the lipids having the best fit would be operative by attracting

together proteins having mismatch of the same sense (Bloom et al. 1991). For the same reason, however, proteins having hydrophobic mismatch of opposite sense should repel each other (Gil et al. 1998).

In the mitochondrial inner membrane, integral proteins of the OXPHOS system are densely packed so that the average distance between complexes may be calculated to be few nanometers (Lenaz 1988). Thus, the presence of protein aggregates of the transmembrane respiratory complexes would not be unexpected on theoretical grounds. Changing the nature of the lipids would dramatically change their matching conditions to the proteins. This could be due to presence and number of electrostatic charges, different lengths of the fatty acyl chains, and of physical state and phases existing at the body temperature. Lipid peroxidation removes *cis* double bonds from the fatty acyl chains, inducing profound biophysical changes in the lipid bilayer (Stark 2005), such as increased disorder and possible breakdown of membrane structure and of lipid-protein interactions (Megli and Sabatini 2003), and presumably affecting the membrane thickness. Therefore, a change in membrane protein aggregation induced by lipid peroxidation can be expected on theoretical grounds.

Since the integral membrane proteins are not fully deformable entities, their insertion in a lipid bilayer can distort the shape of the membrane locally; the lowest energy would be achieved in a system able to induce the least possible mismatch of the protein with the acyl chains in the surrounding lipid domain of *boundary lipids* or *annular lipids* (Jost et al. 1973; Hesketh et al. 1976). The lipid annulus is not a static entity, but rather a dynamic one, where the exchange of lipid molecules with the surrounding bulk phase is very fast (Marsh and Horvath 1998). The annular lipids must not be confused with those lipids that are usually detected in high-resolution crystal structures of membrane proteins and defined *non-annular* or *cofactor lipids* (Lee 2004) (see Sect. III.B.2). In particular, the studies by Ragan (Ragan and

Heron 1978; Heron et al. 1978) and Genova et al. (2008) have shown that reconstitution of a binary I/III mitochondrial protein fraction (R4B) at a high lipid to protein ratio (>10:1) prevents formation of the I-III supercomplex. Thus, it appears that dilution of the proteins with an excess of phospholipids may weaken the forces holding together the respiratory complexes.

Among the lipids, CL and PE are both non-bilayer-forming phospholipids, due to their small polar heads compared with the bulky nonpolar tails (Van den Brink-van der Laan et al. 2004; Osman et al. 2011). The phospholipids in closest vicinity to the protein surface, as well as those in the free bilayer, are highly mobile and free to exchange, but CL is tightly bound being more likely buried within the protein complexes, as in Complex IV and Complex III (Kang et al. 1979; Lange et al. 2001).

The structural studies of the purified mammalian and yeast respiratory supercomplexes by single particle cryoelectron microscopy (Althoff et al. 2011; Mileykovskaya et al. 2012) and cryoelectron tomography (Dudkina et al. 2011) (see Sect. III.A), resulting in three-dimensional density maps of atomic models for these structures, showed the presence of spaces between transmembrane domains of individual complexes which may by filled with phospholipids and suggested that CL may be an essential component for the assembly of supercomplexes.

Further evidence concerning this subject has been collected for the III_2-IV_2 supercomplex in yeast mutants lacking CL (Pfeiffer et al. 2003; Zhang et al. 2002; Mileykovskaya et al. 2005). The putative direct protein-protein interaction of cytochrome oxidase and Complex III in yeast is proposed to involve also one molecule of CL and one of PE, tightly bound in a cavity of the membrane imbedded domain of Complex III (Lange et al. 2001). It has been suggested that these two phospholipids can provide a flexible linkage between the interacting subunits of Complexes III and IV as well as with the ADP/ATP carrier that is also known to exist in physical association with the III-IV

supercomplex (Claypool et al. 2008). Site-directed mutagenesis investigations (Wenz et al. 2009) indicated that CL stabilizes supercomplex formation by neutralizing the charges of lysine residues in the interaction domain of Complex III with cytochrome c oxidase.

In addition, the stability and assembly of Complex IV was found to be reduced in yeast cells lacking Taz1 (Brandner et al. 2005; Li et al. 2007), the orthologue of human Tafazzin, the acyl transferase involved in the maturation of CL (see previous section).

Mutations of Tafazzin in humans result in Barth syndrome, a cardio-skeletal myopathy with neutropenia, characterized by respiratory chain dysfunction. Patient mitochondria display lower CL content and a poly-dispersity in acyl chain composition of CL (Schlame and Ren 2006). BN-PAGE reveals an increase in free CIV monomer and a decrease in the supercomplex $I_1III_2IV_1$ in patient lymphocytes (McKenzie et al. 2006). In a pluripotent stem cell model system of this disorder Dudek et al. (2013) found an impaired remodeling of CL, a dramatic decrease in basal oxygen consumption rate, and in the maximal respiratory capacity; BN-PAGE analyses revealed that decreased respiration coincided with dramatic structural changes in respiratory chain supercomplexes leading to a massive increase in generation of reactive oxygen species.

Significantly, the CL defect in Barth syndrome results in destabilization of the supercomplexes by weakening the interactions between respiratory complexes (McKenzie et al. 2006). Gonzalvez et al. (2013) confirmed that in immortalized lymphoblasts from Barth's syndrome patients the amount of supercomplexes is decreased, as well as the amount of individual complexes I and IV; these changes were compensated by increasing mitochondrial mass.

A recent study from Claypool's group (Baile et al. 2014b) confirmed the absolute requirement for CL, although changes in fatty acid composition did not affect mitochondrial morphology and oxidative phosphorylation, concluding that the mi-tochondrial changes in Barth's syndrome are not due to changes of fatty acid composition, but are probably the result of accumulation of lysoCL and/or the concomitant decrease of fully acylated CL.

The dependence on CL for supercomplex formation was clearly demonstrated in a study by Bazán et al. (2013). By employing a system composed of purified CIII and CIV from *S. cerevisiae* and liposomes of different phospholipid composition, these authors demonstrated, for the first time, the reconstitution of the supercomplexes III_2IV_1 and III_2IV_2 from individual CIII and CIV in proteoliposomes and specific dependence of III_2IV_2 formation on liposomes containing CL in strong preference over other phospholipids.

It is well documented that exposure of mitochondria to reactive oxygen species (ROS) can affect the respiratory activity via oxidative damage of CL, which is required for the optimal functioning of the enzyme complexes (Paradies et al. 2000, 2002; Petrosillo et al. 2003), reviewed by Paradies et al. (2010, 2014a). Genova et al. (2008) demonstrated by flux control analysis that the maintenance of a I-III supercomplex in proteoliposomes is abolished if lipid peroxidation is induced by 2,2′-azobis-(2-amidinopropane) dihydrochloride before reconstitution. Evidently, the distortion of the lipid bilayer induced by peroxidation and the alteration of the tightly bound phospholipids determine dissociation of the supercomplex originally present in the preparation.

C. Biogenesis of Respiratory Supercomplexes

Acín-Pérez et al. (2008) performed pulse-chase experiments, labeling the 13 mtDNA-encoded proteins from cultured mouse cells, and analyzing the time course of their incorporation into respiratory complexes and supercomplexes by 2D electrophoresis.

The labeling of large supercomplexes showed a different pattern depending on whether or not they contained Complex IV. Thus, supercomplexes I/III appear labeled

as soon as after a 0.5 h chase. On the contrary, no labeling of supercomplexes I/III/IV was observed after a 0.5 and 2 h chase despite the fact that fully assembled Complex IV already could be observed. The labeling of the supercomplexes did not reach the characteristic pattern until after a 12 h chase. These results support the idea that supercomplexes are structures formed by the specific and ordered association of previously assembled individual complexes (Acín-Pérez et al. 2008).

The existence of protein factors that regulate the assembly of the different respiratory complexes into respirasomes is fully compatible with this model. A number of independent reports recently described two related *S. cerevisae* proteins (renamed as Rcf1 and Rcf2) that might be important for the assembly between complexes III and IV (Chen et al. 2012; Strogolova et al. 2012; Vukotic et al. 2012; Shoubridge 2012). Rcf1 is a member of the hypoxia inducible gene 1 (HIG1) family, and it has at least two mammalian orthologs, HIG1A and HIG2A. Human HIGD2A partially suppressed the phenotype caused by deletion of the yeast Rcf1 (Vukotic et al. 2012), and siRNA-mediated knockdown of the murine homolog of HIGD2A resulted in a decrease in supercomplexes containing CIV (Chen et al. 2012), suggesting that the mammalian protein is also important in stabilizing CIV-containing supercomplexes. According to Acín-Peréz and Enriquez (2014), however, the role of HIGD2A in supercomplex assembly seems to be marginal, and these factors can therefore be understood as being required for Complex IV function rather than specific supercomplex assembly factors.

In contrast, SCAFI, a recently described protein factor that is required to allow the integration of Complex IV into supercomplexes (Lapuente-Brun et al. 2013), modulates the interaction between Complex III and IV without affecting their assembly or stability, and is thus a true supercomplex assembly factor (Lapuente-Brun et al. 2013).

Moreno Lastres et al. (2012) proposed a different path for supercomplex formation. After depleting cells of OXPHOS complexes by treatment with doxycycline, an inhibitor of mitochondrial translation, they followed complex and supercomplex assembly by BN-PAGE and immunodetection of individual proteins as markers of the overall complexes or subcomplexes. This analysis led them to propose that biogenesis of the respirasome involves the coordinated and sequential association of specific combinations of partially assembled respiratory complexes and free subunits. This model has major implications because it concludes that Complex I cannot be fully assembled nor catalytically active in the absence of Complex III and/or IV. In other words, the respirasome assembly is intimately linked to Complex I assembly and function.

The latter model cannot explain the normal assembly, stability and functionality of Complex I in cells lacking Complex IV (Balsa et al. 2012) and Complex III (Diaz et al. 2012a) in different culture conditions (Enríquez and Lenaz 2014). Nor can it explain either the stability or function of Complex I in fungi that lack Complex III and IV but naturally respire by an alternative oxidase (AOX) (Maas et al. 2009).

Recently, Davoudi et al. (2014) used a transgenic mouse model with decreased expression of BCS1L leading to loss of the Rieske protein of Complex III. In young healthy homozygous mutant mice, liver mitochondria had normal supercomplexes as in wild type; in the same aged mice that had developed hepatopathy, the main supercomplex contained Complex III in a pre-complex form lacking the Rieske protein. No disassembly of Complex I was evident in this model in which ROS generation was not enhanced, supporting the conclusions of Diaz et al. (2012b) that Complex I disassembly in the absence of active Complex III is the result of ROS damage (see Sect. IV.E).

Besides the above mentioned assembly factors required for the biogenesis and assembly of the supercomplexes, to our knowledge one more protein has been recently described that may be involved in the reversible regulation of supercomplex

association and activity: Hatle et al. (2013) identified MCJ/DnaJC15 as a distinct co-chaperone that localizes at the mitochondrial inner membrane, where it interacts preferentially with Complex I and functions as a negative regulator of the respiratory chain by impairing the formation of supercomplexes.

It is also worth mentioning that genetic ablation or overexpression of OPA1 with consequent disorganization of the cristae structure of the inner mitochondrial membrane can affect assembly and activity of supercomplexes (Cogliati et al. 2013).

IV. Functions of Respiratory Supercomplexes

There is now extensive evidence that supercomplexes are not artifacts due to detergent-induced associations, but are present physiologically and have a definite function, providing several physiological advantages.

A. Respiratory Supercomplexes are Functional

The supercomplexes were found to possess the in-gel activities of the individual complexes (Schägger and Pfeiffer 2000, 2001). However, the first demonstration that they are indeed functional and capable of full activity came from the chromatographic isolation of a functional respirasome from *P. denitrificans* (Stroh et al. 2004). The respirasome contained complexes I, III, and IV in a fixed stoichiometry, was enriched in CoQ with respect to the original membrane, but was considerably deficient in cytochrome c_{552}; for this reason, although the activity of NADH cytochrome c reductase was close to that expected from the rate-limiting activity of Complex I, the whole NADH oxidase was strongly impaired with respect to the expected rate.

The respiratory activity of a respirasome isolated from mammalian mitochondria was reported by Acín-Pérez et al. (2008) who performed an extensive study on isolated mouse liver mitochondria and mitochondria from mouse fibroblast cell lines. They demonstrated that BN-PAGE bands containing both CoQ$_9$ (the major CoQ homologue in rodents) and cytochrome c showed complete NADH oxidase activity, as measured using a Clark electrode, whereas a mixture of the bands of the individual complexes I, III, and IV, was inactive. In contrast to previous studies, some bands of supercomplexes also contained Complex II and ATP synthase and showed full respiratory activity from succinate that was sensitive to the specific respiratory inhibitors of all involved complexes. It is, however, possible that co-migrations of complexes II and V are due to overlapping and not to real associations.

A crude mitochondrial preparation (R4B) containing complexes I and III (Hatefi and Rieske 1967) is largely in the form of supercomplex I-III, as shown by BN-PAGE (Fig. 29.5), and exhibits high NADH-cytochrome c reductase activity, demonstrating that the isolated supercomplex is fully functional (Maranzana et al. 2013).

These findings directly confirm that supercomplex organization is compatible with electron transfer; however, it does not exclude the idea that electron transfer is still possible in the membrane in the absence of supercomplex organization, in accordance with the random collision model (see Sect. IV.B.1.a).

B. Kinetic Advantage

Immediately after the discovery of supercomplexes it was proposed that the natural consequence of such assemblies is substrate channeling or enhanced catalysis in inter-complex electron transfer. Substrate channeling is the direct transfer of an intermediate between the active sites of two enzymes catalyzing consecutive reactions (Ovàdi 1991); in the case of electron transfer, this means direct transfer of electrons between two consecutive enzymes by successive reduction and reoxidation of the intermediate without its diffusion in the bulk medium. In such a case, inter-complex electron transfer becomes indistinguishable
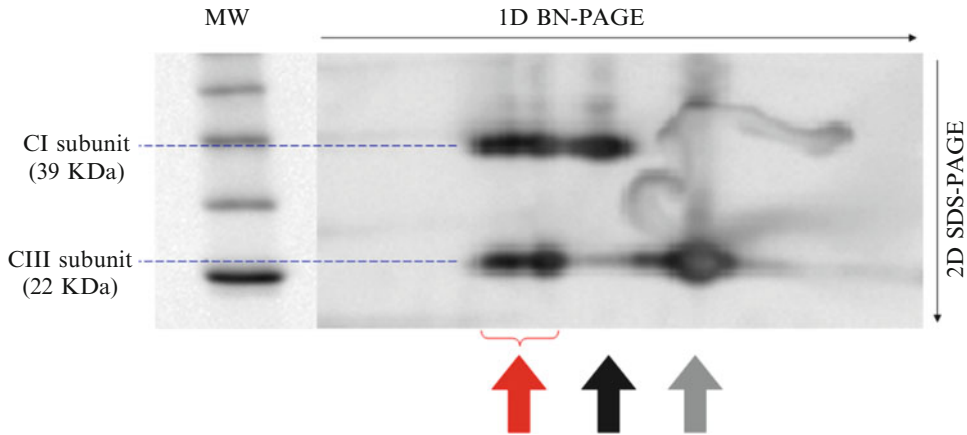
Fig. 29.5. Respiratory supercomplexes and complexes from a crude mitochondrial fraction (R4B) enriched in Complex I and Complex III. The digitonin-solubilized R4B sample (detergent to protein ratio 8:1 w:w) was separated by 2D-BN/SDS-PAGE and resolved by Western blotting followed by immunodetection using monoclonal antibodies (MitoSciences, Inc., Eugene, OR; available for sale through Abcam) specific for single respiratory subunits as follows: NDUFA9 (39 kDa) of Complex I and Rieske protein (22 kDa) of Complex III. Arrows point to monomeric Complex I (*black*), dimeric Complex III (*gray*), and high molecular weight supercomplexes (*red*) containing both Complex I and Complex III.

from intra-complex electron transfer, so that the so-called mobile intermediates, predicted to exhibit substrate-like behavior in the classic view of the random collision model, function more efficiently when buried in the interface between the two consecutive complexes (Lenaz and Genova 2007).

The mechanism of electron transfer in supercomplexes is still uncertain. Ideally, we should have a detailed knowledge of the molecular structure and/or distances of the interacting sites, and this knowledge is still lacking. The extreme models are i) close docking of the active sites with real inter-protein tunneling, up to relatively long distances that may be covered either by important conformation changes, or ii) restricted diffusion (microdiffusion) of the "mobile" components within the space between the two active sites. All of these alternatives have in common obligate channeling between two fixed sites, so that even the last mechanism, microdiffusion, would be quite distinguishable from pool behavior of CoQ or cyt. c. In the latter case, the interaction of the mobile component may stochastically occur with a great number of possible sites located on several different protein targets which can be reached by random diffusion (Genova and Lenaz 2011). In the interaction between Complex I and Complex III within a supercomplex, if the sites are connected by CoQ microdiffusion, it is possible that it takes place within a lipid milieu, although we cannot exclude that the sites are put together by movement of CoQ on the protein or by movement of the protein itself.

Some evidence for possible channeling comes from the 3D structure of the mitochondrial supercomplex $I_1III_2IV_1$ described by Althoff et al. (2011); a unique arrangement of the three component complexes indicates the pathways along which ubiquinone and cytochrome c can travel to shuttle electrons between their respective protein partners (cf. Sect. III.A). In the model of Althoff et al (2011), one of the Complex III monomers faces the lipid bilayer while the other is surrounded by Complex I. Therefore, Coenzyme Q is likely to run a trajectory through a 13 nm gap within the membrane core of the supercomplex which is presumably filled with membrane lipids. It was estimated that approximately 300 lipid molecules would fit the gap that extends between complexes I, III, and IV on the level

of the membrane-embedded portion of the supercomplex (Althoff et al 2011; Dudkina et al. 2011; Chaban et al. 2014).

1. From Complex I to Complex III

The rate of electron transfer between membrane-bound electron carriers depends on their structural organization in the membrane. If two redox enzymes are connected by a mobile redox carrier undergoing long-range random diffusion in the medium, the overall reaction rate would be governed by the frequency of useful collisions between the mobile carrier and its two redox partners. On the other hand, if the system is fixed in a solid state assembly, the frequency of encounters will be dictated by the constraints that determine the interaction between the redox partners (*channeling)*.

a. Evidence of Channeling

The random collision model of electron transfer (Hackenbrock et al. 1986) had been anticipated by an extensive kinetic evidence in the previous decade, that led investigators in the field to advance the notion of a homogeneous and freely diffusible Coenzyme Q pool in the lipid milieu of the inner mitochondrial membrane, whose redox state determines the overall electron flux observed during steady-state respiration. Evidence was provided by the effect of changing the rate of ubiquinol oxidation by means of inhibitor titration of Complex III (Kröger and Klingenberg 1973b), which shows a characteristic convex hyperbolic relationship between the overall rate of electron transfer and the fraction of Complex III inhibited by antimycin. On the contrary, a linear relationship is expected in the case of a stoichiometric association between Complex III and the CoQ-reducing enzymes. However, most available data on CoQ pool concern succinate oxidation (i.e. Complex II and Complex III integrated activity) in submitochondrial particles, whereas fewer data are available for Complex I-dependent activity (reviewed in Lenaz and Genova 2007, 2009; Genova and Lenaz 2011).

Interestingly, Kröger and Klingenberg (1973a, b) already noticed that 10–20 % of CoQ in submitochondrial particles is not reduced by any substrate. More recently Benard et al. (2008) described the existence of three different pools of CoQ in rat liver and muscle mitochondria: one pool is utilized during succinate-dependent steady-state respiration, another (approx. 8 % in muscle and 23 % in liver) is mobilized as a reserve in case of a perturbation to maintain the energy fluxes at normal values (e.g. due to inhibition of the respiratory complexes or in case of mitochondrial diseases), and a third one (approx. 79 % in muscle and 21 % in liver) cannot be mobilized at all. These results are compatible with CoQ compartmentalization, although similar results with NADH oxidation were not provided in order to functionally prove that the fraction of CoQ that is not utilizable for succinate oxidation is channeled within supercomplex I-III.

This compartmentalization prevents significant cross talk between NADH oxidation (Complex I-dependent) (Fig. 29.6a, b) and succinate oxidation (dependent on Complex II) or other flavoenzyme-dependent oxidations (Fig. 29.6c).

Those Complex III molecules that physically interact with Complex I in the formation of supercomplexes are exclusively dedicated to NADH oxidization by a dedicated subset of CoQ molecules ($CIII_{NADH}$), while those that are not bound to Complex I are mainly responsible for oxidation of succinate and other substrates using the free CoQ pool (Lapuente-Brun et al. 2013). Complex I has a very high affinity for Complex III, so that this association is preferred to the free state of either Complex I or Complex III. When a partial loss of Complex III occurs, NADH oxidation by the $CIII_{NADH}$ enzyme is preferentially maintained despite the risk of compromising the oxidation of FAD-linked substrates that, on the contrary, rely on the diminished availability of CIII enzyme in free form.
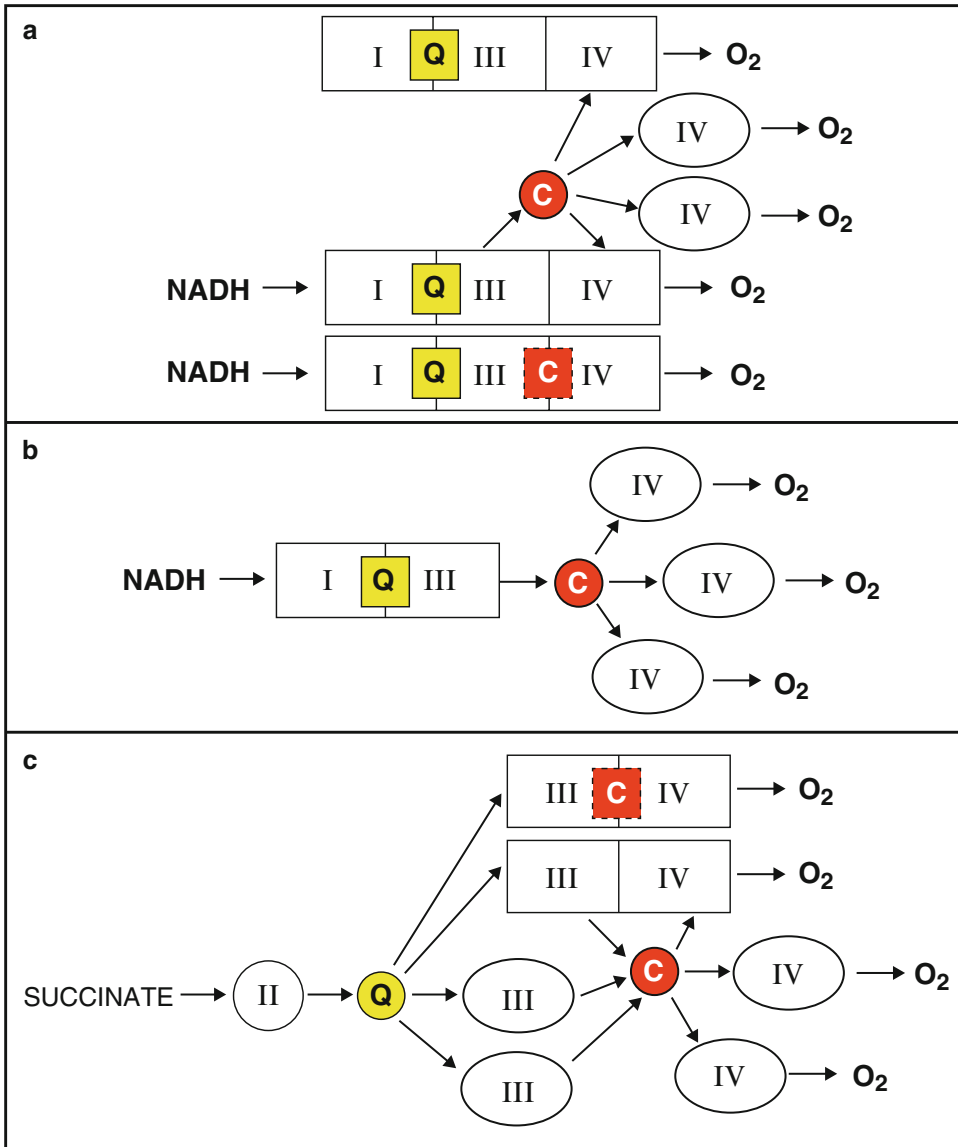
Fig. 29.6. Proposed organization of the respiratory chains in mammalian mitochondria. (**a**) Representation of respirasomes (I-III-IV) and free cytochrome oxidase (IV) molecules; in this model, electron transfer between CI and CIII always occurs by CoQ (Q) channeling, whereas fast dissociation of cytochrome c (C) from CIII may also allow electron transfer by random collisions of free cytochrome c with CIV, either free or bound in other respirasomes. (**b**) scheme of the possible function of the supercomplex formed by CI and CIII; it is postulated that free cytochrome c collides with uncomplexed CIV units. (**c**) Depiction of the mechanism of electron transfer from succinate via free CII and the CoQ pool. This model may also apply to other enzymes feeding electrons to the CoQ pool (see text for details). In this case, the CoQ pool interacts with either free CIII or associated CIII/CIV. The CIII units are shown as monomers for the sake of simplicity (Reprinted from Genova and Lenaz (2013), with permission from De Gruyter Publisher (http://www.degruyter.com/)).

The first studies on the mechanism of electron transfer between Complexes I and III were performed by Ragan (Ragan and Heron 1978; Heron et al 1978). They showed that these Complexes can be mixed in stoichiometric ratios to give rise to a functional

unit having NADH cytochrome c reductase activity; the formation of a stable unit depended on a specific lipid environment.

A more direct comparison of the effect of channeling with respect to CoQ-pool behavior was performed in a simpler experiment. A system obtained by fusing R4B with different amounts of phospholipids and CoQ_{10} (Lenaz et al. 1999) was used to discriminate whether the reconstituted protein fraction behaves as individual enzymes (CoQ-pool behavior), or as assembled supercomplexes depending on the experimental distances between the intramembrane particles. The comparison of the experimentally determined NADH-cytochrome c reductase activity with the values expected by theoretical calculation applying the pool equation (Eq. (29.5), Kröger and Klingenberg 1973a), where V_{obs} is the overall flux observed, V_{red} is the rate of CoQ reduction and V_{ox} is the rate of ubiquinol oxidation,

$$V_{obs} = (V_{red} \cdot V_{ox}) \,/\, (V_{red} + V_{ox}) \quad (29.5)$$

showed overlapping results at phospholipid dilutions (w:w) greater than 1:10, i.e. for theoretical distances >50 nm. On the contrary, pool behavior was not effective and the observed rates of NADH-cytochrome c reductase activity were higher than the theoretical values (Lenaz et al. 1999; Bianchi et al. 2003; Genova et al. 2008) at low protein:lipid dilution (1:1 w:w). This ratio corresponds to the mean nearest neighbor distance between respiratory complexes in mitochondria (Lenaz 1988). Moreover, when the same proteoliposomes at 1:1 lipid:protein ratios were treated with dodecyl maltoside to dissolve the supercomplex organization, the NADH cytochrome c reductase activity fell dramatically, whereas both Complex I and Complex III individual activities were unchanged (Maranzana et al. 2013); an analogous behavior was detected by treating bovine heart mitochondria with the same detergent.

Additional evidence for channeling between complexes I and III derives from the demonstration that selective decrease of Complex III content under a given threshold induces a preferential decrease of electron transfer rate between complexes II and III, while the transfer of electrons between Complex I and III remains unaltered (Lapuente-Brun et al. 2013).

The assumption that supercomplex association has the major function of conferring a rate advantage by substrate channeling arose together with the discovery of supercomplexes (Schägger and Pfeiffer 2000). Although the theoretical basis of channeling by metabolic control analysis was already established (Kholodenko and Westerhoff 1993), the first demonstration of respiratory supercomplex association on a functional basis was achieved some years later (Bianchi et al. 2003, 2004). The study by Bianchi et al. (2004) exploited flux control analysis (Kholodenko and Westerhoff 1993) and the principle that the sum of the flux control coefficients of the individual enzymes in an integrated pathway must equal 1 unless these enzymes form supramolecular units and establish substrate channeling. In the latter case, these enzymes would all be equally rate-limiting and the sum of the control coefficients would be greater than 1 (Kholodenko and Westerhoff 1993). Using specific inhibitor titrations, the flux control coefficients were investigated of the complexes involved in aerobic NADH oxidation (I, III, IV) and succinate oxidation (II, III, IV) in bovine heart mitochondria and submitochondrial particles (Bianchi et al. 2004). Both Complex I and Complex III were found to be rate-limiting for NADH oxidation, kinetics that suggest the existence of functionally relevant association between the two complexes, whereas Complex IV appears randomly distributed. That is, a large excess of active Complex IV exists in free form in the pathway from NADH to oxygen (see Sect. IV.B.2). Moreover, Complex II is rate-limiting for succinate oxidation, clearly indicating the absence of substrate channeling toward Complexes III and IV. This approach

is similar to that of Boumans et al. (1998), who had found that CoQ does not follow pool behavior in yeast mitochondria unless they are treated with chaotropic agents.

Other studies addressed to the functional aspects of supercomplexes using metabolic control analysis (Quarato et al. 2011; Kaambre et al. 2012, 2013) confirmed that the respiratory chain, at least under certain conditions, is organized in functionally relevant supramolecular structures. In digitonin-permeabilized HepG2 cells, it was observed that under conditions of high membrane potential the sum of the flux control coefficients calculated for the activities of Complexes I, III and IV exceeded 1, supporting the proposition that they are complexed in a supramolecular unit Quarato et al. (2011) (see Sect. IV.F.1 for further discussion of the role of membrane potential). In saponin-permeabilized breast and cholorectal tumor samples Kaambre et al. (2012, 2013) observed flux control coefficients for mitochondrial oxidative phosphorylation activities whose sum approached 4, in contrast with lower values for normal tissues; they also interpret the data as due to the presence of supercomplex association.

b. Saturation Kinetics

The finding that Complex I is almost totally associated in a supercomplex with Complex III seems to exclude a role for the CoQ pool in physiological electron transfer between these two complexes. Surprisingly, strong evidence exists that NADH cytochrome c reductase activity follows saturation kinetics with respect to CoQ.

The relation between electron transfer rate and CoQ concentration was seen for NADH and succinate oxidation in reconstituted systems (Norling et al. 1974; Estornell et al. 1992) and in phospholipid-enriched mitochondria (Schneider et al. 1982). Direct titrations of CoQ-depleted mitochondria reconstituted with different CoQ supplements yielded a "K_m" for NADH oxidation by CoQ in the range of 2–5 nmol CoQ mg^{-1} mitochondrial protein (Estornell et al. 1992), corresponding to a CoQ concentration of 4–10 mM in the lipid bilayer.

The presence of saturation kinetics demonstrates that free CoQ in the pool is also necessary for proper channeling between Complex I and Complex III. In fact, the bound inter-complex quinone that allows electron flow directly from Complex I to Complex III must be in dissociation equilibrium with the CoQ pool, so that its amount, at steady state, would be dictated by the size of the pool: this equilibrium explains the saturation kinetics for total ubiquinone exhibited by the integrated activity of Complex I and Complex III and the decrease of respiratory activities in mitochondria fused with phospholipids with subsequent dilution of the CoQ pool. To be in agreement with the experimental observation obtained by metabolic flux analysis, this proposition requires that the dissociation rate constants of bound CoQ be considerably slower than the rates of inter-complex electron transfer via the same bound quinone molecules (Lenaz and Genova 2007). Earlier studies by Heron et al. (1978) also reported that endogenous ubiquinone-10 leaks out of the Complex I-III unit when extra phospholipid is present, causing a decrease in activity that could be alleviated by adding more ubiquinone. It is likely that the function of the large amount of ubiquinone in the natural membrane may be, therefore, to maintain the ubiquinone-10 content in the supercomplex unit when it is formed.

The notion that inter-complex bound CoQ is in chemical equilibrium with CoQ in the pool is sufficient to explain the improved cell bioenergetics upon addition of exogenous CoQ. It is expected that even a slight decrease of CoQ content in the membrane is sufficient to dissociate part of the quinone from the supercomplex, thus decreasing the rate of electron channeling.

This observation may be particularly important in view of the assumption that the

supercomplex association is physiologically dynamic according to the plasticity model of supercomplex association (Acín-Pérez et al. 2008). It also means that, if supercomplexes are dissociated in physiological or pathological conditions, the CoQ content of the membrane becomes less saturating for electron transfer; the obvious consequence is that in this condition an exogenous CoQ supplementation may be extremely useful to sustain a high respiratory rate. The efficacy of exogenous CoQ_{10} supplementation in several disease states has been shown in many studies (cf. Genova and Lenaz 2011).

2. From Complex III to Complex IV

a. Early Kinetic and Biophysical Studies

Although the pool equation has been developed for CoQ between dehydrogenases and bc_1 complexes, the same reasoning is valid for cytochrome c that shares with ubiquinone the postulated role of a mobile substrate-like component of the respiratory chain (Froud and Ragan 1984a, b).

Nevertheless, the role of cytochrome c mobility between Complex III and Complex IV is even more intriguing than that of ubiquinone (Fig. 29.6) (Lenaz 1988). Hackenbrock and coworkers (Gupte and Hackenbrock 1988a, b) observed that under physiological conditions of 150 mM ionic strength, cytochrome c is readily dissociated from the membrane and undergoes three-dimensional diffusion in the inter-membrane space. In reconstituted enzyme systems in the presence of excess cytochrome c, "pool behavior" is followed (Froud and Ragan 1984a); in liposomes consisting of cardiolipin and dimyristoyl lecithin, however, when the latter lipid is in the gel phase, the rotational diffusion of spin-labelled cytochrome c is slowed, suggesting that the cytochrome c pool is bound to cardiolipin molecules, whose lateral and rotational diffusion in the bilayer is adequate to account for electron-transport rates (Froud and Ragan 1984a). Likewise, the rotation rates of

cytochrome oxidase reconstituted in lipid vesicles are not influenced by simultaneous incorporation of Complex III (Kawato et al. 1981), suggesting that Complex III and Complex IV act as independent entities.

However, a similar study in mammalian mitochondrial membranes allowed the conclusion that only about one-half of cytochrome oxidase contributed to the observed rotational relaxation rate, the remainder being relatively immobile, and that the fraction of aggregated protein progressively decreases with decreasing concentration of proteins in the membrane following the fusion of soybean phospholipid vesicles with mitoplasts (Kawato et al. 1980, 1982).

b. Flux Control Analysis

Flux control studies clearly established that CI and CIII behave as a functional supercomplex in bovine heart and rat liver mitochondria, whereas Complex IV does not since it has a low flux control coefficient, despite the BN-PAGE showing that a fraction of Complex IV is physically associated in the respirasome in the same mitochondrial samples (Bianchi et al. 2004; Genova et al. 2008; Lenaz et al. 2010).

The reason for this discrepancy is not clear. The flux control studies allow to conclude that the free molecules of CIV are involved in electron transfer from NADH, and imply that the CIV molecules in the supercomplex do not receive electrons from CIII by channeling of cytochrome c (Fig. 29.6) (Genova and Lenaz 2013, 2014). Of course, branched pathways are difficult to approach by metabolic control analysis and measurements in isolated mitochondria by inhibitor titration of the relevant enzymes inevitably involves using assay conditions (i.e. substrate concentrations) that are not necessarily intended to reproduce in vivo conditions.

Moreover, the non-artifactual nature of the lack of functional channeling shown by the flux control coefficients in mammalian mitochondria is supported by the fact that in

potato tuber mitochondria the respirasome seems to be completely functional in channeling (Genova et al. 2008). The reason for the difference may be in a tighter binding of cytochrome c in the respirasome. Likewise, Boumans et al. (1998) found evidence for cytochrome c channeling in *S. cerevisiae* mitochondria; accordingly, single particle cryo-electron microscopy revealed that the supercomplex consisting of a III_2IV_2 unit (there is no Complex I in this yeast species) is arranged in such way that the distance between the binding sites of cytochrome c, i.e. cytochrome c_1 in CIII and subunit II of CIV (containing the Cu_A), is considerably shorter than in bovine mitochondria (Mileykovskaya et al. 2012). To further confuse the issue, it is also worth noting that Trouillard et al. (2011) recently demonstrated in yeast mitochondria that the time-resolved oxidation of cytochrome c by CIV is a random process not involving substrate compartmentalization.

The purified supercomplex $I_1III_2IV_1$ obtained from bovine heart (Althoff et al. 2011) and some putative respirasomes isolated from rat liver mitochondria (Acín-Pérez et al. 2008) also contained traces of cytochrome c; therefore, it cannot be excluded that substrate channeling on this level occurs also in mammalian mitochondria. It was also found that, after incubating samples of isolated mammalian mitochondria with an excess of cytochrome c, the $I_1III_2IV_1$ supercomplex contains cytochrome c (Lenaz et al. 2010). It is likely that the native supercomplex contains loosely bound cytochrome c that is easily dissociated. The question is, therefore, to understand if such an easy dissociation occurs in the living cell: in other words, this means that if the dissociation rate is of the same order of the respiratory turnover, cytochrome c would exhibit pool behavior even if present in a supercomplex.

Using a different approach, Enriquez' group recently demonstrated that at least part of Complex IV forms a functional supercomplex with channeling of cytochrome c (Lapuente-Brun et al. 2013). In particular, they demonstrated in isolated liver mitochondria that the assembling of Complex IV in supercomplexes defines three types of Complex IV, one dedicated exclusively to receive electrons from NADH oxidation (forming supercomplex I-III-IV), another dedicated to receive electrons from FAD-dependent enzymes (forming supercomplex III-IV) and a third major one that is in free form and that is able to receive electrons from both NADH and $FADH_2$ oxidation.

On the contrary, if Complex IV is maintained permanently detached from supercomplexes by elimination of SCAF1 (Sect. III.C), the maximum respiratory activity of intact cells under glucose-rich medium parallels the maximum respiratory activity obtained in the same cells by feeding directly electrons to Complex IV. In this case, since all Complex IV is in free form and electron transfer takes place via diffusion of the cytochrome c pool, this experiment demonstrates that the cytochrome c pool function is also very efficient.

3. Respiratory Enzymes Not Associated in Supercomplexes

a. Electron Transfer Between Complex II and Complex III

The CoQ pool is certainly required for electron transfer from Complex II to Complex III (Fig. 29.6): in bovine heart mitochondria and submitochondrial particles, Complex II kinetically follows pool behavior in reconstitution experiments (Estornell et al. 1992) and also in intact mitochondria (Stoner 1984); this is in complete accordance with the lack of Complex II-containing supercomplexes as found by BN-PAGE (Schägger and Pfeiffer 2001). Flux control analysis (Bianchi et al. 2004) confirmed that Complex II is the only rate-limiting step in succinate oxidation, and both Complex III and Complex IV have low flux control coefficients in contrast with NADH oxidation (Sect. IV.B.1.a). The existence of small amounts of supercomplexes including Complex II molecules, described by Acín-Pérez et al. (2008) in mouse cell

lines and in mouse liver mitochondria, does not contradict the fact that most of succinate oxidation takes place via the CoQ pool between Complexes II and III.

In contrast with the BN-PAGE and kinetic data outlined above, earlier studies had provided some evidence for a specific association of Complex II with Complex III. Ziegler and Doeg (1959) in Green's laboratory had isolated a succinic dehydrogenase:cytochrome b complex from beef heart mitochondria, that can be dissociated into its components (Complex II and Complex III) and reconstituted therefrom (Hatefi et al. 1962a). At the same time Tsoo King's laboratory isolated a succinate cytochrome c reductase from beef heart Keilin-Hartree particles, that could be dissociated into a soluble succinate dehydrogenase and a particulate bc1 complex (Takemori and King 1964) and reconstituted from the isolated components (King and Takemori 1964). Subsequently, the reconstitution could also be achieved using a detergent-solubilized bc1 complex (Yu et al. 1974).

Further studies (Yu and Yu 1980) yielded similar results using highly purified succinate dehydrogenase and ubiquinol cytochrome c reductase preparations. The effective interaction between the two components of the succinate cytochrome c reductase preparation was demonstrated by Gwak et al. (1986) in the same laboratory. These results indicate that succinate-ubiquinone reductase and ubiquinol-cytochrome c reductase are indeed present in the membrane as a super-macromolecular complex. Strangely enough, no such super-macromolecular complex could be detected between NADH-ubiquinone and ubiquinol-cytochrome c reductases (Gwak et al. 1986).

Another study whose conclusions contradicted the notion of a free Complex II was the kinetic study of Boumans et al. (1998) in yeast, showing that succinate oxidation did not obey pool behavior unless the mitochondria were treated with chaotropic agents.

A likely explanation of these contrasting findings is the existence of very loose but specific contacts between Complex II and Complex III, not giving rise to any kind of channeling even in phosphorylating mitochondria (Stoner 1984). As appears by BN-PAGE, these contacts are lost in frozen mitochondria and in submitochondrial particles, or by digitonin treatment, but may be strongly non-physiologically enhanced by some treatments used for mitochondrial respiratory chain fractionation.

b. Electron Transfer to Complex III from Other Enzymes Using Coenzyme Q

The CoQ pool behavior must fully apply to other enzymes that deliver electrons to CoQ in the respiratory chain (glycerol-3-phosphate dehydrogenase, electron transfer flavoprotein (ETF)-ubiquinone oxidoreductase, dihydroorotate dehydrogenase, choline dehydrogenase, sulfide CoQ reductase and proline dehydrogenase). These enzymes are likely to be in minor amounts and strongly rate-limiting in integrated electron transfer (Lenaz et al. 2010). Few direct studies are, however, available on possible associations of these enzymes. The only study directly addressed to this problem (Rauchová et al. 1997) demonstrated that in brown fat mitochondria the inhibition curve of glycerol phosphate-cytochrome c reductase is sigmoidal in the presence of myxothiazol and antimycin, suggesting the presence of a homogeneous CoQ pool between glycerol phosphate dehydrogenase and Complex III.

Wang et al. (2010) provided evidence of a multifunctional fatty acid β-oxidation (FAO) complex within mitochondria that is physically associated with OXPHOS supercomplexes. They also showed that metabolic channeling of electrons occurs from FAO directly to the respiratory chain since the reduction of cytochrome c is efficiently promoted by the addition of palmitoyl-CoA in a reaction mixture containing excess cytochrome c, CoQ and a sucrose gradient fraction of rat liver mitochondrial proteins enriched in respiratory supercomplexes, thus indicating that the necessary FAO components are also present in the fraction to mediate oxidation of

acyl-CoAs with no accumulation of pathway intermediates. However, Schönfeld et al. (2010) could not find such an association between Complex I and ETF that participates in fatty acid oxidation in rat heart and liver mitochondria, nor could they find any evidence of channeling between fatty acid oxidation and respiratory supercomplexes.

c. Is There a Direct Connection Between Complex I and Complex II?

The hyperbolic relation experimentally found by Gutman (1985) between succinate oxidase and the rate of reverse electron transfer from succinate to NAD^+, involving sequential interaction of complexes II and I by means of CoQ, is in complete accordance with the pool equation. This observation poses a particularly puzzling question (Lenaz and Genova 2007) about how ubiquinol reduced by Complex II interacts from the pool with the CoQ site in Complex I, since Complex I is totally engaged in the supercomplex. The same dilemma applies to the NADH fumarate reductase activity that also involves interaction of Complex I and Complex II (Gutman 1985).

It must be noted that the ATP-driven reverse electron transfer from succinate to NAD^+ occurs in the presence of a high mitochondrial transmembrane protonmotive force that, according to Piccoli et al. (2006), might be the physiological signal causing the structural reorganization of the respiratory complexes. The model hypothesis suggests that the I-III supercomplex would dissociate its constituting complexes under conditions of high $\Delta\tilde{\mu}_H{}^+$, and this would no longer limit the access from the CoQ pool to the binding site in Complex I. This model is however incompatible with the observation reported by Gutman (1985) that NADH-fumarate reductase, assayed at low membrane potential, shares the same pathway of the reverse reduction of NAD^+ by succinate. According to this consideration, the pathways linking Complex II and Complex I during either direct or reverse electron transfer most probably require the interaction between the CoQ pool and Complex I within the supercomplex. If this is true, it must be concluded that Complex I in the supercomplex is somewhat accessible to the CoQ pool (Genova and Lenaz 2014).

C. Supercomplexes and Energy Conservation

There are several reasons why supercomplex assembly might enhance the efficiency of oxidative phosphorylation, although there has been no direct study to address this question.

First, the presence of channeling *per se*, by enhancing electron flow in the respiratory chain, allows a higher rate of proton translocation and hence indirectly of ATP synthesis.

There are additional indications that supercomplex association may provide further kinetic advantages besides substrate channeling. The study of Schäfer et al. (2007b) showed that a supercomplex comprising cytochrome c oxidase ($I_1III_2IV_1$) had higher Complex I and Complex III activities than the supercomplex devoid of the terminal oxidase (I_1III_2); evidently, the presence of Complex IV modifies the conformation of the partner complexes in such a way to enhance their catalytic activity. Likewise, Hildebrandt (2011) showed that supercomplex dissociation abolishes the protective effect of dehydro-ascorbic acid on sulfide toxicity to cytochrome c oxidase, suggesting a conformational effect of supramolecular association on the allosteric properties of cytochrome c oxidase. Such a kinetic advantage would be competent by itself to enhance the creation of the membrane potential; however, it is not known whether such conformational changes also improve the efficiency of proton translocation.

Too little is known presently about the possibility that protein–protein interactions within the supercomplex I–III–IV might affect the subunits that appear to be important in the proton-pumping mechanism of Complex I, Complex III and Complex IV (Genova and Lenaz 2014). The redox

potential span that provides the driving force for proton translocation across the inner mitochondrial membrane depends on the reduction level of the electron donors/acceptors at steady state, as catalyzed by the respiratory supercomplex. It would be of interest to know whether the supercomplex association improves the proton pumping efficiency of the respiratory chain by increasing the redox potential span of the substrates that are channeled within the protein assembly itself, in comparison to the homogenous donor/acceptor pool. However, to our knowledge, a thermodynamic analysis that specifically addresses this problem in the respiratory supercomplex is still lacking, possibly because it is challenging to measure the redox potentials of the ubiquinol/ubiquinone and ferro/ferricytochrome c couples directly in the supercomplex.

There is yet another reason why supercomplexes may enhance the efficiency of energy conservation. As extensively described in Sect. III.B, CL is required for the stabilization of supercomplexes and participates in their structural integrity. In addition, CL is only found at high levels in membranes that pump protons. The high pK(2) (above 8.0) of CL provides a role for its head-group as a proton trap for oxidative phosphorylation. The high pK(2) also indicates that the head-group has but one negative charge in the neutral pH range. Data on the binding of CL to all of the oxidative phosphorylation proteins suggest that the CL may aggregate the oxidative phosphorylation proteins into a patch while it restricts pumped protons within its headgroup domain, supplying protons to the ATP synthase with minimal changes in the bulk phase pH (Haines and Dencher 2002). Adjacent to the respiratory proton pumps, the buffering by CL would increase the voltage component, $\Delta\Psi$, of the electrochemical gradient within the supercomplex. Therefore, in addition to connecting proteins and enhancing efficiency of electron flow, CL could locally contribute to creating a high local $\Delta\Psi$ that is needed to drive the directional ATP export vs. ADP import through the

adenylate carrier across the mitochondrial membrane. Through facilitating respiratory supercomplex assembly and recruiting the adenylate carrier, it has been suggested that, under optimal conditions, CL increases the efficiency of OXPHOS by at least 35 % (Claypool 2009).

D. Modulation of Generation of Reactive Oxygen Species

The possibility that supercomplex assembly prevents excessive ROS generation from the respiratory chain has been suggested in the past, without, however, direct experimental evidence (Panov et al. 2007; Lenaz and Genova 2012; Seelert et al. 2009). In a recent study, Maranzana et al. (2013) induced a primary condition in mitochondrial samples enhancing free Complex I with respect to Complex I bound to the supercomplex; using this approach, the first direct demonstration was obtained that loss of supercomplex organization causes an enhancement of ROS generation by Complex I. The above study showed an enhanced ROS generation by Complex I in two experimental systems in which the supramolecular organization of the respiratory assemblies was destroyed by: (i) treatment either of bovine heart mitochondria or liposome-reconstituted supercomplex I-III with dodecyl maltoside; (ii) reconstitution of Complexes I and III at high phospholipid to protein ratio.

The ROS increase does not appear to be the mere result of Complex I damage as a consequence of the treatment in the presence of detergents, since the NADH-ubiquinone redox activity is only slightly depressed, although Complex I is mostly in its free form. Moreover, the hypothesis that facilitation of electron flow by substrate channeling within the respirasome helps to maintain the redox components of the complexes in the oxidized state, thus limiting ROS formation, cannot be the only explanation. In fact, in the study of Maranzana et al. (2013), ROS production is investigated in the presence of inhibitors (mucidin and rotenone) that prevent electron transfer to any possible accep-
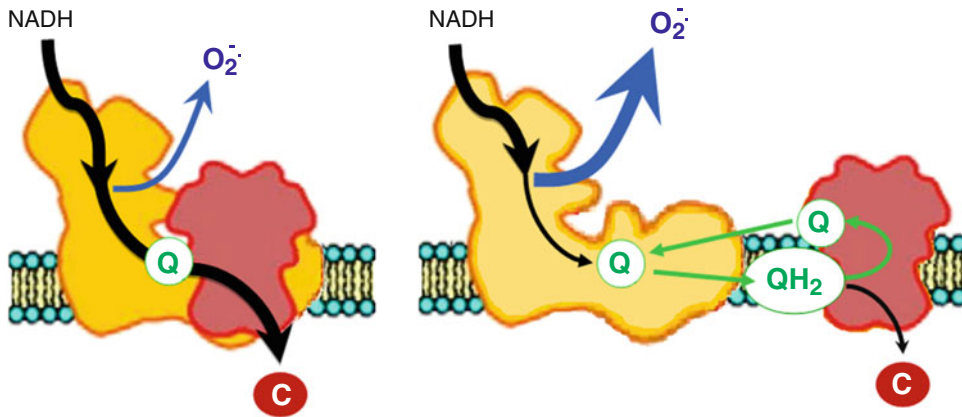
Fig. 29.7. Scheme of electron flux in two models of integrated NADH-cytochrome *c* reductase activity in the respiratory chain. (*Left*) In a supercomplex I_1III_2, any step in the obligatory pathway is regarded as a component of a single enzyme unit where the overall transfer of electrons to the final acceptor is optimized by channeling of the intermediate substrates. Protein-protein interactions modulate the reactivity of the intra-complex redox centers with dioxygen and prevent excessive ROS generation from Complex I (*yellow*). (*Right*) In a linear pathway composed of individual Complex I (*yellow*) and Complex III (*red*), as a consequence of supercomplex disaggregation, the overall electron transfer to cytochrome *c* is mediated by pool behavior of CoQ. The efficiency of electron flux is modified by the absence of protein-protein interactions and superoxide $O_2^-\cdot$ production by Complex I is strongly enhanced.

tor, so that the redox centers in Complex I are maximally reduced both in the situations where supercomplexes are maintained and in the situation where Complex I is free (Fig. 29.7).

The two potential sites for oxygen reduction that exist in Complex I (i.e. FMN and iron-sulfur cluster N2) might distinctly prevail in generating ROS under different conditions (Fato et al. 2009; Hirst et al. 2008; Lenaz and Genova 2010). Possibly, the dissociation of Complex I from Complex III may render the 51 kDa subunit containing the FMN more "loose" allowing it to interact with oxygen (Radermacher et al. 2006). Indeed, the actual shape of the $I_1III_2IV_1$ supercomplex from bovine heart (Schäfer et al. 2007b) suggests a slightly different conformation of Complex I in the supercomplex, showing a higher bending of the matrix arm toward the membrane (and presumably Complex III) which may limit exposure of the redox centers of Complex I to oxygen.

Several observations in cellular and animal models link together supercomplex dissociation and enhanced ROS production.

In mouse fibroblasts expressing the activated form of the k-ras oncogene, a strong decrease of high molecular weight supercomplexes correlating with higher ROS generation was observed in comparison with wild type fibroblasts (Baracca et al. 2010; Lenaz et al. 2010). Moreover, enhanced ROS generation and oxidative stress were found in yeast mutants lacking the supercomplex assembly factor Rcf1 and thus devoid of supercomplexes III-IV (Chen et al. 2012; Strogolova et al. 2012; Vukotic et al. 2012) since the yeast *S. cerevisiae* lacks Complex I; in this case the origin of the extra ROS is presumably Complex III.

Numerous pathological states are accompanied by enhanced generation of ROS (Lenaz 2012, 2014). In an experimental model of heart failure, the decrease of oxidative phosphorylation has been associated with a decrease of respiratory supercomplexes (Rosca and Hoppel 2008, 2009; Rosca et al. 2008).

Lymphoblasts from patients affected by Barth Syndrome, due to genetic loss of tafazzin, an enzyme involved in cardiolipin remodeling, have altered mitochondrial

supercomplexes (McKenzie et al. 2006); likewise, in a yeast experimental model of tafazzin mutation, Chen et al. (2008) observed an increased oxidative stress in response to ethanol.

Aging is also accompanied by a decline of supercomplex association (Gómez et al. 2009; Frenzel et al. 2010); despite some uncertainties and challenges, aging is generally associated with increased ROS and oxidative damage (for recent reviews cf. Cui et al. 2012; Barja 2013). Gómez and Hagen (2012) reason that age-associated destabilization of respiratory supercomplexes may be important for the development of the mitochondrial aging phenotype by means of impaired bioenergetics and enhanced ROS production; in addition Frenzel et al. (2010) on the basis of the 3D-structures of supercomplexes and the close spatial arrangement of the respective electron carrier binding sites (Schäfer et al. 2007b) conclude that less superoxide radical formation is expected to occur in supercomplexes than in randomly distributed individual complexes. These latter studies, however, fail to show which is the causative event (i.e. supercomplex dissociation causing ROS increase or, alternatively, ROS increase causing supercomplex dissociation) or even if they are independent phenomena. [See Chaps. 20 and 21 (Baniulis et al. 2015; Fisher et al. 2015) in this volume on mechanism and consequences of superoxide production in photosynthetic and mitochondrial electron transport chains].

E. Effect of Supercomplex Association on Stability of Individual Complexes

Since the discovery of supercomplexes it was noticed that the stability of Complex I is altered when it is not assembled in the respirasome.

The attempts to isolate Complex I from mutant strains of *Paracoccus denitrificans* lacking complexes III or IV led to the complete dissociation of Complex I under the conditions of BN-PAGE. Reduced stability of free Complex I in those mutant strains was also apparent from an almost complete loss of NADH CoQ reductase activity (Stroh et al. 2004).

Analysis of the state of supercomplexes in human patients with an isolated deficiency of Complex III (Acín-Peréz et al. 2004; Schägger et al. 2004) and in cultured cell models harboring Complex III (Acín-Peréz et al. 2004), Complex IV (Diaz et al. 2006) or cytochrome c (Vempati et al. 2009) depletion, led to the proposal that the formation of respirasomes may be essential for the assembly/stability of Complex I. Genetic alterations leading to a loss of Complex III led to secondary loss of Complex I, therefore primary Complex III assembly deficiencies presented as Complex III/I defects (Lamantea et al. 2002; Acín-Peréz et al. 2004).

D'Aurelio et al. (2006) studied the complementation of mitochondrial DNA (mtDNA) upon fusion of human cell lines, one containing a homoplasmic mutation in a subunit of respiratory chain Complex IV, COX-I, and the other with a distinct homoplasmic mutation in the cytochrome b subunit of Complex III: respiration was recovered in correlation with the presence of supercomplexes containing complexes I, III and IV. From these findings, supercomplex assembly was proposed as a necessary step for respiration.

In a different study, deletion of the *ndufs4* gene, encoding an accessory subunit of Complex I, in mouse tissues results in decreased stability and activity of Complex I: however, the portion of Complex I that is still active is bound in the supercomplexes, indicating that the supramolecular association improves the stability of Complex I (Calvaruso et al. 2011).

Conversely, mutations of Complex I had controversial effects, since in some studies they did not affect the amount of other complexes (Schägger et al. 2004; Pineau et al. 2005), while in others they significantly reduced the amounts of Complexes III and IV (Acín-Peréz et al. 2009a; Ugalde et al. 2004; Grad and Lemire 2004, 2006). The reason for this discrepancy is not known, but might be related to the specificity of the mutation affecting subunits of Complex I, which may

be involved in the contacts with the other complexes.

Animal models of the effects of Complex III and Complex IV mutations on Complex I should prove useful for a better understanding of the role of supercomplexes. In one study, 'knock down' of predicted homologues of COX-IV and COX-Va in the nematode *Caenorhabditis elegans* showed that intrinsic Complex I enzymatic activity is dependent on the presence of Complex IV, despite no overall decrease in the amount of Complex I (Suthammarak et al. 2009). In a further study in *C. elegans* the same group showed that Complex III defects inhibit Complex I by several different mechanisms involving supercomplex destabilization (Suthammarak et al. 2010). Mutant analysis revealed that Complex III affects supercomplex I-III-IV formation by acting as an assembly/stabilizing factor.

Is the stabilization of Complex I by its association in a supercomplex a primary effect on assembly during biogenesis or is it a secondary effect on the stability of the Complex after its assembly? It is possible that both explanations are valid.

Moreno Lastres et al. (2012) report that at early supercomplex assembly stages Complex I is neither fully assembled nor active when it binds to other respiratory chain complexes (cf. Sect. III.C), thus suggesting that the supercomplex constitutes the structural unit in which Complex I arrives at complete assembly.

On the other hand, Enriquez' group (Acín-Peréz et al. 2004; Lapuente-Brun et al. 2013) reported that the assembly of supercomplexes temporally follows the full assembly of the individual complexes and that association of Complex I and Complex III is devoted to the storage and preservation of Complex I since the enzyme stability and not its normal assembly is compromised in the absence of Complex III.

Ghelli et al. (2013) characterized the biochemical properties of cybrids carrying a human cytochrome *b* missense mutation (m.15579A>G) identified in a patient with severe exercise intolerance and multisystem pathological alterations. This mutation does not induce disassembly of the supercomplex containing Complex I. Evidently, there must be specific structural consequences of Complex III mutations in order to disassemble the I_1III_2IV supercomplex with consequent destabilization of Complex I.

Recently Cui et al. (2014) demonstrated that the N-terminal portion of the Rieske protein of Complex III in *S. cerevisiae* is required for stable association with Complex IV in a supercomplex.

From all of these results the evidence would favor the following course of events: misassembled CIII prevents formation of supercomplex; the lack of the supercomplex association induces an enhanced ROS generation from Complex I (see Sect. IV.D), with consequent damage to neighbor molecules and to Complex I itself which is vulnerable to oxidative stress (Choksi et al. 2004), both directly and through the lipid peroxidation, particularly of cardiolipin (Paradies et al. 2004). This series of events can create a vicious circle of oxidative stress and supercomplex disorganization (Fig. 29.8) (Lenaz and Genova 2010).

Indeed, the fragility of free Complex I is not the result of inherent instability of the enzyme, since the isolated Complex I is stable and active for a long time, as evidenced by the early studies of Hatefi and coworkers (Hatefi et al. 1962b), and also by the analysis of Complex I activity in proteoliposomes by Maranzana et al. (2013). However, Complex I shows some fragility due to its sensitivity to the increased production of ROS during the activity of the respiratory chain.

A study on fibroblasts lacking the Rieske iron sulfur protein (Diaz et al. 2012b) also supports the hypothesis that Complex I destabilization in the absence of the Complexes III or IV depends on its particular sensitivity to oxidative damage.

F. Dynamic Nature of Supercomplexes: The Plasticity Model

All the BN-PAGE studies reported in Sect. II.B showed that different types of
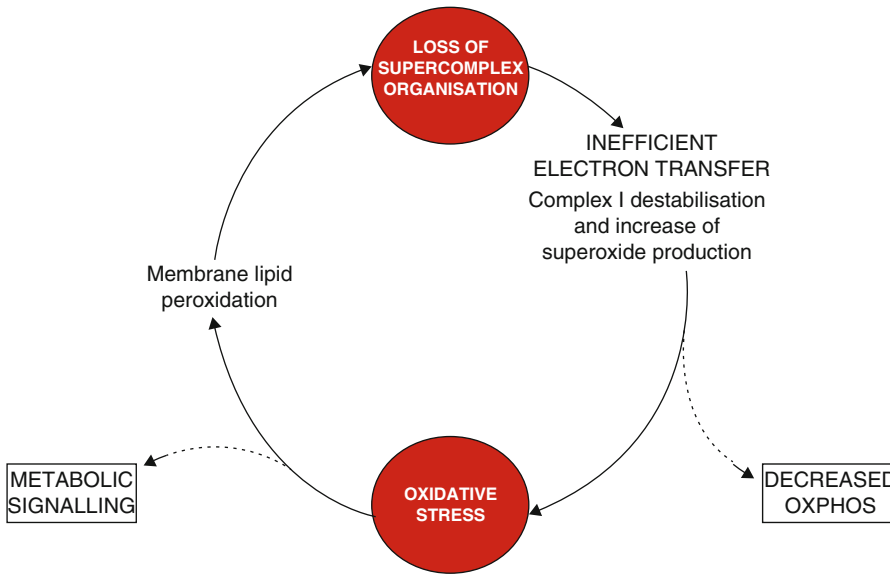
Fig. 29.8. Scheme showing how the loss of supercomplex organization may be involved in a 'vicious cycle' of oxidative stress and energy failure. ROS production by Complex I is enhanced as a consequence of supercomplex disassembly. Membrane phospholipid peroxidation and consequent further loss of supercomplex organization may occur due to mitochondrial oxidative stress, thus perpetuating a 'vicious cycle.' Depending on the amount produced, ROS can also operate as signaling molecules from mitochondria to the cell. See text for explanations.

associations exist between the three core respiratory complexes besides the I+III+IV units or respirasomes, such as I+III or III+IV, which were formed regardless the presence of the third partner of the respirasome, strongly suggesting that they can also be present in vivo. For this reason Acín-Pérez et al. (2008) also concluded that the free complexes likely co-exist with supercomplexes. In this context they proposed an integrated model, *the plasticity model*, for the organization of the mitochondrial electron transport chain. The previous opposed models, solid vs. fluid, would be two extreme functional situations of a dynamic range of different molecular associations between respiratory complexes. The plasticity model and the dynamics of mitochondrial supercomplexes are extensively discussed by Acín-Peréz and Enriquez (2014) in a recent review.

A fundamental prediction of the plasticity model is that, in vivo, the mitochondrial respiratory chain should be able to work both when supercomplexes are present and when the formation of supercomplexes is

prevented. Indeed, several studies in vitro support the view that electron transfer in the respiratory chain can occur in absence of supercomplexes, as described in Sect. IV.B.1.a.

Maas et al. (2009) have been able to express functional AOX in a mutant of Podospora anserina cells lacking complexes III and IV. Under these conditions, Complex I is stable and works perfectly in combination with AOX, which is able to oxidize ubiquinol supplanting the combined role of Complex III, cytochrome c and Complex IV. This demonstrates in vivo that Complex I-dependent respiration does not strictly require supercomplex I-III formation.

The early studies of Hackenbrock in the '80s showed that the intramembrane particles are randomly distributed in the plane of the inner membrane where they diffuse with relatively high diffusion coefficients in the range of 10^{-10} cm^{2}s^{-1} (Gupte et al. 1984; Hackenbrock et al. 1986). Those accurate studies cannot be dismissed as artefacts, therefore they must be explained within the frame of a dynamic equilibrium between su-

percomplexes and randomly diffusing individual complexes. To this respect, the time window of the experimental approaches is of paramount importance and must be taken in account.

The first attempt to follow the temporal dynamics of the respiratory complexes *in situ* was made by Muster et al. (2010) who suggested that supercomplex dissociation into a fully random distribution may not be a fast event. By fusing cells containing mitochondria with respiratory complexes labeled with different fluorescent proteins and resolving their time dependent re-localization in living cells, the authors found that a complete reshuffling of respiratory complexes throughout the entire chondriome in single HeLa cells occurs within 2–3 h by organelle fusion and fission. Moreover, polykaryons of fused cells completely re-mixed their complexes in 10–24 h in a progressive way.

In a recent study, Wilkens et al. (2013) investigated the impact of mitochondrial fusion/fission on the reorganization of OXPHOS complexes in the inner mitochondrial membrane of different HeLa cells tagged with fluorescent proteins (GFP and DsRed-HA). After cell fusion by polyethylene glycol treatment, the outer membrane and matrix proteins mix quickly and homogeneously upon mitochondrial fusion, whereas the mixing and redistribution of the tagged OXPHOS complexes of inner membrane is decelerated. In particular, the comparison of the localization profiles of OXPHOS complexes I, III and IV in the inner membrane compartments clearly showed an overlap: 80 % of CI coincided with 80 % of CIII and CIV, thus supporting the idea of a physical assembly constituted by the three enzymes. Interestingly the diffusion coefficients reported by Wilkens et al. (2013) are of the order of those reported by Hackenbrock et al. (1986).

1. Role of Membrane Electrochemical Potential

A study of the influence of the mitochondrial trans-membrane potential on the flux control exerted by cytochrome *c* oxidase on the respi-ratory indicated that, under conditions mimicking state 4 respiration in intact cells and isolated mitochondria, the control exerted by the oxidase over respiration is decreased relative to endogenous state 3 respiration (Piccoli et al. 2006). The control of Cytochrome c oxidase seems to be exerted by the electrical component of $\Delta\tilde{\mu}_H{}^+$ (Dalmonte et al 2009). More recently, Quarato et al. (2011) extended these studies to the other proton-translocating respiratory complexes (I and III) analyzing the sum of the flux control coefficients, which describe the control that each individual step in a pathway exerts over the global flux of the pathway (Kacser and Burns 1995): under conditions of low potential, the sum exceeded 1, whereas at high potential, the coefficients were much lower.

Although the interpretation of the results in such a complex system is very difficult, the authors suggest that such a change in control strength might be expressed in terms of supercomplex plasticity. In particular, the results showing that the sum of the flux control coefficients of Complexes I, III, and IV exceeds 1, in state 3, were compatible with respirasome organization at lower membrane potential and with a random organization of cytochrome oxidase with respect to other complexes at high membrane potential (state 4); since the respiratory rate is high in state 3 conditions, the supercomplex organization would produce an extra advantage by raising the rate through electron channeling.

The molecular mechanisms leading to the assembly/disassembly of the supercomplexes have been not yet defined, but it is conceivable that, given the membrane-integrated nature of the single complexes, electrostatic/hydrophobic interactions may enter into play in response to $\Delta\tilde{\mu}_H{}^+$. In this respect, recent investigations have revealed a surprising interplay between pH gradients, lipid composition and packing and membrane cristae shape (Khalifat et al. 2008, 2011).

In potato tuber mitochondria, it was found that hypoxia dissociates the supercomplex I-III$_2$-IV into individual Complex I plus Complex III-IV units (Ramirez-Aguilar

et al. 2011). It was shown that the change was due to the acidification of the medium caused by hypoxia and that acidification could reproduce the same effect of hypoxia; moreover, the effect was not due to changes in membrane potential.

2. Role of Post-translational Changes

Mitochondria contain protein kinases and phosphatases and both serine/threonine phosphorylation (Deng et al. 2011; Grimsrud et al. 2012) and tyrosine phosphorylation (Hébert-Chatelain et al. 2012; Hébert-Chatelain 2013; Bellomo et al. 2006) of mitochondrial proteins are important in the regulation of the activity of these organelles (Acín-Peréz et al. 2011a, b).

Phosphorylation of Complex I has been shown to modify the activity of the enzyme and its ROS generating capacity (Bellomo et al. 2006; Iuso et al. 2006; Maj et al. 2004; Papa et al. 2008; Raha et al. 2002) Cyclic AMP-dependent phosphorylation of the 18 kDa subunit of Complex I, encoded by the nuclear NDUFS4 gene, is required for import of the subunit; modulation of subunit phosphorylation by intramitochondrial protein kinase A and phosphoprotein phosphatase contributes to the stability of Complex I and stimulation of its activity (De Rasmo et al. 2008). It is tempting to speculate that the increase of activity of Complex I and the decrease of ROS generation by phosphorylation may be, in part, the result of enhanced stability of the I-III supercomplex. However, to our knowledge, no studies to date have established any correlation between them. Phosphorylation of Complex III subunits has also been reported although its functional role remains undetermined (Zhao et al. 2011).

Complex IV is a target of the intra-mitochondrial cAMP-PKA signaling pathway (Acín-Peréz et al. 2009a, b; Ramzan et al. 2012). It is tempting to speculate that endocrine alterations may affect the assembly state of Complex IV, by hyper- or hypo-phosphorylation of some subunits in the complex. Indeed, cAMP- and PKA-

dependent phosphorylation of Complex IV in heart mitochondria (Rosca et al. 2011) was found to be higher in free Complex IV not associated in supercomplex than in the bound enzyme, suggesting that phosphorylation prevents supercomplex association. Nevertheless, caution must be exerted in the interpretation of the role of post-translational changes in supercomplex formation, since no causal correlation has yet been established.

Recent evidence indicates that thiol-based redox regulation of the respiratory chain activity and especially S-nitrosylation of Complex I could be a strategy to prevent elevated ROS production, oxidative damage and tissue necrosis during ischemia-reperfusion injury (Dröse et al. 2014). It is possible that these redox modifications can also affect the supramolecular structure of the respiratory chain complexes, since they are able to induce important conformational changes; this possibility has not been however as yet explored.

V. A Role of Supercomplexes in Cell Physiology

The supercomplex organization of the mitochondrial electron transport chain imposes functional features to mitochondrial physiology that are a consequence of the different properties that supercomplexes assume with respect to individual cytochrome complexes (Genova and Lenaz 2014).

The dynamic character of the supercomplexes provides a mechanism to optimize the use of available substrates. In fact, glucose utilization as a fuel contributes largely to feed electrons to the respiratory chain through NADH and Complex I, whereas fatty acid oxidation feeds more electrons through a FAD-linked pathway (ETF dehydrogenase). Furthermore, in CD1 mouse mitochondria, fasting reduces maximal Complex I and Complex I+III activities (NADH route) without influencing Complex II or Complex II+III (Lapuente-Brun et al. 2013), and the proportion of Complex III assembled with

Complex I is reduced accordingly. How this change is produced and what would happen if this adaptation was impeded remain to be investigated. At present, no known molecular mechanism can be invoked to explain the exquisite regulation of the balance between Complex III$_{NADH}$ (i.e. Complex III dedicated to Complex I) and Complex III$_{FAD}$. Further understanding of this behavior is of paramount importance.

The modulation of supercomplex assembly may have further consequences ensuring the possibility to modulate ATP levels and ROS production by changing the rate of electron flow in the respiratory chain. Dissociation of supercomplexes would contribute to a low level of NADH oxidation, and hence a high NADH/NAD$^+$ ratio within mitochondria, which could contribute a metabolic switch to the control of the cellular activity. In addition, the plasticity of the respiratory chain supercomplexes can be envisaged as a possible mechanism of intracellular signaling achieved by ROS molecular recognition ("mitochondrial retrograde signaling", Whelan and Zuckerbraun 2013). One notable example is Nrf2 that, in the presence of ROS, is translocated from the cytoplasm to the nucleus, where it stimulates the antioxidant response element (ARE) of cytoprotective genes (Itoh et al. 1999).

Deficiency of respiratory supercomplexes and mitochondrial dysfunction is also correlated with mitochondrial morphology/quality maintenance in the development of the mitochondrial aging phenotype (Gómez et al. 2009; Frenzel et al. 2010; Gómez and Hagen 2012) and in the etiopathology of several diseases (cf. Lenaz and Genova 2012 for a review).

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Part VII

Branched Pathways and Cryptic Cytochromes

Chapter 30

The Interaction Between Cytochrome *f* and Plastocyanin or Cytochrome *c*₆

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Summary

Cytochrome *f* is bound to the cytochrome *b*₆*f* complex embedded in the thylakoid membrane, but plastocyanin is a small protein that diffuses between cytochrome *f* and photosystem I in the thylakoid lumen. The essential feature of electron transfer from cytochrome *f* to plastocyanin is that a weak, transient complex is formed so as to allow both rapid formation of the reaction complex, and rapid dissociation of products. The reaction has been studied with the proteins in solution, using preparations of the large, heme-containing luminal domain of cytochrome *f*, by kinetic methods, NMR and computer simulation. The reaction sites have been identified as small areas of hydrophobic residues on the surface immediately above the heme or Cu atom and including ligand residues (Tyr1 for cytochrome *f* and His87 for plant plastocyanin). A model of the reaction has been developed in which an intermediate encounter complex is formed covering a relatively large area of the surface and serving the purpose of steering the proteins towards a reactive configuration. With plant and algal proteins the main attractive force forming the encounter complex is electrostatic, but the reactive complex depends on desolvation energy to which electrostatics can make little contribution. With cyanobacterial proteins there is considerable species variation. Hydrophobic attraction plays a significant role in formation of the encounter complex as well as the reaction complex and sometimes may dominate. NMR relaxation studies and Brownian dynamics simulations suggest that rather than a single reaction complex, several (encounter)

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configurations contribute to k_{et}. There are additional features governing the reaction in vivo. Plastocyanin diffuses within the tight luminal space which forms a sheet no more than about 10 nm thick, obstructed by the large luminal domains of photosystem II and other complexes. Solution studies with added viscogens suggest that other soluble luminal proteins would cause little inhibition. The lumen is now thought to expand in the light, contrary to previous evidence, raising the intriguing possibility that expansion and contraction of the thylakoid lumen is a significant method of regulation of electron transport. The role of electrostatic attraction in vivo has been tested with *Chlamydomonas* mutants. The surprising result was that charge neutralisation had very little effect on either cell growth or the rate of oxidation of cytochrome *f*, suggesting that the dominant attraction forming the encounter complex in vivo is probably hydrophobic in all oxygenic organisms.

I. Introduction

Electron transfer between cytochrome *f* and plastocyanin or cytochrome c_6 is an essential part of the electron transport system of all oxygenic organisms even though it does not contribute directly to energy conservation. A diffusive process is necessary to connect the cytochrome $b_6 f$ complex to the reaction centre of photosystem I, and for this part of the chain the high redox potential ($\sim +400$ mV) that might be provided either by a cupredoxin or a cytochrome is required. These are soluble proteins, so that diffusion occurs within the lumen of the thylakoid rather than in the lipid phase of the membrane, as for plastoquinone as the intermediate in the reduction of the cytochrome complex by photosystem II. In addition to the high redox potential the other essential feature of the reaction is that it must be very rapid, without formation of a tight complex between cytochrome *f* and plastocyanin. In other words the interaction must be transient so that plastocyanin can rapidly shuttle electrons between cytochrome *f* and the P700 reaction center of photosystem I. In this context 'transient' implies that the complex formed between the two proteins has a lifetime of no more than about 1 ms, and that the binding constant as a rule lies in the range 10^2–10^6 M^{-1}. Understanding this reaction thus depends on detailed knowledge of both electron transfer theory and the factors governing the diffusive interaction between two protein molecules in solution.

Electron transfer is essentially a promiscuous reaction. It depends on quantum mechanical tunnelling between reactant and product wavefunctions, rather than specific activation in a well defined complex, and as such is the simplest kind of chemical reaction and potentially very fast. The theory, which is discussed in more detail in Chaps. 5 and 6 of this volume, leads to a simple statement that the rate constant for electron transfer depends on the product of an electronic factor and a nuclear factor. The electronic factor represents the electronic coupling between the two wavefunctions. For our present purposes the important feature of the electronic coupling, H^2, is that it decays exponentially with distance r between the two redox centers according to Gamow's tunnelling equation

$$H_r^2 = H_o^2 e^{-\beta r}$$

where $\beta = h(2\,mV)^{1/2}$, h being Planck's constant, m the mass of the electron and V the height of the potential energy barrier through which the electron has to tunnel. For tunnelling through protein β is often taken to be 1.4 Å^{-1} (Moser et al. 1995) which leads to the conclusion that to achieve $k_{et} \geq 10^3$ s^{-1} r should be not more than 14 Å. The structures of the two proteins should thus allow this degree of proximity to be achieved by diffusion. The figure of 14 Å^{-1} is only a rough guide, partly because of uncertainty regarding the precise points that r refers to—in the case

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Abbreviations: NMR – Nuclear magnetic resonance; P700 – The P700 reaction center of photosystem I

of heme proteins Moser et al. prefer to measure the distance from the edge of the heme macrocycle rather than from the iron atom.

The nuclear factor, on the other hand, can be described by the activation free energy term of classical transition state theory. $\Delta G^{\ddagger}$ is given by the 'Energy Gap' law derived by Marcus (Marcus and Sutin 1985)

$$\Delta G^{\ddagger} = \frac{\left(\lambda + \Delta G^0\right)^2}{4\lambda}$$

so that it depends on the reorganization energy, λ, and the driving force, that is the standard free energy change for the reaction, ΔG^0. For the reaction between cytochrome *f* and plastocyanin or cytochrome c_6 the driving force is normally small, 10–15 mV for plastocyanin, and even smaller for cytochrome c_6. Both proteins are small, rigid molecules so that λ is mainly the result of the relatively large solvent contribution.

A protein molecule diffuses in solution by a Brownian process. It is continually being buffeted by a myriad of relatively tiny water molecules pushing it this way and that, which results overall in a series of small movements of the protein in a random walk. The rate at which two molecules collide can be calculated with the diffusion equation:

$$k_a = 4\pi N_A r \left(D_A + D_B\right).10^3$$

where N_A is Avogadro's constant, r the sum of their radii and D_A, D_B, are the diffusion constants in $m^2 s^{-1}$. For two molecules the size of plastocyanin the collision rate is found to be in the range 10^9–10^{10} $M^{-1}s^{-1}$ if charges on the proteins are ignored. However, we are interested in contacts with a specific orientation so that the reactive sites become close to each other, and then the great majority of initial contacts are non-productive and k_a for productive contacts becomes orders of magnitude smaller than the above figure. In practice the rate is not as small as one would predict by calculation from the sizes of the reactive sites because when two protein molecules collide in solution they are trapped by surrounding solvent (the solvent cage effect) to form an 'encounter complex' in which they can explore numerous relative conformations by surface diffusion (Northrup and Erickson 1992), as expected from the principle of reduction of dimensionality (Adam and Delbrück 1968). The rate can be enhanced if the two proteins carry complementary charges. The process has been modelled for the interaction of cytochrome *f* and plastocyanin of higher plants, *Chlamydomonas reinhardtii* and the cyanobacterium *Phormidium laminosum* by the Brownian dynamics algorithm with a fair degree of success (Pearson and Gross 1998; Gross and Pearson 2003; Gross 2004; Haddadian and Gross 2005; Gross and Rosenberg 2006). The simulations confirm the very similar performance of plastocyanin and cytochrome c_6 with the *Chlamydomonas* proteins.

The Brownian dynamics algorithm of UHBD (Madura et al. 1995), SDA (Gabdoulline and Wade 1998) or MacroDox (Northrup et al. 1987), mimics the Brownian random walk together with the long-range effect of the interaction of the electrostatic fields of the two proteins, calculated with a finite difference Poisson-Boltzmann equation, and the short-range entropic contribution to binding by hydrophobic desolvation as the two proteins come into contact. The electrostatic calculations emphasize the fact that whereas complementary charges on the two proteins provide a long range attractive force and can exert a steering effect to encourage contact between the two reactive sites, at short range a large desolvation penalty is involved which severely weakens the attraction between a positive and a negative charge and can even result in repulsion.

II. Evolution of Plastocyanin and Cytochrome c_6

Plastocyanin and cytochrome c_6 are functionally interchangeable (Bohner and Böger 1978; Wood 1978). There are only minor differences between the two proteins

in the kinetics of reduction of P700 (Medina et al. 1993; Hervás et al. 1994, 1995; Navarro et al. 1997), and Wood (1978) demonstrated that in vitro cytochrome f from *Chlamydomonas reinhardtii* reduces both plastocyanin and cytochrome c_6 with a rate constant $>1 \times 10^7$ $M^{-1}s^{-1}$, whereas cytochrome c_6 reduces plastocyanin two orders of magnitude more slowly. Why then is there a need for the two proteins? The answer seems to lie in the availability of iron and copper in the environment, which are required for formation of the respective cofactors. Both metals have highly insoluble redox states, oxidized in the case of iron, Fe(III) and reduced in the case of copper, Cu(I), with alternative states soluble and therefore available. The proteins do not normally occur together in any one organism, although many are capable of synthesizing both, under appropriate conditions.

The primitive earth on which life evolved was anoxic. Under these conditions iron, in the form of the highly soluble Fe^{2+}, was freely available. Protoporphyrin IX is a common intermediate in the biosynthesis of both chlorophyll and heme (Hohmann-Marriott and Blankenship 2011) and all known anaerobic photosynthetic organisms depend on a form of chlorophyll in their reaction centers. Thus hemeproteins could be expected to be extremely ancient, preceding the origins of oxygenic photosynthesis and cyanobacteria, and cytochromes occur in every photosynthetic anaerobe today. By contrast, copper would not have been so readily available, because Cu (I), which would predominate under reducing conditions, forms an extremely insoluble sulphide. The earliest oxygenic organisms are therefore more likely to have contained cytochrome c_6 than plastocyanin. Nevertheless, the anaerobic phototroph *Chloroflexus aurantiacus* employs a cupredoxin, auracyanin, in its electron transport system. This organism belongs to the filamentous anoxygenic phototrophs, which show an early branching from all the other groups of photosynthetic eubacteria (Van Driessche et al. 1999). Given that the latter also exhibit the cupredoxin

protein fold, as in the plastocyanin of cyanobacteria (see below for structure), the most parsimonious explanation is that the cupredoxin fold is also ancient.

The relative availabilities of iron and copper on the primitive earth probably persisted until the Great Oxidation Event, about 2.4 billion years ago, brought about a conversion of the reducing atmosphere of the primitive earth to an oxidizing atmosphere as a result of the development by cyanobacteria of water as the electron donor for photosynthetic metabolism, leaving molecular oxygen as the byproduct. This was the first great biological modification of the earth environment. The availabilities of iron and copper would then have been reversed as Fe(III) is highly insoluble in water whereas Cu(II) is readily soluble. By this time iron compounds had been employed in a wide variety of metabolic processes so that cells had to find ways of making iron available, but replacing cytochrome c_6 by plastocyanin would have represented a significant saving. Even so, the aqueous environment was not homogeneous and anaerobic environments would have persisted. To this day aquatic organisms, that is most cyanobacteria and green algae, have in many cases found it an advantage to retain the genes for both cytochrome c_6 and plastocyanin, and use plastocyanin in preference unless copper becomes unavailable in the environment although other algal lineages use only cytochrome c_6.

The controlling factor is the availability of copper rather than iron. By omitting copper from the growth medium of *Chlamydomonas reinhardtii* Wood (1978) was able to change the ratio of plastocyanin to cytochrome c_6 in cultured cells by a factor of at least 5000. On the other hand, *C. mundana*, a species isolated from an anaerobic sewage pool containing free H_2S, contained cytochrome c_6 but no plastocyanin. Sandmann et al. (1983) found a number of species of green algae that were unable to synthesize cytochrome c_6 under copper deficiency, and correspondingly organisms in other groups of algae that were unable to synthesize plastocyanin even when copper was freely available. Merchant

and Bogorad ([1986](#)) demonstrated directly with *C. reinhardtii* that copper in the medium stimulated production of plastocyanin and inhibited production of cytochrome c_6. Plants, like some green algae, have lost the ability to synthesize cytochrome c_6, but it is not clear exactly why this should be so. Although a form of cytochrome c_6, referred to as c_{6A}, has been reported in green algae and plants, with related molecules in cyanobacteria, they have a different function. Cytochrome c_{6A}, as well as the cyanobacterial c_6-like cytochromes, is discussed elsewhere in this volume.

III. Structure of Cytochrome *f*

Cytochrome *f* is a unique protein with no known evolutionary forebears and, having a largely β-sheet structure, is unrelated to other *c*-type cytochromes. Two unusual features should be noted. The first is that it uses the N-terminal residue, almost invariably tyrosine, as a heme ligand, and as the residue is surface exposed it forms a favourable site for electron transfer to acceptors. The second is that cytochrome *f* contains a conserved chain of five internal water molecules (Martinez et al. [1996](#)) which have been suggested to provide a proton channel for operation of the Q cycle in the cytochrome b_6f complex (Ponamarev and Cramer [1998](#); Sainz et al. [2000](#)). Another suggestion is that the buried waters have a structural function (Chi et al. [2000](#); Griffin et al. [2002](#)). A study of mutants in which one or more of the waters have been eliminated by mutation of conserved binding residues within the protein shows that any effects on electron transfer are on cytochrome *f* reduction rather than its oxidation (Ponamarev and Cramer [1998](#)), so the function of the water chain will not be discussed further in this chapter.

Cytochrome *f* is a membrane-bound protein which requires detergent to solubilize it, as well as being an integral component of the cytochrome b_6f complex. The major part of the molecule (the first 250 residues) is exposed to the aqueous phase of the thylakoid lumen and is anchored to the membrane by a single α-helix and a short section of polypeptide chain (14 residues) on the stromal or cytoplasmic side (Gray [1992](#)). This large luminal domain contains the heme prosthetic group, the plane of which lies at about 30° to the membrane plane, as shown originally by EPR (Crowder et al. [1982](#)) and confirmed by the more recent X-ray structures of the whole complex. These structures have been obtained at 3.0 Å resolution for the cyanobacterium *Mastigocladus laminosum* (Kurisu et al. [2003](#)) and 3.1 Å for the green alga *Chlamydomonas reinhardtii* (Stroebel et al. [2003](#)) which is sufficient to resolve α-helices but not to resolve accurately the sidechains of individual amino acids. The most accurate structures have been obtained with the isolated luminal domain which is liberated spontaneously by proteolysis at the membrane surface when leaves of certain crucifers (e.g. turnip, charlock or rape) are homogenized in butanone, or by heterologous expression of the appropriately truncated gene in *Escherichia coli*. Structures for the protein from a higher plant (turnip, *Brassica rapa*), a green alga (*Chlamydomonas reinhardtii)* and the cyanobacterium *Phormidium laminosum* have been reported at a resolution of 1.9–2.0 Å (Martinez et al. [1996](#); Carrell et al. [1999](#); Chi et al. [2000](#)). These structures effectively encompass the whole spectrum of oxygenic organisms, but are remarkably similar. The tertiary structures show virtually identical elongated molecules. The heme group is buried but close to the lumen-exposed surface. The molecule as a whole is acidic in all cases but the major difference is that the plant and algal proteins possess a cluster of basic residues close to the heme (58, 65, 66, 187, 209) which complement an acidic patch in plastocyanin, whereas in the cyanobacterial case several of these basic residues are either acidic or neutral. This truncated, soluble preparation of cytochrome *f* is the form which has been used for nearly all the studies of the interaction with plastocyanin or cytochrome c_6.

IV. Structure of Plastocyanin and Cytochrome c_6

Plastocyanin is a blue copper protein or cupredoxin with a type 1 copper center (Malmström 1970). It has a Greek-key β-barrel structure which is highly conserved throughout all oxygenic organisms, as determined for several species of higher plant, a fern, green algae and several cyanobacteria (Colman et al. 1978; Guss and Freeman 1983; Collyer et al. 1990; Moore et al. 1991; Guss et al. 1992; Redinbo et al. 1993; Bagby et al. 1994; Badsberg et al. 1996; Romero et al. 1998; Xue et al. 1998; Babu et al. 1999; Bond et al. 1999; Inoue et al. 1999a, b; Kohzuma et al. 1999; Shibata et al. 1999; Sugawara et al. 1999; Bertini et al. 2001; Schmidt et al. 2006) either by X-ray crystallography or by NMR to give the solution structure. The structure of the copper center, in which the single copper atom is coordinated in a distorted tetrahedral structure, is also conserved. The three equatorial ligands, the N^δ atoms of His37 and His87 and the S^γ atom of Cys84 lie in a plane slightly below the copper atom, and the axial ligand is the S^δ of Met92 (higher plant numbering system) with an unusually long bond length. His87 is exposed to the surface at one end of the molecule within a patch of hydrophobic residues, and has been favoured as the site for electron transfer to or from its natural redox partners. A second, 'remote' site centered on Tyr83, adjacent to the Cys84 ligand, has been identified by kinetic and NMR experiments observing paramagnetic line broadening with inorganic reactants (Sykes 1985). Tyr83 is located between two groups of acidic residues, 42–45 and 59–61 in higher plants. Positively charged complexes such as $[\text{Co(phen)}_3]^{3+}$ react at this site, whereas negatively charged complexes such as ferricyanide react at the 'northern' site involving His87 (Sinclair-Day and Sykes 1986). NMR experiments with proteins from several different sources have shown conclusively that the electron transfer site with cytochrome f involves His87 (or its cyanobacterial homologue)

(Ubbink et al. 1998; Crowley et al. 2001; Díaz-Moreno et al. 2005a; Lange et al. 2005; Hulsker et al. 2008). The charge properties of plastocyanin from plants and algae are highly conserved. They are all acidic proteins, with a pair of conserved acidic patches which complement the basic ridge of cytochrome f. The larger of these includes residues at positions 42, 43, 44 and 45 whereas the smaller one, which is closer to His87, often includes residues 59, 60 and 61, but is more variable than the large patch. In cyanobacterial plastocyanins, on the other hand, some of the equivalent positions are occupied by basic or neutral residues. A particularly significant residue is Arg93, corresponding to Gln88 in plant plastocyanins, which lies immediately below the hydrophobic patch (Schlarb-Ridley et al. 2002a). The pI varies from acidic to basic, according to species. In particular, plastocyanins from *Synechococcus* sp. PCC 6301 and *Prochlorococcus marinus* have pIs almost as low as those of the plant proteins, but the characteristic plant pattern of acidic patches in plastocyanin and a basic cluster in cytochrome f is lacking.

Cytochrome c_6 is a type 1 cytochrome c which occurs in many cyanobacteria and green algae but not in higher plants. Its structure bears no relation to that of plastocyanin. Nevertheless, one edge of the heme prosthetic group is exposed to the medium and is surrounded by hydrophobic residues, providing a potential reaction site similar to that of H92 in plastocyanin (numbering system of *P. laminosum*). Furthermore, there is a conserved arginine residue, R66, on the edge of this site which has been shown to be essential for reaction of cytochrome c_6 with photosystem I of cyanobacteria (De la Cerda et al. 1999; Molina-Heredia et al. 2001). This residue is in a similar position relative to the reaction site as the conserved R93 in plastocyanin. The reaction of cytochrome f with cytochrome c_6 has not been as thoroughly investigated as with plastocyanin in the mistaken belief that the two c-type cytochromes are not easily distinguished spectroscopically.

V. Structure of Specific Complexes

Electron transfer complexes are inherently unstable; crystallisation has often proved difficult and so far impossible for a complex between cytochrome *f* and plastocyanin, even though the separate proteins have been crystallized. X-ray crystal structures have, however, been obtained for several electron transfer complexes, the first of these being the 1:1 complex between cytochrome *c* and cytochrome *c* peroxidase from yeast by Pelletier and Kraut (1992). This system has become a paradigm for the development of our understanding of electron transfer reactions between proteins in solution. The structure observed in the crystals is very similar both to that of a covalently cross-linked complex which has been shown to be active in electron transfer (Guo et al. 2004), and to the solution structure obtained by NMR (Volkov et al. 2006).

When two proteins interact in solution residues involved in specific binding sites can be identified by observing NMR chemical shift changes due to the change in chemical environment. If one of the proteins contains a paramagnetic nucleus in its active site, such as the ferric atom of oxidized cytochromes, an actual structure can be obtained by observing pseudocontact shifts in the other protein, because these depend upon relative orientation and the distance between the two nuclei. The fact that electron transfer proteins are usually in fast exchange on the NMR time scale, and by assuming that they behave as rigid bodies, the crystal structures of the individual proteins, together with restrained rigid-body molecular dynamics, can be used to calculate the most probable structure for a specific complex. This was first reported for the complex between turnip cytochrome *f* and spinach plastocyanin by Ubbink et al. (1998) and the method has since been extended to several other complexes. More recently the technique has been extended and made more general by measuring paramagnetic relaxation enhancement (T_2) in nuclei of one protein in response to paramagnetic centers containing unpaired electrons introduced at specific sites in the other. Clore et al. have developed the method with several protein complexes (Iwahara and Clore 2006; Tang et al. 2006; Clore and Iwahara 2009) and Ubbink et al. with electron-transfer complexes (Volkov et al. 2006, 2010a, b; Bashir et al. 2010). The effect is highly localized because of the r^{-6} distance dependence and also very sensitive. As a result the technique provides additional, precise information about the structure of the specific complex, and can also identify minor, transiently occupied complexes of the encounter ensemble. The latter aspect will be discussed below in relation to the dynamics of interaction, but for the moment we will concentrate on the structure of the single, specific complex.

The most striking feature of all the complexes is that they include binding of the hydrophobic patch of plastocyanin which contains the copper ligand His87, to a hydrophobic patch on cytochrome *f* which is immediately above the heme group and contains Tyr1 and Phe3. This provides a good coupling pathway for electron transfer between the iron atom of cytochrome *f* and the copper atom of plastocyanin via the copper ligand His87 and the heme ligand Tyr1. The relative orientation of the proteins can differ, however, and in the case of *Phormidium laminosum* it appears to shift the closest contact of His87 from Tyr1 to Phe3 (Crowley et al. 2001). At one orientational extreme there is the complex from the cyanobacterium *P. laminosum*, in which plastocyanin binds 'head-on' to cytochrome *f*, and at the other the higher-plant complex which has a 'side-on' orientation (Ubbink et al. 1998) (Fig. 30.1). The latter corresponds closely to the structure DC2 previously identified by calculation of electrostatic energies by Soriano et al. (1997) and is determined in plants by electrostatic interaction between the basic ridge of cytochrome *f* lying to one side of the heme site, and the acidic patches of plastocyanin. In the cyanobacterium *Nostoc* PCC 7119 (subsequently referred to as *Nostoc*) the binding is also side-on,
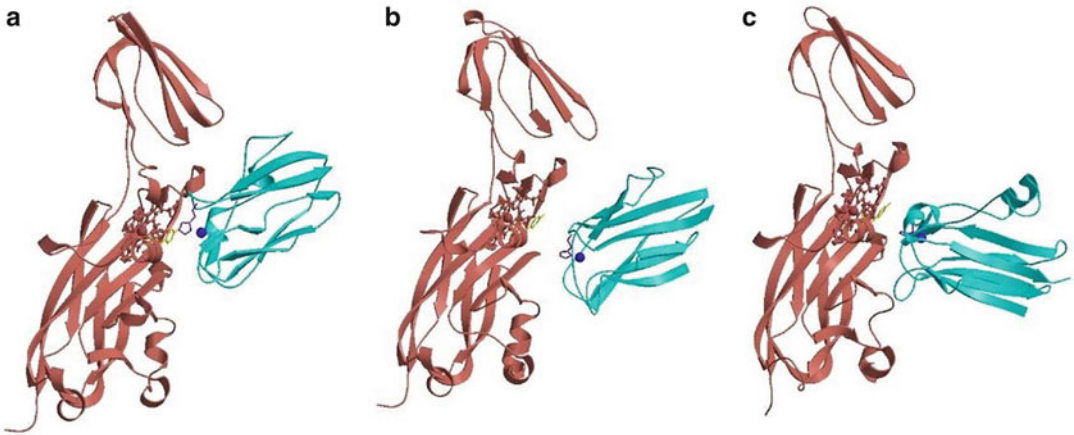
Fig. 30.1. NMR structures of the solution complex between cytochrome *f* and plastocyanin. Higher plant proteins (**a**) *Brassica rapa*, turnip, cytochrome *f* ; *Spinacea oleracea,* spinach, plastocyanin, 2pcf.pdb model 6; cyanobacterial proteins (**b**) *Nostoc* sp. PCC 7119, 1tu2.pdb model 1; (**c**) *Phormidium laminosum*, Crowley et al. 2001). Cytochrome *f*, backbone salmon, heme indianred, heme ligand Tyr1 *yellow*; plastocyanin, backbone cyan, copper *blue*, copper ligand (a, His87, b and c, His92) *purple*. Figure drawn with MolScript (Kraulis 1991) and Raster3D (Merritt and Bacon 1997).

but the charges on the two proteins are reversed (Díaz-Moreno et al. 2005a). The same is true for the complex from another cyanobacterium, *Prochlorothrix hollandica*, in which an involvement of electrostatics in complex formation can be demonstrated by the effects of ionic strength (Crowley et al. 2002; Hulsker et al. 2008). In this case, however, there is no evidence for specific interactions at the interface between acidic residues in cytochrome *f* and basic residues in plastocyanin, as is the case with the *Nostoc* complex. The balance between electrostatics and the relatively short-range hydrophobic interactions can vary, particularly amongst cyanobacteria. In *P. laminosum* electrostatics are much less important and hydrophobic interactions are significant (Crowley et al. 2001; Schlarb-Ridley et al. 2002a). In higher plants and green algae the nature of the hydrophobic patch and the pattern of charged residues is remarkably constant, but in at least three species of fern (*Dryopteris crassirhizoma*, *Nephrolepsis exaltata,* and *Polystichum longifrons*) the distribution of acidic residues in plastocyanin is significantly different (Navarro et al. 2004). Thus, only one of the

four acidic residues in the lower acidic patch of spinach (residues 42–45) remains in ferns which also have several glutamate residues on the edge of the smaller hydrophobic patch. A further difference, which may be significant, is that the highly conserved glutamine residue at position 88 in spinach, adjacent to the copper ligand His87, has become a positively charged lysine residue, where cyanobacteria have a conserved arginine (except in *Gloeobacter* which has lysine).

A significant feature of the hydrophobic interface is that it has a relatively open, loosely packed structure porous to water molecules (Crowley and Carrondo 2004; Bendall et al. 2011). This ensures weak binding and a short lifetime (large k_{off}), in other words the transient nature of the interaction. The idea of 'hot spots' providing most of the binding energy is generally inappropriate for transient complexes. They often involve tryptophan, tyrosine or arginine residues surrounded by a more hydrophobic 'O ring' to exclude water, and tend to be characteristic of tight, static complexes (Clackson and Wells 1995; Bogan and Thorn 1998). Even so, a hot spot involving

Arg13 of cytochrome *c* bound to Tyr39 of cytochrome *c* peroxidase has been identified in the interface between these two proteins in the electron transfer complex (Volkov et al. 2009). This complex has a relatively high binding constant, but in general the magnitude of the binding constant is not a good guide to the kinetic significance of the encounter complex (Worrall et al. 2002).

VI. Dynamics of Interaction

The dynamics of interaction between plastocyanin and the soluble luminal domain of cytochrome *f* have been extensively studied in vitro in the expectation that the properties of the two proteins so revealed would be directly relevant for understanding the reaction in vivo. We will see later that the relevance is, in fact, not straightforward, but for the moment we will concentrate on the solution reaction because there is no doubt that there are important principles that must apply to both sets of conditions.

There are two questions that we need to answer. The first is how the rate of reaction is enhanced from the low value that could be expected for simple diffusional collision when only small patches on the proteins are reactive ($k_2 \sim 10^5$–10^6 M^{-1}s^{-1}) to the experimentally observed rate ($k_2 \sim 10^8$ M^{-1}s^{-1}). The second is how restrictive the distance dependence of electron transfer is on the size of the reactive patches and the nature of the reactive complex between the two proteins.

The first question, appropriately rephrased, is relevant to all cases of specific protein-protein interactions. The most directly relevant to electron transfer systems are those reactions involving enzymes and their specific inhibitor proteins in which the inhibitor must bind extremely rapidly to a specific site in the enzyme to form a very stable complex. This might be either because of the lethality of the enzyme to its parent cell or for physiological reasons. A well studied example of the former is the bacterial ribonuclease known as barnase, which is lethal until excreted,

so that the small inhibitor protein barstar binds rapidly to the intracellular enzyme as soon as it is synthesized. A physiological example is the inhibition by fasciculin of acetylcholine esterase, the terminator of nerve impulses involving acetylcholine. The rate of formation of these enzyme-inhibitor complexes, k_{on}, is often diffusion limited, but dissociation of the complex, k_{off}, is very slow, giving a large binding constant, $K_A = k_{on}/k_{off}$. In electron transfer reactions k_{on} is also usually very fast, although not necessarily diffusion-limited, but k_{off} must also be fast to provide a transient complex with a small K_A.

The model which has been used successfully to explain rapid binding kinetics postulates that the final complex is reached via a transient intermediate complex, the structure of which effectively broadens out the size of the restricted patches through which the two proteins bind to each other in the final complex (Berg and von Hippel 1985; Schreiber and Fersht 1996; Gabdoulline and Wade 1999). This encounter complex is the endpoint of the diffusional interaction which is reached by liquid phase diffusion and may be followed by a rapid two-dimensional surface diffusion. The kinetic advantage provided by the encounter complex depends on relatively long-range inter-protein forces guiding the two proteins towards a productive configuration. In many cases this steering effect is the result of complementary charges on the proteins situated in the region of the target site, but these cannot bring the proteins into close contact because of the strong desolvation penalty that would be incurred in so doing. Movement into the final, specific complex involves short-range hydrophobic and van der Waals forces.

Electron transfer from soluble cytochrome *f* to plastocyanin or cytochrome c_6 is also strongly dependent on electrostatic attraction between the two proteins (Takenaka and Takabe 1984; Anderson et al. 1987; Modi et al. 1992; Meyer et al. 1993; Lee et al. 1995; Kannt et al. 1996; Gong et al. 2000; De Rienzo et al. 2001; Schlarb-Ridley et al. 2002a; Hart et al. 2003; Albarrán et al. 2007).

The steering effect can increase the rate by orders of magnitude by preorienting the two proteins to bring their active sites close enough for electron transfer. The strength of the attraction is strongly dependent on ionic strength, and a pictorial impression of the effectiveness of a charge is given by the concept of the Debye length or screening distance which gives a measure of the way in which the field around an ion is compressed by other ions in the solution. The Debye length is the distance at which the field is reduced by 1/e and is defined by the reciprocal of the Debye-Hückel factor, κ, which in SI units is given by

$$\kappa^2 = \frac{2000 N_A e^2}{\varepsilon_o \varepsilon k_B T} I$$

where I is ionic strength, k_B is Boltzmann's constant, e the charge on an electron and ε_o and ε the permittivity of vacuum and dielectric constant respectively. At high ionic strength $1/\kappa$ is vanishingly small and the field effectively disappears, but at $I = 100$ mM it is about 10 Å which is large enough for the field around a group of charges on a protein surface to be smeared out into a single surface. Simple electrostatics might suggest that the field (and its effect on the rate of electron transfer) would continue to increase exponentially as I is decreased further, but in fact an optimum value of I is normally observed, with decreasing rates as I is lowered still further. Meyer et al. (1993) found the optimum to be at about 40 mM where $1/\kappa$ has a value of about 15 Å. In terms of the encounter complex model this can be explained with the following scheme

$$\text{Pc}_{ox} + \text{Cyt}\,f_{red} \underset{k_{-a}}{\overset{k_a}{\rightleftarrows}} [\text{Pc}_{ox} : \text{Cyt}\,f_{red}]$$

$$\underset{k_{-r}}{\overset{k_r}{\rightleftarrows}} [\text{Pc}_{ox}\text{Cyt}\,f_{red}] \overset{k_{et}}{\rightarrow} [\text{Pc}_{red}\text{Cyt}\,f_{ox}]$$
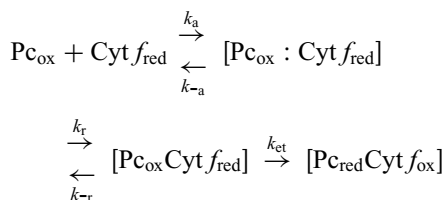
if it is supposed that at low ionic strength the rate constant for rearrangement, k_r, becomes progressively smaller so that the sys-

tem tends to get stuck in the essentially electrostatic encounter complex, represented as $[\text{Pc}_{ox} : \text{Cyt}\,f_{red}]$. The kinetic behaviour of this scheme depends on which of the three steps is rate limiting (Schlarb-Ridley et al. 2005). When k_{et} is fast and initial binding is limiting, the reaction is diffusion controlled, and when, on the other hand k_{et} is limiting the reaction may be described as activation controlled. Taking rearrangement into account, Davidson described the latter type of reaction as 'coupled', that is to say the overall rate is determined not only by k_{et} but also by rearrangement, so that $k_r/k_{-r} < 1$ (Harris et al. 1994; Davidson 1996, 2000). The intermediate case of slow rearrangement but rapid k_{et} is commonly referred to as 'conformationally gated' in the terminology introduced by Hoffman and Ratner (1987).

The existence of the rearrangement step has been reinforced by studies of the effects of viscosity and temperature on several reactions in which electron transfer has been initiated by laser flash photolysis. Millett and coworkers have developed the use of ruthenium complexes as photosensitizers (Mei et al. 1999; Millett and Durham 2002). An early study by Qin and Kostić (1994) involved the accelerated decay of the flash-induced triplet state of zinc-substituted cytochrome c (zinc replacing the iron) in the presence of cytochrome b_5. Quenching is due to electron transfer from the excited triplet state of Zn cytochrome c to ferric cytochrome b_5. Although the reaction is unphysiological it can reveal the dynamics within the protein complex. Electron transfer within the preformed complex was found to be biphasic and sensitive to viscosity, which was interpreted to indicate more than one reactive configuration interconverting by orientational fluctuations. This conclusion agrees with more recent NMR studies of the system by Volkov et al. (2005) who found evidence for a dynamic complex from the small size of chemical shift perturbations on binding. The flash photolysis technique has been used by Hoffman and colleagues in studies, discussed below, of the interaction between cytochrome b_5 and myoglobin.

Hydrophobic forces arise from the entropy increase as two non-polar surfaces approach one another and become dehydrated, and are effective over a shorter distance. This effect normally extends about two water diameters from the surface of a protein, which is sufficient to provide a weak steering effect and a significant rate enhancement by providing pathways into an encounter ensemble. Camacho et al. (2000) have studied this theoretically in relation to the binding of ovomucoid to α-chymotrypsin and elastase. Hydrophobic effects have been shown to be a significant factor in the kinetics of electron transfer from cytochrome *f* to plastocyanin, the best known example being the proteins from *P. laminosum* with which electrostatics have only a minor role to play (Schlarb-Ridley et al. 2002a) and, indeed, ionic strength changes have no detectable effect on the magnitude of chemical shift changes resulting from binding (Crowley et al. 2001). The rate enhancement is not as great as it is for plant proteins when measured under standard conditions at 300 K, but this is compensated by the fact that *P. laminosum* is thermophilic, with a natural growth temperature of 45 °C. Moreover, when electrostatic forces are virtually eliminated at high ionic strength, the *P. laminosum* proteins react an order of magnitude faster than the higher plant proteins (Schlarb-Ridley et al. 2003), which presumably reflects a more effective hydrophobic channelling into the reactive configuration.

It may be worth mentioning that it is not impossible for hydrophobic attraction to extend very much further from a hydrophobic surface (Tsao et al. 1993). This is thought to be an electrostatic effect resulting from the polarization field of water molecules oriented at the surface (Despa and Berry 2007), but is unlikely to be significant for protein interactions.

Electrostatics are not entirely irrelevant in the *Phormidium* system, however. The effects of ionic strength on k_2 reveal a weak electrostatic attraction, but this is the result of a balance between basic and acidic residues on the surface of plastocyanin. The most important basic residue is Arg93, the conserved residue adjacent to the copper ligand, His 92, with minor contributions from Lys46 and Lys53. These residues would provide an attractive force to the acidic surface of cytochrome *f*, but this is offset by a repulsion originating with Asp44 and Asp45 (Schlarb-Ridley et al. 2002a), so that *Phormidium* plastocyanin has not evolved to give the highest possible rate of reaction with cytochrome *f*. Nor can one say that these charge residues are present to satisfy the requirements for rapid reaction with Photosystem I, as the mutants have similar effects on both reactions (Schlarb-Ridley et al. 2002b). The pseudo-neutrality which results for the reaction with cytochrome *f* is important in determining the 'head-on' collision. Thus there is a balance between the steering effects of electrostatic and hydrophobic forces which can vary from one organism to another, at least amongst cyanobacteria.

The failure to detect an effect of ionic strength on the binding constant (K_A) for *P. laminosum* proteins, despite an effect on k_2, was previously explained by the reaction being diffusion controlled, when $k_2 = k_a$ and $K_A = \frac{k_a k_r}{k_{-a} k_{-r}}$ (Hart et al. 2003). One would then expect that an electrostatic attraction influencing the encounter complex would increase k_a and decrease k_{-a} but have opposite effects on k_r and k_{-r} so that k_2 would be stimulated without any significant effect on K_A. Even if the reaction is not fully diffusion controlled and thermal activation makes some contribution to k_2 (Gabdoulline and Wade 2009) this argument is likely to remain the basis of the explanation of the apparent anomaly. A small effect of ionic strength on k_{et} is a possibility in view of the uncertainty surrounding the structure of the single reaction complex (see above).

The second question posed at the beginning of this section, the effective size of the reactive patches, is partially answered by emphasizing the fact that a contribution to electron transfer is significant in any orientation provided the heme-Cu distance

remains below roughly 14 Å. The reaction scheme above assumes that there is a single, specific active complex, represented by [$Pc_{ox}Cyt f_{red}$]. The encounter complex, by contrast, does not have a single configuration but consists of an ensemble of different complexes close to the position of the final complex or, more likely, allows rapid movement between these positions until rearrangement into the reaction position occurs. The loose distance requirement of electron transfer suggests that this concept of the encounter complex is too restrictive and that some encounter configurations might make a significant direct contribution. In an extreme case one might say that there is no single specific configuration responsible for most of the electron transfer. An analysis along these lines has been carried out for the reaction between *P. laminosum* cytochrome *f* and plastocyanin by Gabdoulline and Wade (2009). They modelled the reaction by Brownian dynamics simulation in which they took account of both electrostatic forces and a non-polar desolvation term to allow for close contact orientations. For each configuration the probability of electron transfer was calculated using the semi-classical formulation of Marcus (Marcus and Sutin 1985) with the PATHWAY method of Beratan et al. (1991) to calculate electronic coupling, and the results were summed to give the overall value of k_{et}.

The value of k_{et} has been measured directly by laser flash photolysis in the presence of deazariboflavin to generate a free radical which rapidly reduces cytochrome *f*. The subsequent reoxidation by plastocyanin can then be recorded. Turnip cytochrome *f* reducing French bean plastocyanin gave $k_{et} = 2800$ s^{-1} (Qin and Kostić 1992), but the homologous *Nostoc* proteins gave 13,400 s^{-1} (Albarrán et al. 2005). These figures are surprising. The dominant factor determining k_{et} is the distance between the Fe and Cu atoms, so this should be shorter for *Nostoc* than for the higher plant proteins, remembering that according to Gamow's tunnelling equation with a value of $\beta = 1.4$ Å^{-1} the rate declines by a factor of 10 for every

1.6 Å. In fact, the opposite seems to be the case. The NMR structures give average values of ~16 Å for *Nostoc* (Díaz-Moreno et al. 2005a) and 10.9 Å for turnip/spinach (Ubbink et al. 1998). Although in the latter case plastocyanin was from spinach rather than French bean, the difference in structure would be expected to be small. The relatively large distance for *Nostoc* is probably partially offset by the dynamic nature of the complex, that is to say electron transfer from configurations of the encounter complex is significant, as demonstrated by Scanu et al. (2013) but it is difficult to judge just how important this is. However, a further factor favouring a relatively high value of k_{et} in the higher plant combination of turnip cytochrome *f* and spinach plastocyanin (which favours a single reactive configuration) is the value expected for k_{et} from stopped-flow measurement of k_2, the second-order rate constant, and NMR measurement of the binding constant for which values of 1.8×10^8 M^{-1}s^{-1} and 7×10^3 M^{-1} respectively have been obtained (Kannt et al. 1996). According to a simple kinetic analysis (Bendall 1996; Kannt et al. 1996) k_{et} has a minimum value of k_2/K_A or 2.6×10^4 s^{-1}. For the *Phormidium* system of homologous proteins the measured values are $k_2 = 4.7 \times 10^7$ s^{-1} (Schlarb-Ridley et al. 2002a) and $K_A \sim 10^3$ M^{-1} (Crowley et al. 2001), giving $k_{et} \geq 4.7 \times 10^4$ s^{-1}.

The *Phormidium* system presents further unsolved kinetic problems. In the first place analysis of chemical shift and pseudocontact shift changes described above did not converge on a single structure for the complex and yielded a much larger variation of configurations than did the higher plant proteins (Crowley et al. 2001). The correct interpretation of this situation is uncertain, and one possibility is that there is no single reactive structure but an ensemble of closely related configurations in fast exchange (an encounter complex), each of which has a somewhat lower probability of electron transfer than a single optimum configuration. Each of the calculated structures showed the head-on orientation of plastocyanin relative to cytochrome *f* but the point of closest

approach of the copper ligand, His92, was always to Phe3 rather than Tyr1 (the heme ligand) and gave an average Fe to Cu distance of 15 Å. An interesting comparison can be made with the proteins from *Prochlorothrix hollandica*. The wild type proteins form a defined complex with an orientation similar to that of *Nostoc*, but the plastocyanin has an unusual structure of its hydrophobic interaction site in which G12 has become Y and L14 has become P, compared with plastocyanins from most other sources. Hulsker et al. (2008) characterized the dynamics of both the wild type plastocyanin and the Y12G/P14L mutant with cytochrome *f* by NMR spectroscopy. Chemical shift perturbations and pseudocontact shifts showed that the transient complex formed by the mutant was much more dynamic than that of the wild type, so that the encounter state had become more highly populated. Despite this increase in dynamics the binding constant and overall rate of electron transfer showed little change.

A second problem concerning *P. laminosum* arose from a study of the effects of viscosity on k_2 (Schlarb-Ridley et al. 2005). This rate was previously thought to be diffusion controlled and would therefore be expected to be inversely proportional to the viscosity of the solvent. In practice a series of different viscogens, glycerol, ethane diol and sucrose, gave almost identical rates which depended not on η^{-1} but on $\eta^{-1.8}$. This result was inconsistent with a reaction which was purely diffusion controlled, as was the high temperature coefficient, but is supported by the most recent Brownian dynamics simulations (Gabdoulline and Wade 2009). Several possible, but conflicting, explanations for the power function viscosity dependence remain possible. One would be that each of the viscogens had similar specific effects on the proteins that would inhibit the rate, although plotting against molality rather than viscosity gave markedly different responses. Schlarb-Ridley et al. (2005) attempted to explain the result in terms of a multistep reaction, assuming a single active configuration, which might involve Tyr1 rather than Phe3, and so could be described as a conformationally

gated reaction. It is not clear, however, what effect of viscosity might be expected if no such single configuration exists, as discussed above.

Hoffman and colleagues have made extensive studies of the more extreme case of the reaction between cytochrome b_5 and myoglobin. The native reaction is the reduction of metmyoglobin to its functional form, but the reaction studied has been the triplet quenching of zinc-heme myoglobin by reduction of ferric cytochrome b_5 (Nocek et al. 1997). The interaction is clearly strongly dependent on electrostatic attraction. The problem is that the reaction center of both proteins is the exposed heme edge, so that the two sets of heme propionate groups would be expected to repel each other. If electron transfer were dependent on a single configuration of low energy (simple docking) one would expect neutralisation of the propionate charges on Zn-myoglobin to increase k_2, the second-order process, by increasing the binding constant K_A, but although k_2 was observed to increase 100-fold, there was very little effect on binding (Liang et al. 2002). This could not be explained by the simple docking model but was consistent with a dynamic docking model in which numerous configurations of the complex exist with similar but not identical energies, but only a few, not those with the lowest energy, are effective in electron transfer. The effect of heme neutralisation was to increase the probability of the complex finding the most reactive configurations involving close contact between the two hemes.

The question arising, then, is how to identify the configurations that contribute to the encounter ensemble. Camacho et al. (1999) have calculated three dimensional maps of free energy landscapes for formation of encounter complexes of several protein–inhibitor complexes. Harel et al. (2007) have used double mutant cycle analysis to identify pairs of surface residues of the two proteins that are involved in the transition state for rearrangement of the encounter complex for two enzyme-inhibitor complexes, and in a third case a Type I interferon binding to the extracellular domain of its receptor.

Mutagenesis studies can reveal kinetically significant residues that either enhance or inhibit the rate of reaction. The most powerful technique currently for providing steady-state information about the structure and surface distribution of the encounter complex involves paramagnetic NMR, and has been developed by Ubbink and colleagues for study of electron transfer complexes. As described above, the first method made use of pseudocontact shifts of ^{15}N-labelled nuclei of one protein induced by a paramagnetic cofactor of the other. This provided distance and orientational information for individual nuclei which made it possible to calculate the structure of the single, specific complex. It gave no information about the structure of the encounter complex, but hinted strongly (without being quantitative) at the balance between the encounter complex and the specific complex, that is the size of K_r in Scheme 1, by considering the amplitude of the pseudocontact shifts. In the case of the complex between turnip cytochrome f and spinach plastocyanin it was suggested that the equilibrium lay well over towards the specific complex, with a large K_r, except at low ionic strength when the situation becomes reversed (Ubbink et al. 1998). On the other hand, in the unphysiological reaction between plastocyanin and cytochrome c small diamagnetic chemical shift changes could be observed when cytochrome c was in the reduced state and these were sufficient to identify relatively large binding areas on the surface of each protein, but no additional pseudocontact shifts were observed when the iron atom of cytochrome c was in the oxidized, paramagnetic state (Ubbink and Bendall 1997). This suggested that no single, specific complex was formed, but that the complex consisted of a dynamic ensemble of structures forming an encounter complex, which still yielded a rate constant for electron transfer of 3.5×10^6 $M^{-1}s^{-1}$ (Modi et al. 1992). Similarly dynamic complexes, with small chemical shift perturbations, have been found for cytochrome b_5 and myoglobin (Worrall et al. 2002), cytochrome b_5 and cytochrome c (Volkov et al. 2005), and adrenodoxin and cytochrome c (Worrall et al. 2003).

As described above, measurements of paramagnetic relaxation can be used to calculate the regions of each protein surface that contribute to the encounter ensemble. To achieve this it is necessary to introduce cysteine residues, one at a time, at several different sites distributed over the surface of the protein, and then to label them covalently with a nitroxide spin label. The method has been developed in relation to the reaction between yeast cytochrome c and cytochrome c peroxidase (Volkov et al. 2006, 2010a, b; Bashir et al. 2010). With the wild type proteins 70 % of the lifetime of the complex as a whole is spent in the single, specific configuration and 30 % in the encounter ensemble, but single site mutations of residues shown to be important in the specific form can shift the equilibrium in either direction. Thus the mutation T12A of cytochrome c reduces the encounter form to only 10 % of the total, while R13A increases it to 80 %. A similar qualitative increase in dynamics by mutation was earlier described for *Prochlorothrix hollandica*, as mentioned above (Hulsker et al. 2008). *Nostoc* provides an interesting intermediate case, the only one reported so far for the reaction between cytochrome f and plastocyanin. Pseudocontact shifts were sufficiently large to enable the calculation of a definite orientation (side-on) for the single specific complex, but provided no information about the encounter state (Díaz-Moreno et al. 2005a). More recently paramagnetic relaxation has been used to demonstrate that the complex is, in fact, highly dynamic and that the encounter ensemble must be significantly populated (Scanu et al. 2012). The relative population of the encounter ensemble for an interaction giving rapid electron transfer is likely to depend on whether the distance between redox centers is adequate in the configurations that contribute.

The above considerations lead to some general conclusions about the essential features of the reaction. Above all there is the need for a high k_{off} to allow plastocyanin and cytochrome c_6 to perform their function

of rapid shuttling of electrons between cytochrome *f* and photosystem I. This can be achieved with a suitable structure of the interface in a specific complex. It is composed of a small group of hydrophobic residues in a loose structure which is not sealed off completely from the surrounding solvent (Crowley and Carrondo 2004; Bendall et al. 2011). There are few, if any, hydrogen bonds or 'hot spots' of binding energy.

A high k_{off} implies a much larger k_{on} which is greater by more than one order of magnitude than the simple collisional rate makes possible. This enhancement is achieved by a steering effect which is the result of either long range electrostatic forces or shorter range hydrophobic interactions that bring the two proteins into a relatively loose configuration that forms the encounter complex. Electrostatic steering is the more common, and is probably never completely absent, but hydrophobic steering can be important and may even dominate (especially, perhaps, in thermophilic organisms) although it gives a smaller rate enhancement. The ensemble of configurations of the encounter complex can make a significant contribution to electron transfer if they bring the redox centers sufficiently close together. In extreme cases the specific complex may be dispensed with altogether (that is become just one component of the reactive encounter complex), but such a case has not been described for the reaction between cytochrome *f* and plastocyanin. A more extensive analysis of the *Nostoc* system by Scanu et al. (2013), using the paramagnetic relaxation technique described above, has provided new insight into the nature of the encounter complex in transient interactions. In this organism electrostatic attraction plays a significant part in binding and a specific complex with the side-on orientation has been identified. Nevertheless, electrostatics play no part in the definition of the specific complex because negative charges of cytochrome *f* are distributed over the whole surface of the protein, rather than localised (Albarrán et al. 2007). The similarity of the relaxation data for paramagnetic probes

placed in a variety of positions around the specific site shows that plastocyanin always approaches cytochrome *f* in the same orientation, with the hydrophobic northern site pointed towards the hydrophobic patch near the heme of the cytochrome. The model that results from these observations is that the role of electrostatics is limited to a favourable preorientation of plastocyanin as a result of the strongly localised charges on plastocyanin and delocalised charges on cytochrome *f*. Hence both the encounter complex and the specific complex are stabilized predominantly by hydrophobic interactions. This situation allows rapid electron transfer without strong, specific electrostatic attraction because the activation energy that is required for desolvation of charged residues in passing from the encounter to the specific complex is no longer necessary.

Only a limited comparison can be made between the reactions with plastocyanin and cytochrome c_6 as acceptors because there have been few studies with the latter. Grove and Kostić (2003), however, have used the triplet decay technique to investigate the reaction between cytochrome *f* and Zn-heme cytochrome c_6 from *Chlamydomonas reinhardtii*, and their results show a surprising difference in the behaviour of cytochrome c_6 and plastocyanin as acceptor proteins. With plastocyanin the *Chlamydomonas* proteins behave much like those from higher plants, which is to say that both k_2 and K_A are strongly dependent on ionic strength, indicating electrostatic steering (Soriano et al. 1998). With cytochrome c_6, on the other hand, Grove and Kostić found very little effect of ionic strength on either parameter. It would seem, therefore, that the reaction is predominantly hydrophobic, despite the fact that both proteins have well-defined acidic patches. This conclusion is at odds with that reached on the basis of Brownian dynamics simulations of each protein interacting with cytochrome *f* (Gross and Pearson 2003; Haddadian and Gross 2005) from which models of the structure of the complex were obtained. In the case of the cyanobacterial

proteins from *Nostoc* sp. PCC 7119 an NMR study of the chemical shift perturbations in the complex between cytochrome *f* and cytochrome c_6 tends to confirm the similarity between the behaviour of cytochrome c_6 and plastocyanin, both of which show an interaction between basic groups in the acceptor with acidic groups in cytochrome *f* (Díaz-Moreno et al. 2005b).

Mutagenesis of the reactants in individual species has demonstrated that evolution has not produced sequences that optimise the rate of electron transfer as it occurs in solution in vitro. This suggests that the wild type proteins have already achieved rates considerably in excess of the maximum that may be required in vivo, when other limiting factors presumably come into play, and when other constraints may also be significant. The factors discussed above nevertheless provide the basis of what is possible under in vivo conditions. So we must now turn to the question of what is known about the physiological reaction in vivo.

VIII. The Reaction In Vivo

The detailed studies of the interaction between plastocyanin and the soluble fragment of cytochrome *f* in solution, as described above, provide only a crude model of the physiological process, but it has led to a basic understanding of the physical constraints on the reaction. In the living plant the reaction occurs under conditions far from those of free solution. The in vitro studies pretend that both cytochrome *f* and plastocyanin are free to diffuse in an aqueous medium of infinite extent. In vivo the reaction occurs in the severely confined space of the thylakoid lumen which is a sheet of aqueous solution no more than about 10 nm thick. Moreover, this thin space is crowded with protein complexes anchored to the membrane, especially photosystem II and, to a lesser extent, the cytochrome b_6f complex. The luminal domains of these complexes can occupy much of the trans-luminal dimension. The diffusion space is further complicated in higher plants

by the structure of thylakoids, with differentiation into the appressed membranes of granal stacks and unappressed stroma lamellae. This differentiation is associated with a functional differentiation involving segregation of the major membrane complexes. Most of photosystem II, and all the active form, occurs in the appressed granal membranes, whereas photosystem I is confined to the stroma lamellae and the end membranes and possibly the disc margins of the grana. Fractionation of disrupted thylakoid membranes by phase partitioning has shown that each of the three fractions containing photosystem I has about one-third of the total (Albertsson 2001), but the origin of the fraction corresponding to disc margins according to Albertsson has been questioned (Anderson et al. 2012). The cytochrome b_6f complex, on the other hand, is evenly distributed between appressed and unappressed membranes (Cox and Andersson 1981; Anderson 1982). Connexion between the two photosystems is mostly dependent on diffusion by plastocyanin, as plastoquinone is largely confined to small domains (Joliot et al. 1992; Kirchhoff et al. 2000) and the thylakoid lumen is thought to be one continuous space.

The complexity of the thylakoid membrane suggests diffusion paths for plastocyanin of very different lengths. The longest is that between the cytochrome b_6f complex in the grana and photosystem I in the stroma lamellae, and there is evidence for an increase in granal plastocyanin at the expense of that in stroma lamellae in the light (Haehnel et al. 1989). Linear electron flow, however, is more likely to depend predominantly on diffusion only as far as the disc margins and the end membrane of granal stacks. Diffusion within a granum would be facilitated in the model of thylakoid structure of Shimoni et al. (2005) according to which there are direct connections between the individual sacs of a granum. Photosystem I in the stroma lamellae is primarily concerned with cyclic electron flow and can occur as a supercomplex with the cytochrome b_6f complex (Joliot and Joliot 2002; Iwai et al. 2010). This

provides the shortest possible diffusion path. Diffusion paths also vary with the type of organism. Chloroplasts from higher plants have been the most intensively studied, but green algae, such as *Chlamydomonas reinhardtii*, lack the highly organized separation of thylakoid membranes into grana and stroma lamellae, although some appression of membranes does occur. Thylakoid membranes of cyanobacteria are completely unappressed, as might be expected from the need to accommodate phycobilisomes. Corresponding to the uniformity of the thylakoid membranes, no lateral segregation between the photosystems has been detected (Mullineaux 1999), so that diffusion paths are likely to be short.

The various factors that may affect the rate of diffusion are difficult to evaluate. The confined space represents a reduction in dimensionality which would enhance the rate, but this would be offset by the greater length of the tortuous path available. An attempt was made to gain information about the effect of macromolecular crowding in the lumen by examining the effect of the artificial polymers, Ficoll and Dextran, on the reaction in solution between cytochrome *f* and plastocyanin of the cyanobacterium *Phormidium laminosum* (Schlarb-Ridley et al. 2005). The surprising result was that there was no detectable effect up to a concentration that increased the viscosity threefold. Phillip et al. (2012) have explained a similar lack of effect with the reaction between β-lactamase and its inhibitor protein as the combination of two opposing effects, an enhancement of binding because of the excluded volume, and an inhibition by decreased diffusion rate.

Plastocyanin diffusion may also be influenced by physiological conditions, as well as by thylakoid structure. The total concentration of plastocyanin in the thylakoid is of the order of 1 mM and one could expect a substantial proportion of this to be bound to the cytochrome b_6f complex and photosystem I. Isolated cytochrome b_6f complex has indeed been found to contain plastocyanin (Romanowska and Albertsson 1994). Early experiments with 'broken' chloroplasts from higher plants (i.e. those that have lost their outer membrane and stroma but possess intact thylakoids) showed that photooxidation of cytochrome *f* and photosystem I activity were inhibited at low osmotic pressure (Avron and Chance 1967; Bendall and Wood 1978; Lockau 1979). Lockau showed that this is probably due to a decrease in internal ionic strength, rather than an effect on luminal volume, which would be consistent with the inhibition observed in vitro (but see below). By contrast Cruz et al. (2001) showed that hyperosmotic shock of whole cells of *Chlamydomonas reinhardtii* caused inhibition of light-induced electron transfer through plastocyanin, and electron micrographs suggested that inhibition was associated with a contraction of the thylakoid space. The idea that electron transfer through plastocyanin is influenced by the dimensions of the thylakoid lumen has been followed up by Kirchhoff et al. (2011)). They found that the thylakoid lumen of grana in the chloroplasts of *Arabidopsis* leaves expands from about 5 nm in the dark to 9 nm when illuminated. Modelling demonstrated how the known density of photosystem II complexes in the membrane provided extremely limited space for plastocyanin diffusion, which would be significantly alleviated by expansion of the lumen in the light. Measurements of the redox state of cytochrome *f* and P700 confirmed the limited effectiveness of plastocyanin diffusion and its improvement in the light. These results seem to contradict the long-standing view that the lumen contracts on illumination, which originated with the experiments of Murakami and Packer (1970), but Kirchoff et al. argue that their own use of cryo-electron microscopy gives more realistic results than the slow chemical fixation used by Murakami and Packer. Simulation of the diffusion of plastocyanin within a narrowly confined space such as the thylakoid lumen by Kovalenko et al. (2008) showed a remarkably sharp optimum in the rate of complexing to cytochrome *f* at a lumen thickness of 8 nm. This conclusion is consistent with the experimental result of Kirchoff et al.

Whether or not variations in luminal thickness provide an effective system for regulation of photosynthetic electron transport is not known. Observations need to be extended to other organisms in order to establish the generality of such an effect. In higher plants it seems that plastocyanin diffusion is not normally a limiting factor for linear electron flow but may become so under some circumstances, with variation in lumen thickness an unexpected controlling factor. It is also unlikely to be limiting for cyclic electron flow.

The question now arises as to whether diffusion of plastocyanin in the lumen is influenced by electrostatics as it is in vitro. Experimental work attempting to answer this question is limited. The first point is that as far as plastocyanin is concerned there is little difference in solution between the behaviour of cytochrome f either in its truncated, soluble form, or as a component of the cytochrome b_6f complex, as shown by Illerhaus et al. (2000) for plant preparations. This is consistent with the X-ray structures of the cytochrome b_6f complex that show that in the complex the reaction site of cytochrome f for plastocyanin around Tyr1 is exposed to the lumen (Kurisu et al. 2003; Stroebel et al. 2003).

The question has been most thoroughly investigated with mutants of Chlamydomonas reinhardtii by Soriano et al. (1996, 1998), but their results do not provide a clear-cut answer. Cytochrome f of Chlamydomonas is overall an acidic protein, but it contains a group of five basic residues thought to be important for binding to plastocyanin, Lys58, Lys65 and Lys66 in the large domain which contains the heme, and Lys188 and Lys189 in the small domain. The approach used was to prepare mutant cultures in which all these residues were neutralized or only those of either the small or large domain. Small increases in doubling time of phototrophically grown cultures could be demonstrated, but there was no consistent effect on the half-time for flash-induced oxidation of cytochrome f. These observations suggest that the oxidation of cytochrome f is not rate limiting under these growth conditions although it might become so at an earlier stage of development or under stress (Soriano et al. 1998). There is experimental evidence that the release of oxidized plastocyanin from photosystem I is rate limiting in Chlamydomonas (Finazzi et al. 2005), and the complexity of diffusion paths in the lumen suggests that diffusion of plastocyanin might become limiting under some circumstances. It has also been shown that a small domain deletion mutant is viable, although there is a small increase in doubling time, but no significant effect on flash-induced oxidation of cytochrome f was detected (Gong et al. 2001). The lack of importance of electrostatic attraction in vivo as compared with the in vitro reaction in solution is also consistent with the fact that hydrophobic channelling is also effective. We have already commented above that the charge pattern on the two proteins has not been optimised to give the maximum rate, again suggesting that the reaction is not limiting for overall photosynthesis. Nevertheless, we have to account for the fact that the charge pattern on the two proteins is highly conserved among plants and green algae, but at the present time there is no satisfactory explanation.

The above discussion provides a rough sketch of the interaction between plastocyanin and cytochrome f in vivo, particularly for higher plants and green algae, in so far as Chlamydomonas can be taken as being representative of the latter, but clearly there are many details to be filled in and much that is still probably controversial. Moreover there is very little information concerning cyanobacteria which after all are a major group of oxygenic organisms. Nevertheless a strong impression is beginning to emerge that electrostatics are much less important for the reaction between cytochrome f and plastocyanin than studies in solution indicated (Scanu et al. 2013). The intracellular reaction in Chlamydomonas, at least, can manage quite well without help from electrostatic steering, and there are other indications that the electrostatic properties of the two proteins have not been

optimised to achieve the maximum rate of reaction. This implies that hydrophobic channelling is more generally important than has hitherto been recognized. It also indicates that the ridge of basic residues in cytochrome *f*, which is such a consistent feature of most, possibly all, higher plants and green algae, has another function which has not been clearly identified.

Studies of the behaviour of the purified proteins in solution have emphasized the need for a fast k_{off} to maintain the shuttle property of plastocyanin, which is of the essence of the reaction in vivo, and this property of the proteins does not depend on salt bridges in the reaction site. Although much of the luminal plastocyanin is likely to be bound to the cytochrome b_6f complex this must be in fast exchange with free plastocyanin. From the evolutionary point of view a fast k_{off} would not have been difficult to achieve. Whereas for many protein-protein interactions of physiological importance the key factor would have been the slowing down of k_{off}, for transient electron transfer reactions it would have been the development of hydrophobic channelling to establish a sufficiently rapid binding to a more or less specific reaction site to provide a modest increase in K_A. Short-range hydrophobic interactions seem to be adequate for this purpose, and would have been achieved at an early stage in the evolution of oxygenic photosynthetic organisms. The establishment of a rapid reaction between the cytochrome b_6f complex and plastocyanin is part of the essential background for efficient photosynthesis, but does not seem to present a limitation to the flexibility of thylakoid organisation in response to fluctuating illumination (Anderson et al. 2012).

Acknowledgements

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Cytochrome c_6 of Cyanobacteria and Algae: From the Structure to the Interaction

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Summary

Cytochrome c_6 is a small, globular and soluble hemeprotein—found in algae and cyanobacteria, but not in higher plants—that usually serves as a redox carrier between the cytochrome b_6f complex and photosystem I in the photosynthetic electron transport chain. In cyanobacteria, in particular, it can also donate electrons to cytochrome c oxidase and can thus serve as a switch between photosynthesis and respiration as these two processes share the same cellular location.

Cytochrome c_6 can be replaced by the blue copper protein plastocyanin in many cyanobacteria and eukaryotic algae, which are all able to synthesize one or the other protein depending on the relative availability of iron and copper. Such a metabolic versatility lets these organisms rapidly adapt to environmental changes. Although the three-dimensional structures of cytochrome c_6 and plastocyanin are evolutionary unrelated, their similar physicochemical properties and surface features make them easily exchangeable within each organism. Plants,

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however, only synthesize plastocyanin but not cytochrome c_6 as the increase in atmospheric molecular oxygen throughout the Earth's life made copper more readily available than iron.

This chapter also focuses on cytochrome c_6 and its physiological transient interactions with photosynthetic or respiratory membrane-complexes, namely cytochrome b_6f, photosystem I and cytochrome c oxidase. In all three cases, an optimal coupling between redox centers within the adducts is essential for electron transfer although the interactions of cytochrome c_6 with its partners must be weak enough to guarantee a rapid turnover. So rather than forming a well-defined system, the two partners adopt multiple conformations within encounter complexes that exchange among them with low activation barriers to eventually yield a final productive orientation. Accordingly, cyanobacterial and algal cytochrome c_6-involving dynamic ensembles result from a balance between hydrophobic contacts and electrostatic interactions, thus allowing cytochrome c_6 to scan the surface of its partner.

I. Introduction

In oxygenic photosynthesis, either cytochrome c_6 (Cc_6) or plastocyanin (Pc) carries out the electron transport from the cytochrome b_6f complex (Cb_6f) to photosystem I (PSI). The structures of these two small metalloproteins are unrelated, but the analogies between their surface properties make them exchangeable in organisms that are able to synthesize both. Indeed, the two proteins show similar dissociation constants towards their physiological partners (De la Rosa et al. 2002, 2011; Díaz et al. 1994a; Díaz-Quintana et al. 2003, 2008).

A few years ago, we proposed that Cc_6 is the most ancient of the two soluble carriers. The reducing nature of the primitive Earth's atmosphere favored the availability of iron rather than copper (De la Rosa et al. 2002, 2011). This context eventually reversed as the concentration of molecular oxygen heightened due to photosynthetic activity, and Pc started to replace Cc_6. In soils, copper was not a limiting element

for plants and Pc became a constitutively synthesized protein. Many cyanobacteria and eukaryotic algae still maintain their ability to adapt to changing aqueous environments by synthesizing either of the two metalloproteins. Recently, Cc_6-like proteins have been reported, and they are described in the final chapters in this book.

In this chapter, we summarize the major features that enable Cc_6 as a functional and efficient electron carrier in algae and cyanobacteria. In addition, we sum up our current knowledge on the interactions of Cc_6 with its physiological partners.

II. Cytochrome c_6 Structure and Properties

Cc_6 is a typical type-I c-cytochrome (Moore and Pettigrew 1990) with a folding different from other cyanobacterial cytochromes (Navarro et al. 1995; Frazao et al. 2001). Cc_6 shows a characteristic globular fold comprising four α-helical regions separated by loops with different sequence lengths. The first of these regions is split into two helices (Ia and Ib) because of covalent binding to heme. Figure 31.1a shows an overlay of the structures of Cc_6 from several cyanobacteria and algae. Figure 31.1b shows a ribbon diagram of Cc_6 from *Monoraphidium braunii* (pdb 1ctj; Frazao et al. 1995) colored according to residue conservation. A structure-based sequence

alignment is shown in Fig. 31.1c. Most of the identities between Cc_6 sequences in the UNIREF database (Suzek et al. 2007) fall in the range between 0.2 and 0.8 per amino acid residue. Besides those involved in binding the heme cofactor covalently, the most conserved residues (>85 % of sequences) are the hydrophobic ones that surround the heme group or help helices pack. In particular, three conserved aromatic amino acids form a core that stabilizes the interaction between helices Ia and IV. These residues are a Phe at helix I, a Tyr from helix IV and a Trp at the C-terminus. At the protein surface, only the patch surrounding the heme-lodging cleft is highly conserved. Notably, a Lys is the only invariable charged residue in the alignments. This residue makes a salt bridge with the solvent accessible carboxyl of propionate 6 and is located in the Ω loop joining helices III and IV.

In all Cc_6 species, helix IV packs with helices Ia and III, whereas helices Ib and 2 do not interact with another regular structure. The analysis of the 12 aligned structures in Fig. 31.1c indicates that helices III and IV pack according to the canonical side chain "ridges and grooves" model (Chotia et al. 1985). The average angles between these two elements are $52.4 \pm 1.1°$, and the distance between their main axes is 8.4 ± 0.1 Å. Helix IV also packs with helix Ia but, in this case, they are almost perpendicular. Average angle and distance between the main axes of these two elements are $82.5 \pm 1.4°$ and 7.3 ± 0.3 Å, respectively. These values correspond to an "extended" or non-canonical "ridges and grooves" packing model, which is made possible by the presence of an invariable Gly residue in helix Ia that faces another low-volume residue in helix IV. This slot-to-slot helix packing is conserved in all of the Cc family. In the PDB structures of Cc_6 from green algae, the nearby residue in helix IV is either Ala or Gly, while the cyanobacterial, brown, and red algal structures show either Asn or Asp instead. A still larger variability is found among the sequences deposited at UNIREF: In addition to the residues above, Glu, Gln, Ser and Thr are also found. No-tably, when the residue at that position is Asp, it makes a salt bridge with a positively charged residue in helix Ia. The D70R mutation in Cc_6 from *Nostoc* destabilizes the protein as it turns the salt bridge into a repulsive interaction (Lange et al. 2003). The Hammond behavior (Matouschek and Fersht 1993) associated with this mutant indicates that it needs to set more native interactions to arrange the two helices properly along the folding pathway.

The heme cofactor is located in a cleft of the protein. The peptide binds the cofactor by two thioether bridges involving the two Cys residues from a CXXCH motif. This motif is conserved in all type-I c-cytochromes. The proximity of the two Cys strains the macro-cycle, bringing on a major B_{1u}-symmetry distortion of the equatorial plane to yield a ruffled structure (García-Heredia et al. 2011). The geometry of the iron first coordination sphere fits a distorted octahedron. Nitrogen atoms from the tetrapyrrole are the equatorial ligands while the imidazole Nε2 nitrogen from the CXXCH motif and a thioether sulfur (Sδ) from a conserved Met act as axial ligands. The last residue is located in the conserved Ω loop joining helical regions III and IV. An octahedral distortion comes from the distance between Sδ and Fe (2.15 ± 0.003 Å), which is slightly longer than those between the nitrogen atoms and the metal (ranging from 1.96 to 2.00 Å) (Díaz-Moreno et al. 2006a).

Physical-chemical properties affecting the functionality of the protein depend on the amino acid composition, the tertiary structure of the protein and the nature of the cofactor. The pK_a value for the alkaline transition is 9.3 (Dikiy et al. 2002), identical to that of mitochondrial Cc (García-Heredia et al. 2010), which can be drastically modified upon nitration (Díaz-Moreno et al. 2011; García-Heredia et al. 2012; Ly et al. 2012).

As Cc_6 is a photosynthetic electron carrier, its surface and redox properties are of major importance to assure efficient binding to physiological partners and electron transfer between cofactors, respectively. The redox potential of Cc_6 is ca. 330 mV at pH 7.

Fig. 31.1. Sequence and structure conservation within the Cc_6 family and related proteins. (**a**) Overlay of α-carbon traces of the structures of Cc_6 from the green algae *Monoraphidium braunii* (PDB: 1ctj; Frazao et al. 1995), *Scenedesmus obliquus* (1c6o; Schnackenberg et al. 1999), *Cladophora glomerata* (1ls9; Dikiy et al. 2002) and *Chlamydomonas reinhardtii* (1cyi; Kerfeld et al. 1995), the red alga *Porphyra yezoensis* (1gdv; Yamada et al. 2000) , the dun alga *Hizikia fusiformis* (2zbo; Akazaki et al. 2008), and the cyanobacteria *Synechococcus sp.* (4eic; Bialek et al. 2009), *Synechococcus elongatus* (1c6s; Beissinger et al. 1998), *Phormidium laminosum* (2v08; Worrall et al. 2007) and *Arthrospira maxima* (1kib; Kerfeld et al. 2002). The structures of Cc_{6c} from *Synechococcus sp.* (4eie; Bialek et al. 2009) and Cc_{6A} from the plant *Arabidopsis thaliana* (2ce0; Marcaida et al. 2006) are overlaid for comparison. Traces are shown in *blue* except for sequence insertions, which are *orange* for *Chlamydomonas*, *red* for *Synechococcus sp.* and *bright green* for *Arabidopsis* Cc_{6A}. *Insertions* are marked by *arrows with the same color code*. The heme group is represented by *green sticks*, and iron by an *orange ball*. Helical regions are labeled and highlighted with colored cylinders. (**b**) Ribbon diagram of Cc_6 from *Monoraphidium braunii*, colored according to conservation scores from a Consurf computation

It is similar to that of Pc, the alternative soluble electron carrier. In part, this value is determined by the His-Met axial coordination. As expected, replacement of the thioether ligand by a more basic group, such as imidazole or thiolate, drops the midpoint potential drastically. The observed values for M58H and M58C Cc_6 mutants are −140 and −235 mV, respectively (Kranich et al. 2009; Díaz-Moreno et al. 2014). Still, the value for wild-type Cc_6 differs from those reported for other c-type cytochromes showing a His-Met axial coordination of the metal cofactor.

A more polar heme environment stabilizes the oxidized form, thereby lowering the redox potential of the protein. This depends on structural features such as solvent accessibility of the porphyrin ring or the polarity of side chains and hydrogen bonding close to the cofactor moiety (Dikiy et al. 2002). Notably, the heme moiety is mostly buried inside the protein: it only exposes methyl 3 and vinyl 4 residues at ring C, and the carboxyl group of propionate 6 at ring D. Further, no water molecule dwells near the porphyrin inside the protein. In its turn, side chain polarity modulates the midpoint potential depending on the neighboring region of the macrocycle. Thus, removing the internal aromatic ring from the Ω loop, as in the F67A mutant by De la Cerda et al. (1999) decreases the redox potential by 37 mV. In addition, the conserved glutamine at helix III is responsible for the large differences in the redox potential between Cc_6 and the cryptic cytochrome c_6-like homologues (Marcaida et al. 2006; Worrall et al. 2007) that show a hydrophobic residue in its place. The side chain amide of this Gln stacks on top of ring A of the heme.

It is worth mentioning that the NMR structures of the two redox forms of *Monoraphidium* Cc_6 (PDB: 1ced, Banci et al. 1996; PDB: 1a2s, Banci et al. 1998) barely show differences in the backbone (RMSD 0.89 Å). Still, the two structures differ in the orientation of several side chains. These belong to His30 and Gln53, along with several hydrophobic residues located near the porphyrin ring. In fact, the Gln53 side chain is the one lying on top of the heme ring A. However, X-ray Diffraction (XRD) structures of the two forms of Cc_6 from *Scenedesmus* (PDB: 1c6o, 1c6r; Schnackenberg et al. 1999) show no change as regards the side chains in these structures. Indeed, the RMSD for all the heavy atoms is 0.8 Å and that of the backbone is 0.39 Å.

Surface properties are essential for the functionality of Cc_6. The surface is mostly hydrophilic, showing a small hydrophobic patch near the heme cleft (Fig. 31.2). As its analog Pc, the heme protein displays an asymmetric charge distribution leading to a well-defined dipole moment (Frazao et al. 1995). This is common to all the Cc_6 family, though its magnitude changes from one organism to another as does the isoelectric point. The torque induced by the opposite electrostatic interactions with the surface of the physiological partners may be responsible for the dynamics of the encounter complex. Indeed NMR data shows that even when the complex is highly dynamic, Cc_6 always shows the rim of the heme cleft to the surface of its partner (Díaz-Moreno et al. 2005a, b, 2014).

Fig. 31.1. (continued) (Landau et al. 2005) on the full UNIREF dataset. The orientation is similar to that of the *left image* in panel (**a**). Conserved residues are in *blue*, variable in *red*, and those not evaluable in *green*. The side chains of the most conserved residues are represented by cyan sticks. (**c**) Sequence alignment based on the above structural overlay. Consensus sequence is colored in *blue* for fully conserved residues, *cyan* for partially conserved residues and *black* for those showing variability. Individual sequences are colored according to their hydrophobicity index (Kyte and Doolittle 1982). *Residues in bold* are in close vicinity to the heme porphyrin (Graphics created with UCSF Chimera (Pettersen et al. 2004)).
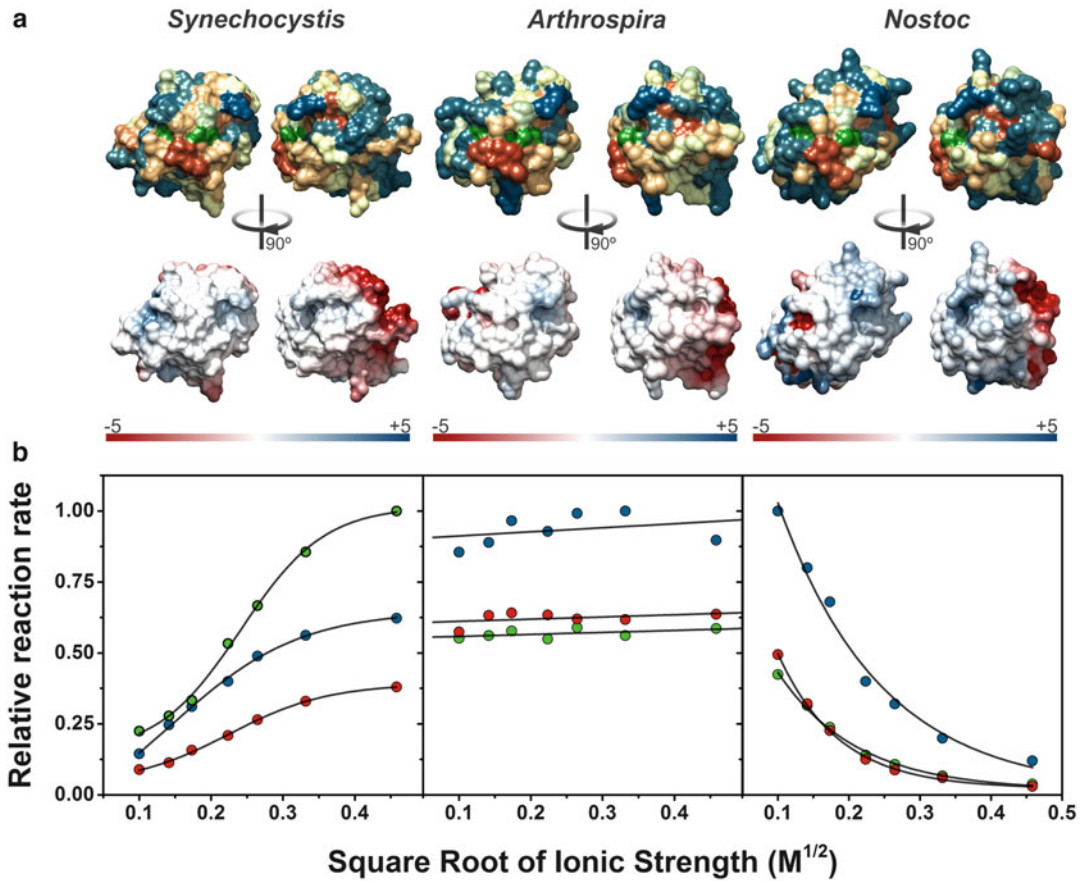
Fig. 31.2. Surface properties and functionality of Cc_6. (**a**) Surface solvation properties and electrostatic potential maps of Cc_6 from *Synechocystis* sp. PCC 6803, *Arthrospira maxima* CS-328 and *Nostoc* sp. PCC 7119. The *top row* shows hydropathy maps according to their Kyte and Doolittle (1982) score. Hydrophobic residues are shown in *brown*, hydrophilic are *blue* and the heme group is *green*. Below the hydropathy profiles, the surface electrostatic potential maps are shown with similar orientations. The scales are in k$_B$$T/e$ units. Computations were performed with DelPhi (Nicholls and Honig 1991) at 150 mM ionic strength. (**b**) Ionic strength dependencies of the electron transfer rates from the three cytochromes to PSI from *Synechocystis* (*green circles*), *Arthrospira* (*red circles*) and *Nostoc* (*blue circles*), as reported by Hervás et al. (2005).

III. Cc_6-Involving Photosynthetic Complexes

The electron transfer from the Cb_6f complex to PSI is carried out by Pc and Cc_6 (Díaz et al. 1994b; Díaz-Quintana et al. 2003). Most cyanobacteria and green algae synthesize either Pc or Cc_6 depending on the availability of copper and iron, their respective cofactor metals (De la Rosa et al. 2002; Durán et al. 2004). Higher plants only contain Pc, although a Cc_6-like protein has been identified in *Arabidopsis* (Gupta et al. 2002),

but it is unable to transfer electrons to PSI (Molina-Heredia et al. 2003).

A. Cc_6 as the Electron Donor to PSI

PSI is the electron acceptor for Cc_6 (Hope 2000). Solar energy absorbed by PSI sparks the translocation of an electron from either Cc_6 or Pc at the lumenal face of the membrane to flavodoxin and ferredoxin at the stroma (Medina et al. 1992; Casaus et al. 2002). The membrane-embedded assembly comprises a heterodimeric core bearing the

intrinsic pigments and cofactors for excitation energy and electron transfer. A peripheral antenna system provides additional pigments for effective light-harvesting. The X-ray coordinates of PSI from cyanobacteria (PDB code: 1jb0; Jordan et al. 2001; PDB code: 4kt0; Mazor et al. 2013) and plants (PDB code: 3lw5; Amunts et al. 2010) are available. The structural features of PSI have been described by Nelson and Mazor in another chapter in this volume.

An indenture of the luminal surface of the PsaA-PsaB heterodimer provides the binding site for Pc and Cc_6. It is located around the pseudo-C_2 axis of the reaction center, close to the P700 cofactor. There, two solvent-exposed helices form a hydrophobic patch to interact with the electron donors. Each one contains a highly-conserved Trp residue, essential for the binding and the subsequent electron transfer process (Sun et al. 1999; Sommer et al. 2004). Two acidic residues in these helices seem to destabilize the complex in algae, to warrant an efficient turnover of Pc (Kuhlgert et al. 2012). In addition, the cyanobacterial and eukaryotic complexes differ in the donor binding site. Compared to the prokaryotic N-terminal domain of PsaF, the eukaryotic domain has an 18-residue insertion. This spans the first helical region of the subunit and provides four additional Lys residues to the donor binding site. In eukaryotes, this basic patch of PsaF and the acidic regions of the soluble carriers undergo long-range electrostatic attractions. This favors the subsequent hydrophobic interaction with the reaction center core. However, the N-terminus of the lumenal domain of the prokaryotic PsaF subunit lies more distant from the C_2 axis. These changes account for the different abilities of the distinct PsaF subunits to bind the donor. Indeed, inserting a Lys-rich helix into *Synechococcus elongatus* PsaF makes the cyanobacterial PSI behave as the plant complex when reacting with algal Pc.

The mechanism for the electron transfer from Cc_6 to PSI has been widely analyzed using fast-kinetics approaches. Light oxidizes the special chlorophyll pair P700 in PSI to trigger charge separation within the reaction complex. Then, the electron transfer reaction from any donor to the oxidized $P700^+$ species is recorded by UV-Visible spectroscopy (Díaz-Quintana et al. 2003). The changes in the redox state of the P700 cofactor are measurable at either 698 nm (Hiyama and Ke 1972) or 820 nm (Mathis and Sétif 1981). Pulsed laser devices provide a fast excitation of the pigment, keeping the monitor dead times to within the sub-microsecond time scale.

Early kinetic analyses of P700 reduction used Pc as the electron donor. Notably, collision-limited models mismatched the reaction mechanism. Indeed, the reaction showed a two-exponential trace with half-times of 12 and 160 μs (Bottin and Mathis 1985). The fast and slow phases fitted to first-order and second-order reactions, respectively. Hence, the initial phase was attributed to direct electron transfer in a preformed Pc/PSI complex and the second one to a binding-limited, two-step reaction model reported by Tollin et al. (1986). The first analyses using Cc_6 (Hervás et al. 1992, 1994; Medina et al. 1993) displayed a similar behavior.

Further insight into the reaction with proteins from different sources demonstrated the variability of its performance under different conditions. To integrate this information, we first proposed a single three-step kinetic model that accounted for three observable kinetic behaviors (Hervás et al. 1995). Thus, the properties of the reaction partners and the experimental conditions determine the limiting step in each case.

The initial, second-order binding step limits the rate of the slow exponential decay at low protein concentrations. Viscosity affects the kinetics of this phase. According to the observed *in vitro* ionic strength profiles of kinetic rates, this event is influenced by long-range electrostatics. In some cyanobacteria such as *Synechocystis sp.* PCC 6803, this is the limiting step (Hervás et al. 1994, 1995, 1996). This is due to the subsequent phenomena lasting much less than the lifetime of the complex.

The encounter complex is highly dynamic, and suffers conformational re-arrangements that determine the probability for the intramolecular electron transfer. This heterogeneity of the encounter is responsible for the biphasic ionic strength dependencies of kinetic data (Watkins et al. 1994). In fact, the PSI reduction rate in eukaryotic organisms shows a bell-shaped ionic strength profile (Hervás et al. 1995). The drift across the encounter conformation landscape affects the relative amplitudes of the distinct exponential phases within the reaction trace. This step may limit the rate of the slow phase at saturating donor concentrations. In most green algae and plants, a rearrangement step is needed to fit all the kinetic data (Hervás et al. 1995; Sigfridsson et al. 1995). However, in other cases as in the green alga *Monoraphidium*, this event cannot be easily discriminated (Hervás et al. 1995), and the data are fit by the classical two-step mechanism (Yoneda and Holwerda 1978; Tollin et al. 1986).

Finally, donor dissociation is another important event in the reaction, but little is known about the dissociation of Cc_6 from PSI. Still, double-flash excitation experiments and site-directed mutagenesis have shown that oxidized Pc dissociation may limit the donor turnover demanded in photosynthesis (Drepper et al. 1996; Kuhlgert et al. 2012). An efficient turnover demands differences in the affinity of the partner towards the two redox forms of Cc_6. This may be due to some structural change in the partners during the reaction, or just to a change in electrostatics. Dissociation may be favored by the charge transfer during the reaction, as it affects the electrostatic interactions between the two partners. The donor gains a positive charge upon the electron transfer, and we may speculate that the two conserved acidic residues of helices at the binding site of PSI cause mismatching in the complex once P700 has been reduced.

The above schema helps to understand the reaction kinetics under different conditions. For instance, the effects of electrostatics on a reaction can be inferred from its ionic strength profiles. Usually, depending on the organism, Cc_6 holds either attractive or repulsive long-range electrostatic interactions with its partners under *in vitro* conditions (Fig. 31.2b). Sometimes, as in *Arthrospira*, Cc_6 reduces PSI in an ionic strength independent way (Hervás et al. 2005; Fig. 31.2b). Moreover, the sign of the interaction and the reaction mechanism in cyanobacteria rely on the surface properties of Cc_6 and they are independent of those of PSI (Hervás et al. 2005; Fig. 31.2b).

Thus, changes in the ionic strength or pH may decrease the encounter affinity while easing the re-arrangement (Hervás et al. 1998; Molina-Heredia et al. 1998, 1999). A single mutation may affect one or more events along the reaction pathway, thereby modifying the kinetic behavior (Molina-Heredia et al. 1999; Olesen et al. 1999). Notably, protein charge modifications showed that the collision-limited kinetics depend on the charge configuration of the protein (De la Cerda et al. 1999). The kinetics of Cc_6 oxidation by PSI in *Synechocystis* correlate with the size of the positive region surrounding the heme cleft. Single mutations also serve to determine that a conserved Arg residue (Arg67) plays an essential role in the binding event (De la Cerda et al. 1999; Molina-Heredia et al. 1999) and that this feature is common to Pc (Molina-Heredia et al. 2001).

As opposed to the interaction between Cf and Cc_6 (see below), little is known about the conformation of the electron-transfer complex between Cc_6 and PSI. To the best of our knowledge, co-crystallization trials have failed, and the complex is too large to be characterized by NMR. Still, Transverse Relaxation-Optimized SpectroscopY (TROSY) NMR has provided a clue that Cc_6 interacts with PSI using the same surface patch as with Cf (Díaz-Moreno et al. 2005b). Notably, most of the NMR signals from ^{15}N-labeled Cc_6 from cyanobacteria are still monitored in the presence of an excess of PSI trimers. This clearly indicates that the complex is highly dynamic. All the Cc_6 signals should broaden

beyond the detection limits if the binding to PSI is tight, given the long diffusional correlation time of the reaction center. In addition, X-ray absorption spectroscopy (XAS) experiments have shown that binding to PSI induces subtle changes in the heme iron. Indeed, the absorption edge of Fe^{2+} Cc_6 shifts slightly towards lower energies (Díaz-Moreno et al. 2006a).

B. Cc_6 as the Electron Acceptor from the Cb_6f Complex

1. The Structure of Algal and Cyanobacterial Cb_6f

The hetero-oligomeric Cb_6f complex contains eight tightly-bound polypeptide subunits that couple the electron transfer to proton translocation, generating the proton electrochemical potential gradient necessary for ATP synthesis. The three-dimensional crystal structure has been determined for the Cb_6f complex from the green alga *Chlamydomonas reinhardtii* (Stroebel et al. 2003), and the cyanobacteria *Mastigocladus laminosus* (Kurisu et al. 2003) and *Nostoc sp*. PCC 7120 (Baniulis et al. 2009). On the one hand, the first two Cb_6f crystallographic structures are essentially identical despite the great evolutionary distance between the prokaryote (*Mastigocladus*) and the eukaryotic organelle (*Chlamydomonas*). Both structures reveal the presence of a chlorophyll molecule and a β-carotene in unusual surroundings, whose functions are still unclear. On the other hand, the main difference between the cyanobacterial Cb_6f crystallographic structures is the acetylation of the *Nostoc* Rieske Fe-S protein at the N terminus, a post-translational modification uncommon in cyanobacterial membranes and electron transfer proteins (Baniulis et al. 2009).

Cytochrome f (Cf) is a subunit of the Cb_6f complex, anchored to the thylakoid membrane by a C-terminal transmembrane helix leaving a 28-kDa soluble portion exposed to the lumen with a clear two-domain structure. The large domain harbors the heme, and the small domain possesses a patch of charged residues. Cf is considered an unusual *c*-type cytochrome because of its β-sheet structure, elongated form and particular, heme axial coordination with the amino group of the N-terminus residue, Tyr1 (Martínez et al. 1994; Stroebel et al. 2003; Kurisu et al. 2003; Díaz-Moreno et al. 2006b; Baniulis et al. 2009).

2. Kinetics of Electron Transfer Between Cf and Cc_6

Fast-kinetics studies combined with Brownian dynamics using a *Chlamydomonas* Zn-Cc_6 derivative and Cf have been reported (Grove and Kostic 2003; Grove et al. 2012), concluding that the hydrophobic forces are crucial not only for protein docking but also for reactivity. The association of both redox metalloproteins is only weakly enhanced by electrostatic interactions. The kinetic rate constants are invariant with ionic strength over a wide range.

Such studies reveal that both cytochromes associate in broad, dynamic ensembles of configurations whose exchange is possible because they are close in energy. As the ensemble is driven by non-directional hydrophobic interactions that allow easy rearrangement, the oxidoreduction mechanism between Cf and Cc_6 can be true, coupled, gated, or a combination of the three. In the first case, the electron transfer is the limiting step. In the gated mechanism, rearrangement is the reaction bottleneck. Finally, in the coupled electron transfer, rearrangement is thermodynamically unfavorable, but faster than the electron transfer event (Grove et al. 2012).

3. Structural Model of the Cf and Cc_6 Complex

NMR-based structural approaches using heme proteins from several cyanobacterial sources unveil differences in the specificity, not only for Cf-Pc complexes (Díaz-Moreno et al. 2005c, d), but also for the Cf-Cc_6 complexes. The acidic Cc_6 from *Synechococcus elongatus* forms a non-

specific and highly dynamic adduct with *Phormidium* C*f*, which has a negative net charge (Crowley et al. 2002). On the contrary, the interaction site of the basic C*c*$_6$ from *Nostoc sp.* PCC 7119 when binding to C*f* from *Phormidium* or *Nostoc sp.*, which is also negatively charged, consists of a predominantly hydrophobic patch surrounding the heme (Crowley et al. 2002; Díaz-Moreno et al. 2005a), as previously described in the reactive *Nostoc* C*c*$_6$-PSI complex (Díaz-Moreno et al. 2005b). The presence of positive residues near the heme cleft of *Nostoc* C*c*$_6$ also favors the attractive electrostatic interactions with either *Phormidium* C*f* or *Nostoc* C*f*.

The binding constant of the physiological C*c*$_6$-C*f* interaction from *Nostoc* is identical to that of the C*c*$_6$-C*f* cross-complex, suggesting that *Nostoc* C*c*$_6$ binds to *Nostoc* and *Phormidium* C*f* in a similar way. However, significant differences are found between the homologous (*Nostoc* C*c*$_6$ and *Nostoc* C*f*) and the heterologous (*Nostoc* C*c*$_6$ and *Phormidium* C*f*) complexes. For instance, Arg64 of C*c*$_6$, whose functional and structural relevance has been emphasized with PSI, its other redox partner (Molina-Heredia et al. 2001; Díaz-Moreno et al. 2005b), shows chemical-shift perturbations within the native complex but not within the non-physiological one.

The NMR-restraint docking simulations of *Nostoc* C*c*$_6$-containing adducts are similar (Crowley et al. 2002; Díaz-Moreno et al. 2005a) and resemble the models of the C*f*-C*c*$_6$ complex from the green alga *Chlamydomonas* (Gross and Pearson 2003) and of the cross-complex between turnip C*f* and *Monoraphidium* C*c*$_6$ (1jx8 PDB code). In all these complexes, C*c*$_6$ interacts with charged amino acids at the small domain of C*f* (Fig. 31.3a). A positively charged ridge of positive charges on the small domain of C*f* interacts with a cluster of negative charges on C*c*$_6$ in green algae while the opposite is true for cyanobacteria, despite the fact that the cyanobacterial C*c*$_6$ does not possess such well-defined, complementary charged patches.

In silico data on the *Chlamydomonas* C*c*$_6$-C*f* complex shows not only the relevance of hydrophobic and electrostatic interactions in bringing the two heme proteins sufficiently close as to allow efficient electron transfer (Gross and Pearson 2003; Haddadian and Gross 2005) but also the key role of the C*f* small domain in guiding C*c*$_6$ to dock with C*f*, which suggests that C*c*$_6$ explores different positions on C*f* (Haddadian and Gross 2006).

This finding, along with the kinetic study demonstrating that multiple conformations of C*c*$_6$ contribute to electron transfer within the C*f*-C*c*$_6$ complex (Grove et al. 2012), is in agreement with recent paramagnetic relaxation enhancement (PRE) NMR data (Díaz-Moreno et al. 2014), which are not compatible with a well-defined *Nostoc* C*f*-C*c*$_6$ complex (Díaz-Moreno et al. 2005a). In fact, the interaction can be described as a highly dynamic ensemble comprising multiple orientations without any one orientation being predominant (Fig. 31.3b). Then, after an electrostatic pre-orientation to bring both cytochromes closer, an ensemble of orientations are formed with extensive hydrophobic contacts between the heme groups, which must be sufficiently close to allow rapid electron transfer. The importance of hydrophobic contacts in the interactions of C*c*$_6$ with photosynthetic C*f* (Crowley et al. 2002; Díaz-Moreno et al. 2005a; Grove et al. 2012) and PSI (Molina-Heredia et al. 1999, 2001; Díaz-Moreno et al. 2005b), as well as with its respiratory partners (Hart et al. 2008), have been corroborated using NMR, kinetic measurements and docking calculations.

An efficient electron transfer within the C*f*-C*c*$_6$ ensemble is as important as a high off-rate to avoid reduction of the electron flow rate. To make the turnover high enough, C*c*$_6$ binds to C*f* with relatively low affinity and with no dominant stereospecific orientation; multiple contacts make the encounter complex avoid dissociation before electron transfer takes place. Then, an ensemble of conformations, governed by a gradual increase and decrease of the hydrophobic interactions between both cytochromes during association and dissociation, ensures a low
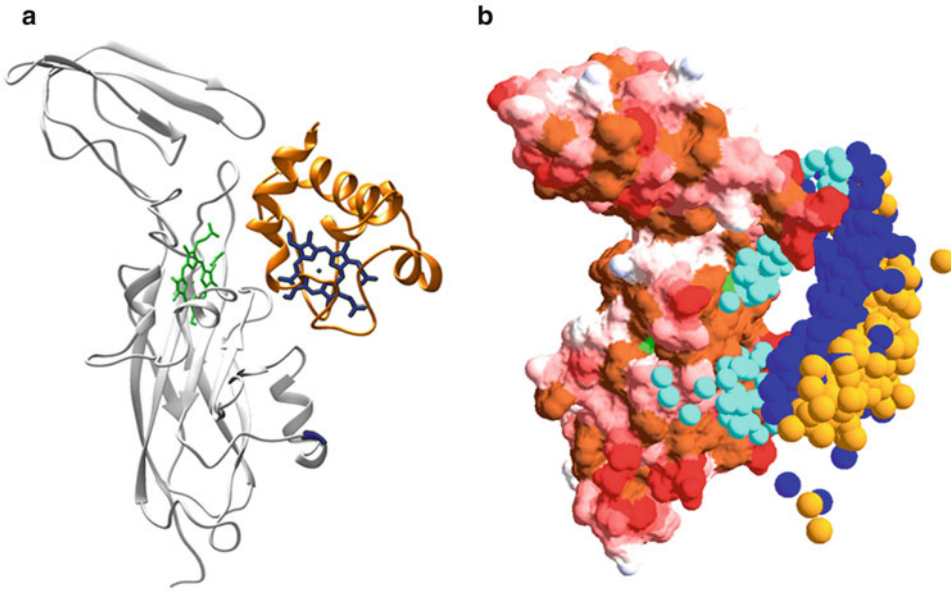
Fig. 31.3. Structural models of the complex between Cf and Cc_6. (**a**) The well-defined Cf-Cc_6 structure as calculated by NMR-restraint docking. Cf and Cc_6 are represented by *light grey* and *orange ribbons*, respectively. Heme groups are shown as *green sticks* for Cf and *blue sticks* for Cc_6. (**b**) The ensemble of Cc_6 around Cf as determined by PRE NMR restraints. Cf is shown in surface representation according to its electrostatic potential. Negative potential is displayed in *red*. Non-polar and Tyr residues are shown in *brown* and the heme, in *green*. The cyan spheres indicate the positions of the spin label oxygen atoms in the ensemble structures. The *yellow-orange* and *blue spheres* represent the centers-of-mass and iron atoms of Cc_6 in the ensemble, respectively. Images have been adapted from Díaz-Moreno et al. 2005a (panel **a**) and Díaz-Moreno et al. 2014 (panel **b**) (Figure was created with Chimera software (Pettersen et al. 2004)).

transition state barrier that accelerates the disruption of the Cf-Cc_6 complex (Díaz-Moreno et al. 2014).

IV. Cc_6-Involving Respiratory Complexes

In cyanobacteria, oxygenic photosynthesis and aerobic respiration occur within a single prokaryotic cell. The cyanobacterial thylakoid membrane contains not only photosynthetic but also respiratory electron transport complexes forming transport chains that intersect with each other and share the plastoquinone pool, the Cb_6f complex and the soluble electron carriers Pc and Cc_6 (Fig. 31.4).It has been firmly established that Cc_6 or Pc, at the thylakoid lumen, are able to reduce both PSI and Cytochrome c Oxidase (CcO). However, the
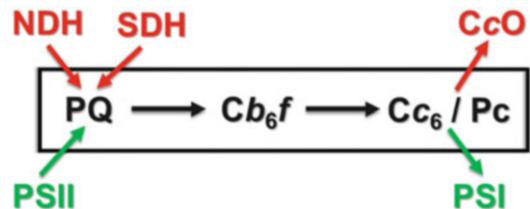
Fig. 31.4. Schematic representation of the intersecting photosynthetic and respiratory electron reactions in thylakoid membranes of cyanobacteria. Electron transfer complexes specifically involved in photosynthesis are Photosystem II (PSII) and PSI, which are shown in *green*, whereas those specific for respiratory electron flow include NADPH DeHydrogenase (NDH), Succinate DeHydrogenase (SDH) and CcO, which are represented in *red*. Plastoquinone (PQ), Cb_6f, Pc and Cc_6 are shared by both metabolic pathways. The *arrows* represent electron transfer reactions.

nature of the periplasmic carrier transferring electrons to CcO at the plasma membrane, which separates the cytosol from the

periplasm and contains only respiratory components, is still under discussion. So far, only Cc_6 has been identified at the periplasmic space of cyanobacteria (Obinger et al. 1990).

A. The Structure of Cyanobacterial CcO

No structural information is yet available for any cyanobacterial CcO complexes, but they can be modeled by using the high-resolution structures of the PDB as templates. Such three-dimensional structures are from *Paracoccus denitrificans* (Koepke et al. 2009; Harrenga and Michel 1999; Ostermeier et al. 1997), *Rhodobacter sphaeroides* (Svensson-Ek et al. 2002; Qin et al. 2006, 2009), *Rhodothermus marinus* (Srinivasan et al. 2005), *Thermus thermophilus* (Williams et al. 1999; Soulimane et al. 2000; Liu et al. 2009; Lyons et al. 2012; Tiefenbrunn et al. 2011; Luna et al. 2012) and *Bos taurus* (Tsukihara et al. 1996, 2003; Yoshikawa et al. 1998; Muramoto et al. 2007; Suga et al. 2011).

Cyanobacterial CcO belongs to the superfamily of heme-copper oxidases and transfers electrons from Cc_6 to oxygen. CcO is usually an aa_3-type protein complex, whereas those described for *T. thermophiles* are aa_3- or ba_3-type. It is composed of three core subunits (Fig. 31.5). Subunit I (also called CtaD) ligates heme a and the binuclear center formed by heme a_3 and Cu_B. Subunit II (CtaC) ligates the Cu_A center. Subunit III (CtaE) does not ligate any redox centers, but could play a role in complex stability and assembly.

The assembly of the Cu_A cofactor in the CtaC of eukaryotic CcO is performed by Sco proteins, which work as copper transporters (Herrmann and Funes 2005; Cobine et al. 2006; Banci et al. 2011). The Sco proteins are also found in prokaryotic organisms where their function goes beyond the assembly of the Cu_A center. Sequenced prokaryotic genomes reveal that ca. 12 % of them code for Sco proteins but not for Cu_A-containing proteins (Banci et al. 2007). It has been proposed that such Sco proteins in bacteria, namely *Neisseria gonorrhoeae* and

Rhodobacter capsulatus, protect periplasmic proteins from oxidative damage (Seib et al. 2003; Borsetti et al. 2005). Unlike eukaryotes and most bacteria, archaea and cyanobacteria do not contain either the Sco proteins or their metallochaperones, although the CcO complexes harbor Cu_A cofactors. Therefore, the mechanism by which the copper delivery to CtaC occurs in cyanobacteria, if one exists, is still unknown.

B. Kinetics of Electron Transfer Between Cc_6 and CcO

In the thylakoid lumen, Cc_6 can deliver electrons to two distinct acceptors, namely PSI and CcO, with similar Cc_6-mediated reduction rates within the same organism. However, there are some discrepancies in the oxidation rate of Cc_6 by CcO among different organisms. The Cc_6-CcO electron transfer kinetics in the cyanobacterium *Nostoc sp*. PCC 7119 (Navarro et al. 2005) is one order of magnitude higher than that reported for the interaction of *Paracoccus* bacterial cytochrome c_{552} with the extrinsic Cu_A-binding domain of the CcO subunit II (Lappalainen et al. 1993). The electron flow in the *Nostoc* Cc_6-CcO system is two to three orders of magnitude higher than that observed for the interaction of *Synechocystis sp*. PCC 6803 Cc_6 with the Cu_A-binding domain of the CcO subunit II (Paumann et al. 2004). Of particular interest is the fact that the electron transfer constant of the *Nostoc* Cc_6-CcO system resembles the constant recently estimated in human mitochondrial complexes between Cc and CcO (Rodríguez-Roldán et al. 2008).

The ionic strength dependence shown by the Cc_6-CcO system relates to the cyanobacterial organism. The oxidation of Cc_6 by CcO in *Synechocystis* occurs without the formation of any electrostatically governed and kinetically detectable complex between these proteins (Paumann et al. 2004). On the contrary, the *Nostoc* Cc_6-CcO adduct is impaired by added salt, which reveals the existence of strong electrostatic attractive interactions, as previously reported for other respiratory Cc and CcO systems (Hazzard et al. 1991;
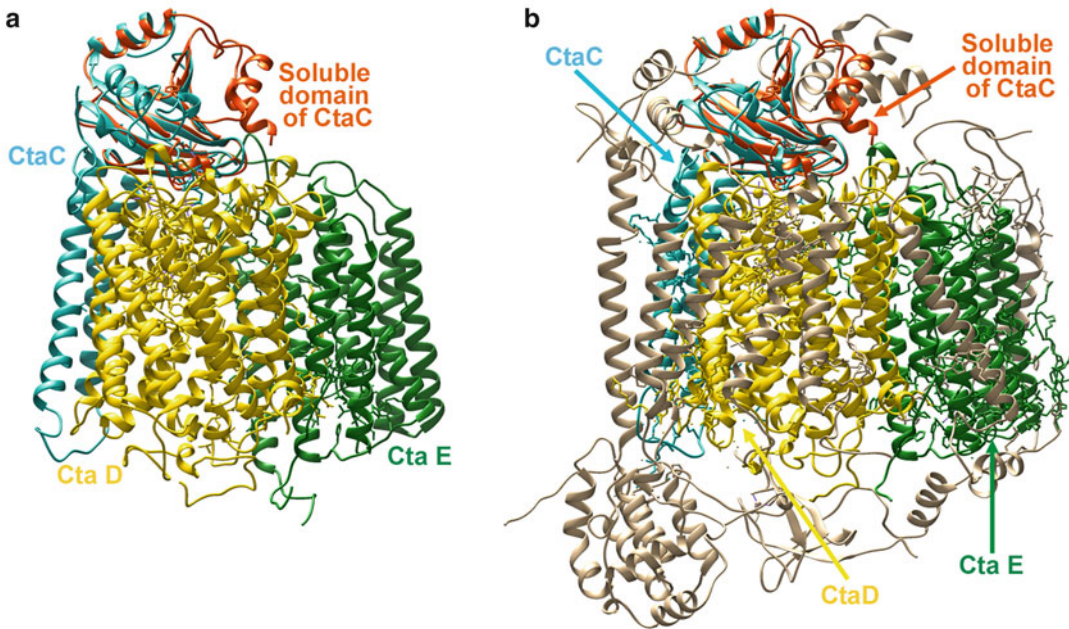
Fig. 31.5. Comparison between Bacterial and Eukaryotic CcO Structures. (**a**) *Rhodobacter sphaeroides* CcO (PDB: 1 m56; Svensson-Ek et al. 2002). (**b**) Bovine heart CcO in the fully reduced state (PDB: 2eij; Muramoto et al. 2007). The CtaC subunit is colored in *light blue*, CtaD in *yellow* and CtaE in *green*. The remaining subunits, only present in eukaryotic CcO complexes, are shown in *grey*. The soluble domain of the CtaC subunit from *Phormidium laminosum,* in *orange*, has been aligned with respect to that from *Rhodobacter* and from *Bos taurus* in panels **a** and **b**, respectively (Figure was created with Chimera software (Pettersen et al. 2004)).

Lappalainen et al. 1993; Wang et al. 1999; Maneg et al. 2003, 2004; Navarro et al. 2005; Rodríguez-Roldán et al. 2008).

Such differences between *Nostoc* and *Synechocystis* Cc_6-CcO could be due to the use of the entire membrane-bound CcO complex in the case of *Nostoc*, yielding an affinity binding constant similar to that of the human mitochondrial Cc-CcO complex. However, these differences could be inherent to these cyanobacteria. Kinetic discrepancies are also observed in the interaction of Cc_6 with PSI, with the *Nostoc* system exhibiting high electron transfer rates and strong ionic strength effects as compared with *Synechocystis* (Díaz-Quintana et al. 2003).

C. Structural Model of the Cc_6 and CcO Complex

In cyanobacteria, there is only one docking simulation analysis of Cc_6 and its physio-logical electron acceptor, the Cu$_A$-binding

domain of CcO subunit II, from *Phormidium laminosum* (Hart et al. 2008). The resulting models show seven different docking orientations, with the heme to copper distances being close enough to guarantee an efficient electron transfer. The lowest-energy docking solution is similar to that observed for the docking between Pc and CcO (Hart et al. 2008). Both complexes are mainly mediated by hydrophobic and non-ionic contacts, thus resembling the NMR restraint-based model proposed for the interaction between cytochrome c_{552} and the ba_3-type CcO subunit II from *Thermus thermophilus* (Muresanu et al. 2006). This is in agreement with previous studies on the photosynthetic electron transfer complexes formed by Cc_6 or Pc with PSI in *Phormidium*, which are independent of ionic strength (Schlarb-Ridley et al. 2002a; Hervás et al. 2003). However, the available structural and functional data on the *Phormidium* Pc-Cf adduct shows an apparent contradiction on the role of elec-

trostatics in the interaction between the two metalloproteins. Whereas the ionic strength does not have any effect on the binding equilibrium according to NMR analysis (Crowley et al. 2001), the electron transfer between Pc and Cf is slightly affected by the addition of salt (Schlarb-Ridley et al. 2002b; Hart et al. 2003). Molecular Dynamics (MD) simulations harmonize such discrepancies showing changes in dynamics of Cf caused by repulsive electrostatic interactions with Pc. Unlike the complexes in *Phormidium* and *Thermus*, the complex between the soluble cytochrome c_{552} domain and CcO from *Paracoccus denitrificans* (Flöck and Helms 2002), as well as the mitochondrial Cc-CcO complex (Roberts and Pique 1999) are exclusively driven by electrostatic steering.

A plausible explanation in *Phormidium* CcO is the lack of acidic residues close to the ligands of the copper center and to the electron accepting Tyr. These acidic residues should guide the proteins to the correct docking site via long-range electrostatic interactions in bacterial and mitochondrial CcO complexes but not in the cyanobacterial complexes. Therefore, the cyanobacterial

surface-exposed ligands of the copper center and the Tyr residue are on a relatively non-polar surface, leading to a docking mainly dominated by hydrophobic contacts (Hart et al. 2008).

The orientations of the mitochondrial Cc-CcO and bacterial cytochrome c_{552}-CcO complexes are quite different with respect to the cyanobacterial Cc_6-CcO complex (Fig. 31.6). In *Phormidium,* Cc_6 samples a region on the CcO subunit II surface that would be occupied by a loop surrounding the copper center in other organisms. This loop becomes more prominent in *Paracoccus* and mitochondria, shifting to Cc or to cytochrome c_{552} towards a different spatial location to decrease the steric hindrance. Actually, the C-terminal α-helix extension at the cyanobacterial CcO subunit II prevents the docking of Cc_6 in a similar relative position as Cc or cytochrome c_{552}. The relative orientation of c_{552} when interacting with CcO in *Thermus termophilus* clashes with that of CcO in *Phormidium*, although the cytochromes are roughly located in the same space region but with different orientations (Fig. 31.6).
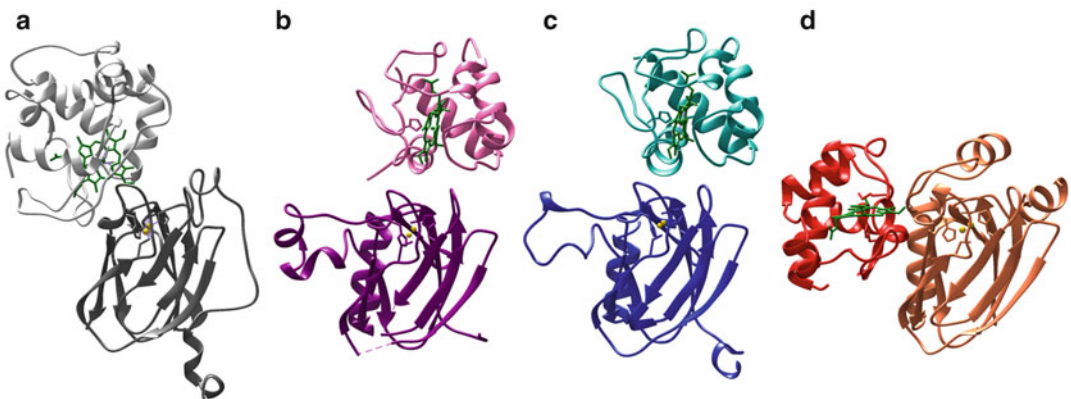
Fig. 31.6. The lowest energy docking solutions of CcO CtaC and *c*-type Cytochromes. (**a**) Cytochrome c_{552} (*light grey*) and the soluble domain of ba_3-type CcO subunit II (*dark grey*) from *Thermus thermophilus*. (**b**) The soluble cytochrome c_{552} domain (*pink*) with the soluble domain of CcO subunit II (*purple*) from *Paracoccus denitrificans*. (**c**) Mitochondrial Cc (*cyan*) bound to the soluble domain of CcO subunit II (*blue*). (**d**) Cc_6 (*red*) and the soluble domain of aa_3-type CcO subunit II (*orange*) from *Phormidium laminosum*. All the CcO soluble domains are equally oriented with respect to their copper centers. Heme groups are represented by *green sticks*, and copper centers by *yellow spheres*. Proteins are represented by ribbons (Figure was created with Chimera software (Pettersen et al. 2004)).

The finding of alternative conformations in *Phormidium* Cc_6-CcO docking calculations suggests that the proteins form an ensemble of interactions rather than a well-defined and fixed complex governed by multiple contacts, as previously reported for the mitochondrial Cc-CcO (Roberts and Pique 1999) and the cytochrome c_{552} domain with CcO from *Paracoccus denitrificans* (Flöck and Helms 2002). These electron transfer complexes require an optimal coupling between redox centers by weak and unstable associations to guarantee a rapid dissociation and an efficient turnover. As already described for other cyanobacterial photosynthetic Cc_6-involving interactions (see Sect. III), the ensemble of orientations between Cc_6 and CcO in *Phormidium* might also be dominated by hydrophobic contacts, rather than by electrostatic interactions.

V. Conclusions and Perspectives

Photosynthesis and respiration in cyanobacteria are two redox processes sharing the same cellular location and several redox components, but occurring at different time and under distinct conditions. Cyanobacteria require aerobic respiration for the generation of energy in darkness and under certain stress conditions, which impair photosynthesis. A concerted regulation of the whole electron transfer process is crucial, with Cc_6 facilitating a rapid switching between photosynthesis and respiration.

To bring the redox centers within the Cf-Cc_6, Cc_6-CcO and Cc_6-PSI adducts sufficiently close is thus essential, but the formation of a well-defined complex is not required if multiple orientations exist in which electron transfer can occur. Such a specific complex is not desirable from the point of view of fast dissociation, because a well-defined state has a lower free energy than all similar states and thus a higher transition state energy to dissociate. Further discussion of Cf interactions with Cc_6 or Pc and of cyanobacterial terminal oxidases may be found in subsequent chapters in this volume.

Acknowledgements

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Chapter 32

Electron Partitioning in Anoxic Phototrophic Bacteria

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Summary

Photosynthetic cells make major contributions to many important processes on this planet, including solar energy capture, nitrogen or carbon dioxide sequestration and production of useful biocommodities. The sheer number of photosynthetic cells also makes them significant contributors to global nutrient cycling, especially in aquatic ecosystems. For each of these activities, photosynthetic cells need efficient systems for production and distribution of reducing power among the myriad of cellular pathways that depend on reductant. This chapter focuses on the partitioning of reductant in purple nonsulfur photosynthetic bacteria. It summarizes known membrane and cytoplasmic enzymes and pathways that need the reductant produced via photochemical activity (quinol in these organisms). These observations illustrate that quinol is used to provide reducing power to a variety of crucial processes (cellular biosynthesis, maintenance of a proton motive force) and key assimilatory pathways (carbon dioxide and nitrogen fixation), depending on the availability of nutrients. We also summarize data illustrating that cells use a variety of pathways to recycle excess reductant. Finally, we illustrate how the recent use of genomic and computational approaches to the analysis of these and other photosynthetic organisms has provided testable predictions and considerable new insight into the partitioning of reductant that is produced from solar energy capture.

I. Introduction

Photosynthetic organisms are among the most abundant organisms on earth. Their unique ability to harvest solar energy and use it to fix atmospheric carbon dioxide is at the foundation of the global food chain. Consequently, photosynthetic organisms have been the attention of intense study from chemical, energetic and biological perspectives. Other chapters in this volume illustrate that photosynthesis depends on the interplay of many components. This chapter focuses on the cellular distribution of electrons that are required to support energetic and biosynthetic processes in photosynthetic organisms. Specifically, we describe recent work analyzing this process in anoxic phototrophic bacteria, particularly the purple nonsulfur bacteria (PNSB).

PNSB contain some of the most thoroughly characterized bioenergetic pathways (Blankenship et al. 1995; Blankenship and Hartman 1998). The photosynthetic apparatus of Gram-negative bacteria is typically housed in the intracytoplasmic membrane, a specialized bioenergetic membrane that is the functional equivalent of the plant chloroplast thylakoid (Tavano and Donohue 2006). These bacteria convert light energy into chemical energy by using specialized light-harvesting complexes to capture photons. Once light is captured they couple light-driven oxidation of pigments in a reaction center complex to electron transfer through membrane-bound enzymes (Meyer and Donohue 1995). The ultimate product of photochemistry in the PNSB reaction center—quinol (QH_2)—diffuses from the reaction center through the membrane where it binds to and is oxidized by the cytochrome bc_1 complex to form a trans-membrane H^+ gradient that supports ATP synthesis (Fig. 32.1) (Meyer and Donohue 1995). The reaction center of these bacteria lacks an O_2-evolving complex to reduce the photochemically-oxidized reaction center complex. Instead, periplasmic proteins

(cytochrome c_2, iron-sulfur- or non-heme iron proteins) or another integral membrane protein complex acts in a cyclic electron transport chain to reduce the light-oxidized reaction center complex to its ground state (Fig. 32.1) (Meyer and Donohue 1995). PNSB lack photosystem I, so NADH is not formed directly from light energy conversion (Meyer and Donohue 1995). Instead, a proton-translocating NADH:quinone oxidoreductase (complex I) uses the proton gradient to pass electrons from quinol to NAD^+, producing the NADH needed for anabolic reactions (Fig. 32.1) (Herter et al. 1998; Tichi et al. 2001). The photosynthetic apparatus of PNSB is the ancestor of the photosystem II complex in modern plants and oxygenic phototrophs (Blankenship et al. 1995; Blankenship and Hartman 1998). The photocycle of PNSB is arguably the best understood bioenergetic membrane process in terms of structural, functional and genetic information (Blankenship et al. 1995; Blankenship and Hartman 1998).

PNSB are typically facultative anaerobes capable of several well-studied energetic and metabolic lifestyles (McEwan 1994). Individual species of PNSB are able to metabolize a wide variety of electron-rich carbon sources under anaerobic conditions in the presence of light (Imam et al. 2013). To maintain redox balance, cells grown under these photoheterotrophic conditions require a means to recycle excess reducing power (Richardson et al. 1988; McEwan 1994; Dubbs and Tabita 2004; McKinlay and Harwood 2010b). In many species of photosynthetic bacteria excess reducing power can be dissipated or recycled through poly-β-hydroxybutyrate (PHB) synthesis (McKinlay and Harwood 2010a; Yilmaz et al. 2010; Kontur et al. 2011), the Calvin-Benson cycle (assimilating CO_2 even in the presence of organic carbon) (Dubbs and Tabita 2004), H_2 synthesis (Gest and Kamen 1949; Gest and Blankenship 2004), and respiration of alternative electron acceptors (Richardson et al. 1988). Products of uncharacterized genes also appear to recycle excess reducing

power under photoheterotrophic conditions in some species, suggesting there are yet to be identified additional uses for excess reducing power in these cells (Tavano et al. 2005).

Below, we summarize new information on the pathways used to partition or recycle reducing power in PNSB, focusing on studies or models that have been developed to analyze this in *Rhodobacter sphaeroides* and other photoheterotrophic species (Mackenzie et al. 2007; McKinlay and Harwood 2010a; Imam et al. 2011, 2013). The analysis of electron partitioning has been facilitated by prior knowledge of cytochromes and other electron carriers (Meyer and Cusanovich 1985; Bartsch et al. 1989; Meyer and Donohue 1995), the availability of complete genome sequences (Larimer et al. 2004; Mackenzie et al. 2007; Kontur et al. 2012b), the ability to generate mutants by a variety of techniques (Donohue and Kaplan 1991) and the incorporation of synthetic (Jaschke et al. 2011; Work et al. 2012; Tikh et al. 2014) or systems biology (Callister et al. 2006; McKinlay and Harwood 2010a; Imam et al. 2011, 2013, 2014, 2015a, b, c; Berghoff et al. 2013) techniques to analyze or model the metabolic or regulatory activities of these bacteria.

II. Competition for Electrons of the Quinone Pool

During anoxic phototrophy, reduced quinones (quinol) are responsible for shuttling electrons from the reaction center to the cytochrome bc_1 complex (Fig. 32.1). However, they also interact with a number of other integral membrane complexes that can alter the quinone:quinol ratio. Because the quinone pool is connected to many metabolic processes, it serves as a dynamic measure of cellular redox state. A number of energy-conserving (e.g. photosynthesis, H_2 oxidation) or energy-consuming (e.g. CO_2 fixation, N_2 fixation) pathways are regulated by the RegB/RegA (PrrB/PrrA)
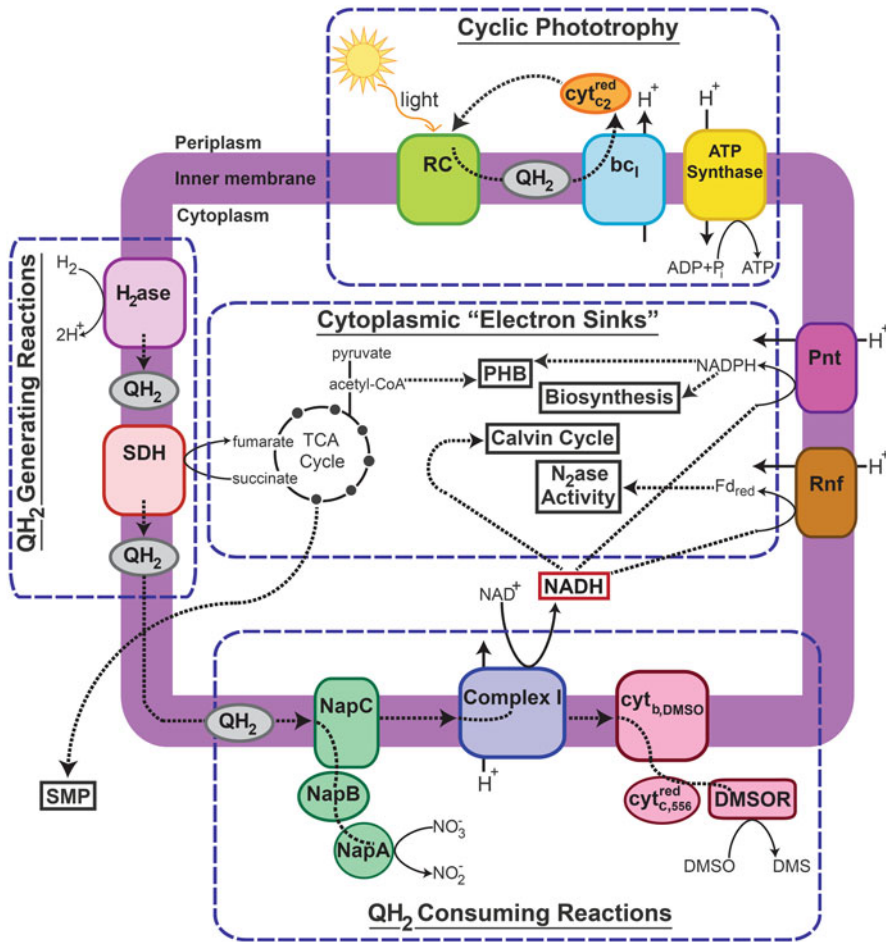
Fig. 32.1. Phototrophic electron transfer pathways in PNSB. During cyclic electron transfer, the reaction center (RC) reduces quinone to produce quinol (QH_2), which donates electrons to the cytochrome bc_1 complex (bc_1). Electrons are transferred to cytochrome c_2 (cyt_{c2}^{red}) and are then carried back to the reaction center. This process generates a proton motive force (H^+) that can be used by ATP synthase for ATP formation. Quinol generating reactions include uptake [NiFe] hydrogenase (H_2ase) and succinate dehydrogenase (SDH). Quinol consuming reactions include nitrate reductase (NapABC) and DMSO reductase (DMSOR), which requires a *b*-type cytochrome ($cyt_{b,DMSO}$) and a *c*-type cytochrome ($cyt_{c,556}^{red}$). Complex I also performs a quinol consuming reaction that uses the generated proton motive force to synthesize NADH. NADH is used in a variety of electron sinks to balance cellular redox components. It can be shuttled directly into the Calvin-Benson-Bassham cycle for CO_2 fixation, or electrons can be transferred from NADH to ferredoxin via the Rnf complex (Rnf), and the reduced ferredoxin (Fd_{red}) can transfer electrons to nitrogenase (N_2ase). Electrons can also be transferred from NADH to $NADP^+$ via pyridine nucleotide transhydrogenase (Pnt), and the resulting NADPH can be used for biosynthetic reactions or combined with acetate to synthesize poly-β-hydroxybutyrate (PHB). The cell can also excrete compounds, called soluble microbial products (SMP), to balance redox.

two-component regulatory system, which alters gene expression potentially in response to changes in the redox state of the quinone pool or electron transport chain (Elsen et al. 2004; Oh et al. 2004; Swem et al. 2006; Wu and Bauer 2010). Furthermore, many studies of PNSB show that mutations that alter

redox balance result in photoheterotrophic growth defects, which can be corrected by addition of the terminal electron acceptor dimethylsulfoxide (DMSO), which accepts reducing equivalents from quinol (Shaw et al. 1999). Thus, the redox state of the quinone pool is linked to a variety of critical

processes. Here, we discuss the enzymes that contribute to or utilize electrons from the quinone pool during phototrophic growth.

A. Quinol Producing Reactions

In addition to the photochemical reaction center, other conserved respiratory enzymes can contribute electrons to the quinone pool. The first is the tricarboxylic acid (TCA) cycle enzyme succinate dehydrogenase, which transfers electrons from succinate to quinone (Fig. 32.1). While little work has investigated the role of succinate dehydrogenase in maintaining the quinone pool during phototrophy, its expression and synthesis has been observed during photoheterotrophic growth in *R. sphaeroides* (Callister et al. 2006; Arai et al. 2008). Metabolic modeling of *R. sphaeroides* predicts succinate dehydrogenase is essential for photoheterotrophic growth on succinate, but not for growth on many other substrates, including pyruvate or glucose (Imam, unpublished).

In addition to succinate dehydrogenase, many PNSB use [Ni-Fe] hydrogenases to oxidize H_2 and produce quinol (Vignais and Billoud 2007). Uptake [Ni-Fe] hydrogenases are often distinguished from other hydrogenases by their bimetallic [Ni-Fe] active site, and because they are membrane-bound respiratory enzymes (Vignais and Colbeau 2004). Uptake hydrogenases can transfer electrons to the quinone pool (via a *b*-type cytochrome), conserving the energy of this reaction by generating a proton motive force (Fig. 32.1) (Porte and Vignais 1980). Many environmental signals regulate the expression of uptake hydrogenases in PNSB. Uptake hydrogenase expression is activated in the presence of H_2 by a cytoplasmic, H_2-sensing regulatory hydrogenase (HupUV/HoxBC) and a two-component signal transduction system (HupT/HoxJ and HupR/HoxA) (Dischert et al. 1999; Vignais et al. 2005; Rey et al. 2006). Uptake hydrogenase expression is negatively regulated by the presence of O_2, and by the global anaerobic regulator RegB/RegA

(PrrB/PrrA) during conditions where the cell has other (non-H_2) sources of electrons, such as photoheterotrophic growth (Elsen et al. 2000; Vignais 2009; Donohue and Kiley 2010; Wu and Bauer 2010). In diazotrophs, uptake hydrogenase gene expression is often co-regulated with nitrogen fixation genes, since nitrogenase produces H_2 as a byproduct of the nitrogen fixation reaction (Elsen et al. 2000; Hallenbeck and Benemann 2002). Related to this, work in *Rhodospirillum rubrum* and specific strains of *R. sphaeroides* have used hydrogenase-deletion strains to increase yields of nitrogenase-mediated H_2 production during photoheterotophic growth (Kern et al. 1994; Kim et al. 2006; Kars et al. 2008; Kontur et al. 2011). While a hydrogenase-deletion mutant is able to grow photoheterotrophically, the enzyme is required for photoautotrophic growth with H_2 as the electron source (Xu et al. 1989; Rey et al. 2006; Kontur et al. 2011).

In addition, the *R. sphaeroides* genome is predicted to encode three other quinol-producing enzymes. The first (RSP_1777) is annotated as an FAD-binding flavoprotein-quinone oxidoreductase. Homologs of this protein can be found in many PNSB genomes. The second (RSP_3381) is a predicted NADPH-quinone oxidoreductase, homologous to the cytoplasmic *Escherichia coli* QOR enzyme, for which protein crystal structures have been described (Thorn et al. 1995; Shimomura et al. 2003). Lastly, RSP_2673 is predicted to encode a quinoprotein glucose dehydrogenase (glucose:quinone oxidoreductase). The *E. coli* homolog of this enzyme is membrane-bound and contains a pyrroloquinoline quinone cofactor (Cozier et al. 1999; Anthony 2004). The physiological roles of each of these proteins in PNSB remain to be experimentally tested.

B. Quinol Consuming Reactions

During phototrophic growth, quinol generated from the reaction center carries electrons to the cytochrome bc_1 complex. However, quinones can also be reduced

during central carbon metabolism (succinate dehydrogenase) or from alternative electron donors such as H_2 (via uptake hydrogenase) during phototrophic growth (Fig. 32.1). As a result, the quinone pool can become over-reduced, halting cyclic electron transfer and slowing or even preventing growth (McEwan 1994). Consequently, under phototrophic growth conditions, electron transfer reactions that maintain the redox state of the quinone pool are essential.

One way that quinol can be oxidized is by transferring electrons to a terminal electron acceptor. DMSO and trimethylamine N-oxide (TMAO) are organic intermediates of the sulfur and nitrogen cycles, respectively, that are commonly found in surface waters (Andreae 1980; Gibb and Hatton 2004). Both compounds can act as terminal electron acceptors under photoheterotrophic conditions, where electrons are transferred from quinol through a membrane-bound cytochrome b, to a periplasmic cytochrome c, and finally to DMSO or TMAO via the periplasmic molybdoenzyme DMSO reductase (DMSOR) (Fig. 32.1) (McEwan et al. 1983, 1985, 1989; McEwan 1994; Shaw et al. 1999). DMSOR expression in $R.$ $sphaeroides$ and $Rhodobacter$ $capsulatus$ is controlled by the FNR (fumarate and nitrate reduction) homolog, FnrL, in response to O_2 tension and by the two component regulatory system DorSR in response to the presence of DMSO (ZeilstraRyalls et al. 1997; Mouncey and Kaplan 1998; Tavano et al. 2004). Thus, DMSOR is only synthesized under anaerobic conditions in the presence of DMSO (Mouncey et al. 1997). DMSOR activity is required for anaerobic respiration with DMSO/TMAO in the dark. However, while the enzyme is active during phototrophic conditions in the presence of DMSO and provides an alternative quinol consuming reaction, it is not essential for growth under these conditions (Mouncey et al. 1997). Finally, the addition of DMSO to cultures can act as an electron sink to balance redox components and rescue growth phenotypes predicted to result from an altered redox state of the quinone pool (see below).

Many PNSB also contain complete or partial denitrification pathways. Denitrification is a dissimilatory process, whereby nitrate (NO_3) is reduced to nitrogen gas (N_2) (Zumft 1997). Denitrification can be used to generate a proton motive force for ATP synthesis in the absence of O_2; however, that is not considered its only function in PNSB. Four enzymes are required for the conversion of nitrate to N_2, but many species of PNSB encode only partial denitrification pathways (Michalski and Nicholas 1988; Shapleigh 2009). Moreover, there is no apparent phylogenetic trend among the distribution of denitrification enzymes in these bacteria (Shapleigh 2009). The current hypothesis is that many PNSB encode partial denitrification pathways that serve to dissipate excess reducing power during phototrophic conditions, rather than to conserve energy for ATP synthesis. For example, $R.$ $capsulatus$ strains that encode nitrate reductase or nitrous oxide (N_2O) reductase can grow photoheterotrophically on reduced substrates that typically do not support growth (such a butyrate) with the addition of nitrate or nitrous oxide (Richardson et al. 1988). Furthermore, work in $R.$ $sphaeroides$ strain DSM158 demonstrates that nitrate reductase activity increases during photoheterotrophic growth on highly reduced substrates, suggesting it plays a role in redox balance (Gavira et al. 2002). There are three types of nitrate reductases (Nas, Nar, Nap), none, some, or all of which can be found in any given purple nonsulfur bacterium (Richardson et al. 2001). Two of these enzymes, Nar and Nap (Nap, Fig. 32.1), accept electrons from the quinone pool (Richardson et al. 2001; Shapleigh 2006). However, the other enzymes involved in denitrification (nitrite reductase, nitric oxide reductase, and nitrous oxide reductase) accept electrons from reduced cytochromes generated by the cytochrome bc_1 complex (Shapleigh 2006). This allows denitrification enzymes to act as sinks for over-reduced components of cyclic phototrophic electron transfer reactions (quinol, reduced cytochromes). While the

transcriptional control of denitrification genes is complex, they are, in part, positively regulated by RegB/RegA (PrrB/PrrA), again suggesting a role for this pathway in balancing cellular redox state (Laratta et al. 2002; Imam et al. 2014, 2015b).

PNSB can also alter the redox state of the quinone pool by moving reductant from the membrane to the cytoplasm. This is typically accomplished by complex I, which utilizes the proton motive force to drive electron transfer from quinol to NAD^+, synthesizing NADH (Fig. 32.1) (McEwan 1994; Herter et al. 1998). Once generated, cytoplasmic NADH can be used by a variety of pathways to support biosynthesis, or this excess reductant can be dissipated via a variety of "electron sinks" (discussed below). In *R. capsulatus* and *R. sphaeroides*, complex I is required for photoautotrophic and photoheterotrophic growth, where it synthesizes NADH to oxidize the quinol pool. The phenotypes of complex I mutants can be rescued by the addition of the terminal electron acceptor DMSO (Dupuis et al. 1997; Tichi et al. 2001; Spero et al. 2015), suggesting that the growth defect of these strains is also associated with an alteration in the redox state (over-reduction) of the quinone pool. Thus, the requirement for complex I during phototrophy is predicted to be conserved across PNSB. Indeed, genomic analyses indicate that all bacteria that use solely a quinol-generating type-2 reaction center (purple bacteria, filamentous green nonsulfur bacteria) are predicted to encode complex I. However, bacteria that have a reduced ferredoxin-generating type-1 reaction center (cyanobacteria, green sulfur bacteria) do not encode complex I, but rather complex I-like enzymes predicted to use electron donors other than NADH (Bryant and Frigaard 2006; Battchikova et al. 2011; Spero et al. 2015; see also the chapter by Shikanai and Aro in this volume). These correlations support the hypothesis that complex I of PNSB serves to recycle quinol that is produced from photochemical activity in the reaction center. Interestingly, genomic analyses also find that a small number

(<4 %) of sequenced bacteria are predicted to encode two complex I isozymes, including all sequenced strains of *R. sphaeroides* and *Rhodopseudomonas palustris* (Spero et al. 2015). While the roles of these isozymes are currently unknown, gene expression studies in *R. sphaeroides* show the two complex I operons are differentially expressed in the presence or absence of O_2 (Arai et al. 2008). However, both of the complex I operons are expressed (at different levels) during photoheterotrophic growth. Thus, additional experiments are needed to determine the roles of these complex I isozyme in maintaining the cellular redox balance.

III. The Role of Cytoplasmic "Electron Sinks" During Photoheterotrophic Growth

When complex I transfers excess reducing power from membrane-bound quinol to NAD^+, the resulting NADH can be shuttled into a variety of cytoplasmic "electron sinks" to maintain the NAD^+:NADH ratio. Alternatively, the reducing power in NADH can be transferred to another electron carrier (ferredoxin, $NADP^+$) and used to support other cellular processes (Fig. 32.1). Because complex I is required for phototrophic growth (Dupuis et al. 1997; Tichi et al. 2001), it is predicted to supply a large fraction of reducing power for biosynthesis or for passage to electron sinks. However, it is not known the extent to which electrons directed into electron sinks originate from complex I-mediated NADH synthesis, as opposed to other NADH-generating catabolic processes. Below we describe known electron sinks that are important for photoheterotrophic growth in PNSB.

A. The Calvin-Benson-Bassham Cycle

Excess NADH can be directed into the Calvin-Benson-Bassham (Calvin) cycle, which utilizes this reducing power and ATP to fix CO_2 into organic carbon

(Hallenbeck et al. 1990; Dubbs and Tabita 2004; McKinlay and Harwood 2010b, 2011). Though the role of the Calvin cycle during autotrophic growth has been known for a long time, its importance for photoheterotrophic growth is a more recent finding. The Calvin cycle is required for many photoheterotrophic growth conditions in *R. sphaeroides*, *R. capsulatus*, and *R. palustris* (Hallenbeck et al. 1990; Paoli et al. 1998; McKinlay and Harwood 2010b). Here, it is proposed that the Calvin cycle acts an electron sink, oxidizing NADH so it can be used in catabolism (Fig. 32.1). Calvin cycle mutants can be rescued by the addition of exogenous DMSO, or in some organisms (*R. palustris*, *R. capsulatus*) by increasing the expression of another enzyme, such as nitrogenase, that can act as electron an sink (Hallenbeck et al. 1990; Tichi and Tabita 2000; McKinlay and Harwood 2010a). Additionally, an *R. sphaeroides* Calvin cycle mutant is able to grow on acetate, where the ethylmalonyl-CoA pathway that is used to assimilate acetate also balances the redox state of the pyridine nucleotide pool by oxidizing NADPH (Laguna et al. 2011). However, *R. palustris* uses the glyoxylate cycle to assimilate acetate, which produces NADH, leading to a requirement for the Calvin cycle during photoheterotrophic growth on acetate (Laguna et al. 2011).

In *R. palustris*, ^{13}C-metabolic flux analysis has demonstrated the extent to which the organism depends on the Calvin cycle for redox balance (McKinlay and Harwood 2010b). During growth on acetate, 22 % of the consumed acetate is released as CO_2, most of which (67 %) is re-assimilated via the Calvin cycle. While most of the acetate carbon is used in biosynthetic pathways, only half of the reduced electron carriers generated during acetate catabolism are re-oxidized via biosynthetic pathways. Under these conditions, the Calvin cycle re-oxidizes almost all of the remaining reduced electron carriers (McKinlay and Harwood 2010b). Thus, most of the CO_2 produced during acetate metabolism is subsequently assimilated in order to maintain cellular

redox state. A similar study found that the *R. palustris* Calvin cycle oxidizes a large fraction of reduced electron carriers (38–55 %) during photoheterotrophic growth, regardless of the oxidation state of the provided carbon substrate (McKinlay and Harwood 2011). These observations demonstrate the important role of the Calvin cycle as an electron sink during photoheterotrophic growth.

B. Nitrogenase Activity

In the absence of a preferred nitrogen source such as ammonium, photoheterotrophically growing PNSB activate expression of their N_2 fixation system, which can be used to convert N_2 to ammonia and thus dissipate excess reductant. N_2 fixation, which is performed by the nitrogenase enzyme, requires large amounts of ATP and reductant, according to the following reaction (McKinlay and Harwood 2010a):

$$N_2 + 8e^- + 8H^+ + 16ATP \rightarrow 2NH_3 + 1H_2$$
$$+ 16ADP + 16P_i$$
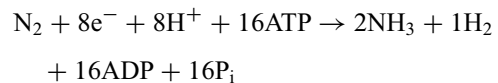
However, nitrogenase can also function in the absence of N_2. For example, when cells are grown in the presence of a fixed nitrogen source (such as glutamate), the reducing power consumed by nitrogenase activity is used to synthesize H_2, according to (McKinlay and Harwood 2010a):

$$8H^+ + 8e^- + 16ATP \rightarrow 4H_2 + 16ADP + 16P_i$$
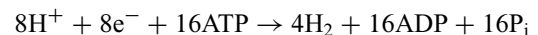
The electron donor to nitrogenase is not known in many PNSB, but because N_2 fixation requires low potential electrons, it is proposed that reduced ferredoxin is used for this purpose (Yoch and Arnon 1975; Jouanneau et al. 1995; Biegel et al. 2011; Kontur et al. 2011). Electrons are predicted to be transferred from NADH to ferredoxin by the _Rhodobacter_ _n_itrogen _f_ixation (Rnf) complex, which uses the proton motive force to drive the reaction (Fig. 32.1) (Schmehl et al. 1993; Biegel et al. 2011).

The role of nitrogenase-mediated H_2 production as an electron sink varies depending on the carbon substrate provided to the cells. When *R. sphaeroides* was grown in the presence of glutamate as a nitrogen source, nitrogenase activity (measured as H_2 production) was highest when cells were grown photoheterotrophically on lactate, and lowest when grown on glycerol (Kontur et al. 2011). In addition, an *R. sphaeroides* nitrogenase mutant grows very poorly on a reduced carbon source such as lactate, where nitrogenase activity is presumably important for redox balancing. Meanwhile, the nitrogenase mutant has the same growth rate as the wild type on glycerol, although no H_2 is produced by the mutant (Kontur et al. 2011).

In *R. palustris*, Calvin cycle mutants grow when nitrogenase is active, suggesting that this enzyme plays a substantial role in maintaining cellular redox state in this organism. When wild type *R. palustris* is grown photoheterotrophically on acetate in the absence of nitrogenase activity (i.e., on a preferred nitrogen source), half of reduced electron carriers generated during catabolism are re-oxidized by biosynthetic pathways, and the Calvin cycle re-oxidizes most of the remaining reduced electron carriers (McKinlay and Harwood 2010b). In contrast, when nitrogenase is active, electrons are diverted from the Calvin cycle into H_2 production. Under these conditions, half of the reduced electron carriers are still re-oxidized by biosynthetic pathways, but the Calvin cycle re-oxidizes a smaller fraction of reduced electron carriers (14 %) while nitrogenase activity re-oxidizes the remaining (34 %) reduced electron carriers (McKinlay and Harwood 2010b). The fraction of electrons that are diverted to nitrogenase or the Calvin cycle is regulated at the transcriptional level and depends on the carbon substrate, since transcription of the *R. palustris* Calvin cycle genes depends on the supplied carbon source (McKinlay and Harwood 2010b, 2011). Furthermore, the partitioning of electron flow to nitrogenase in *R. palustris* was influenced by how the carbon substrate was metabolized. Thus, the role of nitrogenase activity in cellular redox balancing depends on the fraction of electrons diverted away from the Calvin cycle, and how the reductant is produced by carbon metabolism (McKinlay and Harwood 2011).

C. Other Cytoplasmic Electron Sinks

While the Calvin cycle and nitrogenase are well-documented major electron sinks in PNSB, several others exist. Polyhydroxyalkanoic acids (PHA) are carbon storage polymers, the most common of which is poly-β-hydroxybutyrate (PHB). PHB is synthesized in bacteria when carbon and energy, specifically acetyl-CoA and NAD(P)H, are in excess but nitrogen, sulfur, or phosphorous are limiting (Fig. 32.1) (Hustede et al. 1993; Steinbuchel and Fuchtenbusch 1998; Madison and Huisman 1999). *R. sphaeroides* and *R. rubrum* produce the highest levels of PHB when growing photoheterotrophically on acetate (which feeds directly into the PHB pathway), where it can account for 40–70 % of dry cell weight (Hustede et al. 1993; Khatipov et al. 1998). Despite the large contribution of PHB to cell mass, mutant strains defective in PHB synthesis still grow photoheteroptrophically on acetate. To compensate for the loss of PHB as an electron sink, these mutants produce significantly more H_2 than wild type cells when grown on acetate under conditions where nitrogenase is active (Hustede et al. 1993). While PHB mutants have been made in a variety of PNSB with the goal of diverting excess reductant towards nitrogenase-mediated H_2 production, these strains show little or no increases in H_2 production as a function of growth conditions (Lee et al. 2002; Franchi et al. 2004; Kim et al. 2006; Yilmaz et al. 2010). The largest differences in H_2 production between wild type and PHB mutants were seen when strains were grown on substrates such as acetate or glucose, which typically result in the highest amount of PHB production in wild-type cells (Hustede et al. 1993; Yilmaz et al. 2010). These results indicate that there are still many unknown aspects of electron partitioning among different pathways in PNSB.

Additionally, photoheterotrophically grown cells will often excrete metabolic intermediates referred to as soluble microbial products (SMP) (Fig. 32.1) (Yilmaz et al. 2010). For example, *R. sphaeroides* grown photoheterotrophically on lactate excreted almost 20 % of the provided carbon in the form of SMP by late stationary phase (Yilmaz et al. 2010). A large fraction of the SMPs are central metabolic intermediates, such as pyruvate, succinate or fumarate (Yilmaz et al. 2010; McKinlay and Harwood 2011). These SMP can be thought of as a way to dissipate excess reducing power, or incompletely oxidized carbon skeletons that are not used to support biosynthetic activity and thereby "lost" to the cells. However, in some cases, a fraction of the SMP are eventually consumed by cells when other nutrients become limiting (McKinlay and Harwood 2011).

IV. Recycling Reducing Power Captured in Pyridine Nucleotides

In phototrophic and other cells, biosynthetic reactions oxidize a large fraction of reduced electron carriers. For many anabolic processes, NADPH serves as the electron donor. Below, we discuss the major enzymatic routes of NADPH synthesis in *R. sphaeroides*, and the growth conditions that require these enzymes.

A. Role of Transhydrogenases in Maintaining Redox Balance of the Pyridine Nucleotide and Glutathione Pools

NADPH is an important cofactor used as a source of reducing power in a wide array of crucial anabolic processes including the Calvin cycle, fatty acid biosynthesis, and photosynthetic pigment biosynthesis, amongst others (Nelson and Cox 2005). NADPH also plays an important role in cellular resistance to oxidative and other stresses, where it provides reducing equivalents to maintain reduced pools of glutathione,

thioredoxin and other antioxidants (Hickman et al. 2002; Brumaghim et al. 2003). Thus, the $NADP^+$:NADPH ratio needs to be tightly controlled for normal cellular function.

Pyridine nucleotide transhydrogenase plays an important role in maintenance of the cellular $NAD(P)^+$:NAD(P)H ratios, catalyzing the reversible exchange of reducing equivalents between pyridine nucleotides, NADPH and NADH (Bragg et al. 1972; Rydström et al. 1976; Bragg 1998; Sauer et al. 2004):

$$H^+_{periplasm} + NADP^+ + NADH \leftrightarrow NADPH$$
$$+ NAD^+ + H^+_{cytosol}$$
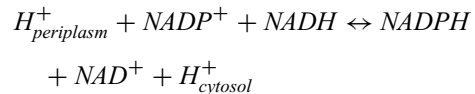
Two forms of transhydrogenases have been identified: (i) the energy-dependent membrane-bound pyridine nucleotide (NADH/NADPH) transhydrogenase (PntAB); and (ii) the energy-independent soluble pyridine nucleotide (NADH/NADPH) transhydrogenase (STH or UdhA) (Voordouw et al. 1983; Sauer et al. 2004). PntAB is a widely-distributed heterotetrameric $\alpha_2\beta_2$ enzyme complex, which catalyzes a reversible reaction based on the direction or magnitude of the proton gradient across the cytoplasmic membrane (Bragg et al. 1972; Bragg 1998; Cao et al. 2011). On the other hand, STH is a less commonly found flavoprotein (Boonstra et al. 1999; Sauer et al. 2004; Cao et al. 2011). Only a few γ-Proteobacteria, such as *E. coli*, possess both forms of this enzyme (Boonstra et al. 1999; Sauer et al. 2004).

Both PntAB and STH have been extensively studied in *E. coli* by metabolic flux analysis and by analyzing the phenotypes of mutants under different growth conditions (Sauer et al. 2004). PntAB has been shown to typically function in the forward direction (i.e., synthesis of NADPH) and thus its expression is induced when there is a demand for NADPH (Sauer et al. 2004). In *E. coli*, PntAB is required for optimal growth on carbon sources whose metabolism does not directly result in the net production of NADPH, such as glycerol, where PntAB functions to provide the necessary NADPH for biosynthe-

Table 32.1. Growth phenotypes of transhydrogenase deletion strains in *E. coli* and *R. sphaeroide*[a].

Growth substrate	*E. coli*		*R. sphaeroides*	
	$\Delta pntAB$	$\Delta udhA$	$\Delta pntAB$ (photo)	$\Delta pntAB$ (aero)
High NADPH producing (e.g., acetate in *E. coli*)	++	−−	?	?
Moderate NADPH producing (e.g., glucose)	+−	++	++	++
NADPH-neutral (e.g., glycerol or succinate)	+−[b]	++	+−[b]	++
NADPH consuming (e.g., acetate in *R. sphaeroides*)	?	?	−−	+−

[a]Observed growth phenotypes on different categories of carbon sources displayed by $\Delta ptnAB$ deletion stains in *E. coli* and *R. sphaeroides* and a $\Delta udhA$ deletion strain in *E. coli*. (++), Normal growth rate relative to wild type cells; (+−), reduced growth rate relative wild type cells; (−−), No growth

[b]During growth on NADPH-neutral carbon sources such as glycerol or succinate, a long lag is observed in both *E. coli* and *R. sphaeroides* before growth commences

sis (Table 32.1). Conversely, during growth on substrates whose metabolism results in the production of excess NADPH (such as acetate, which is metabolized via the TCA cycle and glyoxylate shunt), PntAB is not required. Under these conditions, STH expression is induced, and the enzyme catalyzes the conversion of NADPH to NADP$^+$ with the concomitant production of NADH (Sauer et al. 2004; Lee et al. 2010). STH activity is essential under these conditions, since a *sth* deletion strain is incapable of growth on acetate. Thus, through the use of these two enzymes, *E. coli* is able to maintain NAD(P)$^+$:NAD(P)H homeostasis. In addition to PntAB and STH, other enzymes such as glucose-6-phosphate dehydrogenase (Zwf), 6-phosphogluconate dehydrogenase (Gnd) and isocitrate dehydrogenase (Icd) are also known to make significant contributions to the cellular NADPH pool under specific conditions (Sauer et al. 2004; Lee et al. 2010).

B. PntAB Is Required for Normal Photosynthetic Growth in Rhodobacter sphaeroides

In photosynthetic organisms, NADPH has additional roles. In oxygenic phototrophs it provides reducing equivalents required for CO_2 fixation via the Calvin cycle (Allen 2003), and in all phototrophs, including PNSB, NADPH is required for the biosynthesis of tetrapyrroles, photopigments and lipids of the photosynthetic apparatus. The role of PntAB during anoxygenic phototrophy has been studied in *R. sphaeroides* by assessing the growth phenotypes of deletion mutants and by flux balance analysis (Imam et al. 2013). Unlike *E. coli*, *R. sphaeroides* does not possess a known homolog of STH, so PntAB is likely central to the maintenance of the cellular NAD(P)$^+$:NAD(P)H ratios. During photosynthetic growth on substrates whose metabolism results in the net production of NADPH (such as glucose), PntAB is not required for growth (Imam et al. 2013), as NADPH produced through the Zwf reaction is sufficient to support normal photosynthetic growth (Table 32.1). Conversely, during growth on substrates that require a net consumption of NADPH (e.g. acetate metabolized via the ethylmalonyl-CoA pathway) (Alber 2011), PntAB is essential for photosynthetic growth (Imam et al. 2013). During growth with NADPH-neutral carbon sources (i.e. substrates whose metabolism does not result in the net production or consumption of NADPH) such as succinate and most of the carbon sources known to support *R. sphaeroides* growth, the loss of PntAB activity results in a significantly reduced photosynthetic growth rate, indicating that PntAB is needed to produce NADPH under most conditions (Imam et al. 2013).

Under aerobic respiratory conditions, the loss of PntAB activity has much less impact on *R. sphaeroides* (Table 32.1),
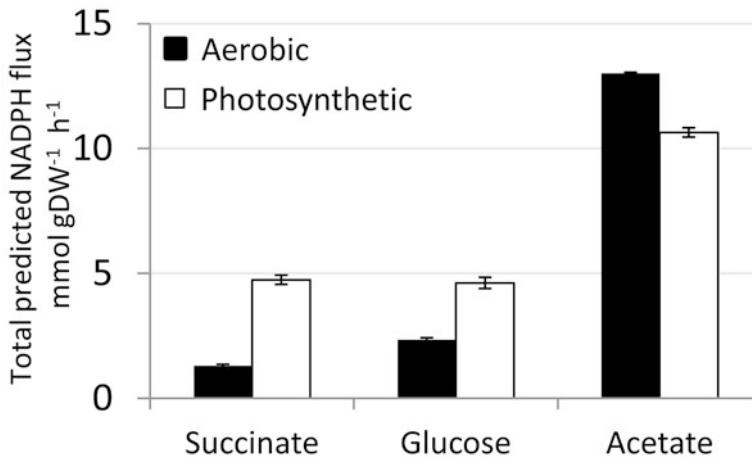
Fig. 32.2. Comparison of the predicted NADPH flux during photosynthetic or aerobic respiratory growth. Predictions were made for the total NADPH flux during growth of *R. sphaeroides* with succinate, glucose or acetate under photosynthetic and aerobic respiratory conditions. The larger predicted NADPH demand under aerobic conditions compared to photosynthetic conditions during growth with acetate is due to a larger predicted uptake rate of acetate under aerobic conditions (Figure adapted from Imam et al. (2013)).

suggesting there is a diminished need for either NADPH or activity of this enzyme under these conditions (Imam et al. 2013). However, it is also possible that other NADPH producing pathways are active aerobically but not photosynthetically. To assess the amount of NADPH required under individual conditions, a genome-scale metabolic model of *R. sphaeroides*, iRsp1095 (Imam et al. 2011), was used to predict the flux through the PntAB reaction in aerobic and photosynthetic cells that use different growth substrates (Fig. 32.2). This analysis indicated that, in general, more NADPH was required for growth under photosynthetic than aerobic conditions, consistent with the observation that the PntAB mutant had more severe growth defects during photosynthetic conditions (Imam et al. 2013).

Interestingly, more NADPH was predicted to be required for aerobic growth on acetate than photosynthetic growth on any other substrate (Fig. 32.2). This prediction was unexpected, as a strain lacking PntAB activity was able to grow aerobically on acetate (Imam et al. 2013). This suggested that alternative routes existed for aerobic NADPH synthesis, which were either not functional or insuffi-

cient to support photosynthetic growth. Indeed, the iRsp1095 model indicated that at least 6 other reactions could potentially contribute to the NADPH pool in *R. sphaeroides* under any growth condition (Table 32.2). Subsequent RNA abundance analysis showed that the genes encoding the enzymes that catalyze some of these reactions were differentially expressed in response to the loss of PntAB activity under aerobic conditions, depending on growth substrate (Table 32.2), suggesting they might function to provide NADPH to compensate for the loss of PntAB (Imam et al. 2013).

C. Zwf Is an Alternative Route for NADPH Synthesis in Rhodobacter sphaeroides

The central carbon catabolism enzyme glucose-6-phosphate dehydrogenase (Zwf) is one of the alternative routes for NADPH synthesis predicted by the iRsp1095 model to be used by *R. sphaeroides* in the absence of PntAB activity (Table 32.2). iRsp1095 predicts that succinate is converted to glucose-6-phosphate via gluconeogenesis, which could then be metabolized via the Entner-Doudoroff glycolytic pathway,

Table 32.2. Reactions predicted by iRsp1095 to be involved in generation of NADPH in *Rhodobacter sphaeroides*.

Enzyme name	Gene identifier	Reaction catalyzed	EC number	Glucose[a]	Acetate[a]
PntAB	RSP_0239 & RSP_0240	$NADP^+ + NADH + 2 H^+[p] \Rightarrow NADPH + NAD+ + 2H+$	1.6.1.2		
NADPH-ferredoxin reductase	RSP_1939	Reduced ferredoxin $+ NADP+ + H+ \Longleftrightarrow$ Oxidized ferredoxin $+ NADPH$	1.18.1.2		↑
Zwf	RSP_2734	D-Glucose 6-phosphate $+ NADP+ \Longleftrightarrow 6PGL + NADPH$	1.1.1.49		
Malic enzyme	RSP_1217 | RSP_1593	(S)-Malate $+ NADP+ \Rightarrow$ Pyruvate $+ CO2 + NADPH$	1.1.1.40	↑	
THF dehydrogenase	RSP_0661	mlthf $+ NADP+ \Longleftrightarrow$ methf $+ NADPH$	1.5.1.5	↑	
Icd	RSP_0446 | RSP_1559	Isocitrate $+ NADP+ \Longleftrightarrow$ 2-Oxoglutarate $+ CO2 + NADPH$	1.1.1.42	↑	
Glutamate synthase	RSP_1146 & RSP_1149	2L-Glutamate $+ NADP+ \Longleftrightarrow$ L-Glutamine $+$ 2-Oxoglutarate $+ NADPH + H+$	1.4.1.13		

6PGL D-Glucono-1,5-lactone 6-phosphate
mlthf 5,10-Methylenetetrahydrofolate
THF dehydrogenase 5,10-methylene-tetrahydrofolate dehydrogenase
[a] Genes differentially expressed in response to the loss of PntAB under aerobic conditions during growth on either glucose or acetate. Upward arrows indicate upregulation

generating NADPH via Zwf. Mutational analysis showed that this route is indeed employed by *R. sphaeroides* under both aerobic and photosynthetic conditions. While loss of either only PntAB or Zwf had little impact on aerobic growth, loss of both PntAB and Zwf resulted in significantly impaired aerobic growth on succinate (Imam et al. 2013). The residual growth of the PntAB and Zwf double deletion strain under aerobic conditions, likely indicates that other alternative NADPH generating reactions might exist under these conditions. Further analysis showed that Zwf also makes a significant contribution to the NADPH pool during photosynthetic growth on succinate. While loss of PntAB resulted

in significantly impaired photosynthetic growth on succinate, loss of both PntAB and Zwf resulted in a complete loss of growth on succinate (Imam et al. 2013).

In sum, these data indicate that PntAB and Zwf are the two major sources of NADPH during photosynthetic growth in *R. sphaeroides*. In addition, it appears that PntAB plays a crucial role in maintenance of NADPH:NADH homeostasis in *R. sphaeroides* and possibly other phototrophic bacteria, with added importance under photosynthetic conditions. As is the case in other well-studied bacteria, PntAB appears to generally function in the direction of NADPH production in *R. sphaeroides*.

Conditions of excess NADPH production have yet to be assessed in *R. sphaeroides*, but given its lack of a STH homolog, such conditions could be detrimental as the NADPH pool would become over-reduced. However, experiments to search for alternative reactions or pathways that could recycle excess NADPH have yet to be performed.

V. Modeling the Metabolism of Photosynthetic Organisms

As highlighted in the previous sections, computational models of metabolism can play an important role in guiding the analysis of metabolism and electron flow through one or more pathways. By considering the genes, proteins, reactions and metabolites involved in individual pathways or the entire metabolic network of an organism, models have been used to make accurate qualitative and quantitative predictions about outcomes of growth conditions and genetic perturbations, which have led to novel discoveries and strategies for strain improvements (Oberhardt et al. 2009; McCloskey et al. 2013). Many approaches are utilized for modeling of cellular metabolism, including kinetic (Schallau and Junker 2010), ensemble (Tran et al. 2008; Tan and Liao 2012) and constraint-based (Varma and Palsson 1994; Palsson 2000) models. In recent years, constraint-based models (CBMs) have found widespread use.

A. Constraint-Based Modeling

Constraint-based modeling assumes that the flux of metabolites through a network at steady state is dependent on enzyme capacity, the stoichiometry of the reactions that make up the network, and their inherent thermodynamic constraints (Varma and Palsson 1994; Palsson 2000). As a consequence, constraint-based modeling bypasses the need for kinetic parameters, which are often difficult to obtain. Simulations can be carried out with CBMs using flux balance analysis (FBA) (Varma and Palsson 1994) and related approaches, which can be used to make experimentally testable qualitative or quantitative predictions about growth rates, phenotypes, electron flow, flux distributions and production rates of specific metabolites.

B. Constraint-Based Models for Photosynthetic Organisms

Manually curated genome-scale CBMs have recently been constructed and applied for analysis of several photosynthetic bacteria, including *R. sphaeroides*. An initial genome-scale CBM for *R. sphaeroides* (iRsp1095) was later refined using high-throughput phenotypic data to generate a larger, more comprehensive model called iRsp1140, which accounts for 1140 genes (~26 % of the *R. sphaeroides* genome) and includes 1416 reactions and 878 metabolites (Imam et al. 2013). iRsp1095 and iRsp1140 have been used to study the various *R. sphaeroides* lifestyles, analyze its metabolic capabilities, predict the fate of gene deletions, study H_2 production and electron transport, identify alternative electron sinks, as well as general analysis of the metabolic network structure (Imam et al. 2011, 2013). Recently, the impact of light intensity on steady state photosynthetic growth has been used to extend and improve the modeling of photosynthetic growth in *R. sphaeroides* (Imam et al. 2015a).

Other photosynthetic organisms for which manually curated genome-scale CBMs have been reconstructed include: *Synechocystis* sp. PCC 6803 (Fu 2008; Montagud et al. 2010), *Cyanothece* sp. ATCC 51142 (Vu et al. 2012), *Dinoroseobacter shibae* DFL12T (Rex et al. 2013), *Chlamydomonas reinhardtii* (Chang et al. 2011), *Arabidopsis thaliana* (Poolman et al. 2009; de Oliveira Dal'Molin et al. 2010; Saha et al. 2011), *Zea mays* (Dal'Molin et al. 2010; Saha et al. 2011), and *Oryza sativa* (Poolman et al. 2013). These models have guided research in a variety of ways, including predicting the effects of gene deletions during photosynthetic growth and predicting

the flux of carbon and electrons during photosynthetic growth.

C. Challenges for Metabolic Modeling of Photosynthetic Cells

CBMs for photosynthetic organisms appeared initially in ∼2009, and have not yet undergone the sort of extensive refinements seen for models of non-phototrophic bacteria (e.g. *E. coli*). However, improving computational tools for genome annotation and automated network reconstruction are speeding up the process of CBM reconstruction and refinement (Overbeek et al. 2005; Aziz et al. 2008; Henry et al. 2010).

A unique challenge of constraint-based modeling in photosynthetic organisms is the measurement of the amount of light used by the cell. Given that a significant amount of exogenous light can be reflected or dissipated as heat, measurement of this parameter can prove difficult. In addition, the light source used can also impact cell growth and modeling predictions. To accurately measure light uptake in *Cyanothece*, custom photobioreactors were built that allowed quantitative measurements of incident and transmitted light, and provided light sources of the specific wavelengths used by the *Cyanothece* photosystems (Vu et al. 2012). Using these bioreactors, they were able to accurately measure light uptake for *Cyanothece* sp. ATCC 51142 and used it as a modeling constraint to study how incident light affects growth rate. In another attempt to quantitatively use supplied light in modeling, one study (Chang et al. 2011) developed a novel approach for modeling light emitted by different sources. These so called "prism reactions" enable decomposition of different light sources into wavelength ranges that correspond to the optimal absorption wavelength ranges of the various light utilizing reactions in *C. reinhardtii*. Thereby photons of different wavelength ranges supplied by a given light source can be apportioned to the different light utilizing components (e.g. photosystems) that can absorb them. Using

this approach, researchers were able to model *C. reinhardtii* growth on 11 different light sources, including light emitting diodes, solar, incandescent and fluorescent light sources (Chang et al. 2011). Future experimental and computational developments should further improve our ability to model light uptake in photosynthetic organisms.

D. Metabolic Modeling as a Tool for Rational Design of Photosynthetic Organisms

Many photosynthetic microbes are metabolically versatile and naturally capable of producing compounds of socio-economic value. For instance, *R. sphaeroides* is capable of producing large amounts of H_2 gas (Mackenzie et al. 2007; Yilmaz et al. 2010; Imam et al. 2011). Further, given its ability to increase membrane synthesis, *R. sphaeroides* also produces more ubiquinone (Lu et al. 2015), fatty acids or membrane lipids per cell than many other bacteria (Kien et al. 2010; Lemmer-Christensen et al. 2015), which can serve as a biocommodity (Khan et al. 2015), pharmaceutical (Lemke et al. 2014), or food supplement (Kien et al. 2010). *R. sphaeroides* and several other species of PNSB also have the native ability to accumulate PHB, which can be used in the manufacture of biodegradable plastics (Khatipov et al. 1999). However, significant genetic, genomic or synthetic biology improvements in the capabilities of these organisms to produce these value-added commodities will be required before they can have an economic impact (Jaschke et al. 2011; Desai and Atsumi 2013; Tikh et al. 2014).

In this regard, another important application of CBMs is in the development of strategies for improving the performance of photosynthetic systems. These strategies could involve genetic changes to remove or add genes or reactions to the metabolic network. Approaches such as FBA, minimization of metabolic adjustment (MOMA) (Segre et al. 2002) and regulatory on/off minimization of metabolic flux changes (ROOM) (Shlomi et al. 2005) have been used to make

predictions of the effects of genetic perturbations to metabolic flux distributions in non-phototrophs (Alper et al. 2005; Park et al. 2007). In addition, several other approaches have been developed specifically geared towards strain optimization for improving metabolite production such as optknock (Burgard et al. 2003), optGene (Patil et al. 2005), optORF (Kim and Reed 2010), and optStrain (Pharkya et al. 2004), which have been used with varying levels of success. Recent efforts have been able to produce transcriptional regulatory network models that can predict previously unidentified genes that are involved in the low oxygen, photosynthetic, central carbon and other major pathways of PNSB (Imam et al. 2014, 2015b, c). In addition to providing new insight into these important transcriptional regulatory networks, they illustrate the need to integrate these predictions into CBMs. Despite their potential to improve performance of photosynthetic systems, to date, these modeling strategies have yet to be successfully applied or combined in any phototroph. As CBMs and transcriptional network models for phototrophs become more detailed and improve in their predictive accuracy, one would expect that these approaches and others developed in the future will prove to be successful for improving desired traits and production capabilities in PNSB.

VI. Concluding Remarks and Future Directions

The analysis of PNSB and other photosynthetic bacteria has provided unique insights into photochemistry, bioenergetics, the assimilation of atmospheric nutrients (N_2 and CO_2) and the production of compounds of industrial value (Blankenship et al. 1995; Meyer and Donohue 1995; Blankenship and Hartman 1998; Steinbuchel and Fuchtenbusch 1998; Dubbs and Tabita 2004; Gest and Blankenship 2004; Kien et al. 2010; McKinlay and Harwood 2010a; Kontur et al. 2012a). Each of the above areas has in common the production or use of electrons. Despite the wealth of biochemical and mechanistic information on individual pathways or processes that are summarized in studies cited in this and other chapters in this volume, there are still many unanswered questions about the coordination of electron partitioning within and between different pathways in photosynthetic and other organisms. Future analyses of PNSB and other photosynthetic bacteria have the ability to provide such knowledge about the pathways used for electron partitioning under different conditions.

Our ability to globally understand how cells divert electrons among different pathways is challenged by the number of membrane bound or soluble enzymes or electron carriers that can be present under given metabolic conditions (e.g. photosynthetic, respiratory, aerobic, or anaerobic). Despite this complexity, the integration of genomic and modeling approaches with traditional biochemical, genetic and physiological tools has the ability to predict, test and provide new and important insights into electron partitioning by photosynthetic organisms. Obtaining such a systems-level understanding can allow us to increase the utility of photosynthetic organisms for the production of food, fuel and other products of societal importance in the twenty-first Century.

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Cytochrome c_{6A} of Chloroplasts

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Summary

Cytochrome c_{6A} must be an important protein, as it is conserved across plant and green algal lineages, yet its function remains as mysterious as when it was discovered over 10 years ago. The protein is similar in sequence to conventional cytochrome c_6 proteins, which are well known as alternatives to plastocyanin in transferring electrons from the cytochrome $b_6 f$ complex to Photosystem I in the light reactions of photosynthesis. Yet cytochrome c_{6A} has a dramatically lower redox midpoint potential than conventional cytochrome c_6, meaning that it is an unsuitable electron acceptor from cytochrome f. The structure of cytochrome c_{6A} has been determined, and mutagenesis studies have pinpointed a single substitution compared to cytochrome c_6 that is responsible for at least 100 mV of the lowering of the midpoint potential—a remarkable effect for a single residue. Another striking feature of cytochrome c_{6A}, absent from cytochrome c_6, is a 12-residue insertion in a loop region of the protein. This insertion has two conserved cysteine residues. The function of these is unknown, but

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may be structural rather than catalytic. Cytochrome c_{6A} is able to transfer electrons to plastocyanin. Transcriptomic and proteomic studies have provided remarkably little insight into the protein's function. Ironically, the recognition of cytochrome c_{6A} in plants—from which cytochrome c_6 was long thought to be absent—has now led to the recognition of a previously unsuspected class of cytochrome c_6-like proteins in cyanobacteria, designated cytochrome c_{6B} and cytochrome c_{6C}.

I. Introduction

A. Cytochrome c$_6$ Family

Cytochrome c_{6A} is a low molecular weight protein that is clearly homologous, based on primary sequence, to the cytochrome c_6 family of proteins. However, cytochrome c_{6A} differs from the conventional cytochrome c_6 proteins in aspects of its primary structure, in redox and other biochemical properties, and in function. The conventional cytochrome c_6 proteins have been known for many years to function in photosynthetic electron transfer between the cytochrome b_6f complex and Photosystem I (PSI). The established view was that some cyanobacterial and algal lineages rely on cytochrome c_6 for this stage in the electron transfer chain, while others use either cytochrome c_6 or, especially under copper-replete conditions, the copper-containing protein plastocyanin. In addition it was accepted that land plants no longer have a cytochrome c_6, having completely replaced it with plastocyanin (Sandmann et al. 1983; Ho and Krogmann 1984; Kerfeld and Krogmann 1988). Cytochrome c_6 and plastocyanin were believed also to function in electron transfer to cytochrome oxidase in organisms with a cytochrome oxidase in the same compartment as the photosynthetic electron transfer chain, i.e. in photosynthetic bacteria (Peschek et al. 2004; Hart et al. 2005).

B. Discovery of Cytochrome c$_{6A}$

It was therefore very surprising when the existence of a modified form of cytochrome c_6 in plants was reported in 2002 independently by two groups (Gupta et al. 2002a; Wastl et al. 2002). Wastl et al. reported genomic and expressed sequence tag (EST) database searches that showed the existence in land plants of sequences encoding a protein with clear homology to cytochrome c_6, including the heme-binding motif —CXXCH— and a potential iron-ligating methionine, both of which were found in algal cytochrome c_6 proteins. They also noted the existence of an insertion of 12 amino acids, including two cysteines, in all the novel cytochrome c_6 proteins that was not found in the conventional ones, and suggested this was indicative of a recently acquired function, perhaps in redox regulation or signalling. They predicted, based on homology models, that the newly recognised protein would have significantly different surface charge distributions from conventional cytochrome c_6, again suggesting a different function. In addition, they noted that sequences encoding the novel protein were found in EST collections from non-photosynthetic, as well as from photosynthetic, tissue and suggested that this might indicate a function for the protein alternative or additional to any role in photosynthesis (Wastl et al. 2002).

Gupta et al. (2002a), by contrast, argued on the basis of a more detailed functional study that the novel protein serves as a substitute for plastocyanin, especially under copper-deficient conditions, in much the same way as conventional cytochrome c_6. They recognized the existence of sequences encoding the novel cytochrome in yeast two-hybrid screens of an *Arabidopsis* cDNA

library for sequences encoding proteins interacting with a chloroplast immunophilin which was subsequently identified as the thylakoid-located immunophilin FKBP13 (Buchanan and Luan 2005). Based on RNA blot hybridizations and immunoblots, they reported that transcripts for the protein and the protein itself were found in leaves, but not in roots, and that when the gene was expressed under a powerful promoter, although transcripts were seen in both tissues, the protein accumulated only in leaves. Immunoblot analysis of chloroplast fractions from over-expressing lines indicated that the protein could be found in the thylakoid lumen, consistent with a putative targeting sequence at the N-terminus of the protein. A chloroplast location was subsequently supported by Nimmo (2011). Gupta et al. also reported that recombinant protein could restore oxygen evolution in inside-out thylakoid preparations in vitro, and that T-DNA insertion mutants of *Arabidopsis* lacking the cytochrome c_6 were phenotypically normal. In addition, they reported that RNAi lines designed to down-regulate expression of both plastocyanin genes of *Arabidopsis* were viable, although impaired in growth, but that the combination of cytochrome c_6 knockout and plastocyanin RNAi was lethal. On the basis of these experiments they concluded that the new cytochrome c_6 was a functional substitute for plastocyanin, although they noted that less severe reduction of plastocyanin levels achieved by copper deprivation ultimately led to death of wild type plants, and did not cause elevation of transcript levels for cytochrome c_6.

Subsequently, Wastl et al. (2004a) reported that the genome of the eukaryotic green alga *Chlamydomonas* contained a sequence encoding the novel cytochrome c_6, as well as a sequence encoding the conventional form of the protein. Phylogenetic analysis showed that the novel *Chlamydomonas* protein formed a monophyletic group with those identified from land plants, consistent with the fact that they all contained the 12 amino-acid insertion. Reflecting their monophyletic nature, Wastl et al. proposed

the name cytochrome c_{6A} for this family of proteins in preference to a previous proposal of the name cytochrome c_X, arguing that the latter name was potentially confusing (the term had already been used for a different cytochrome), and did not reflect the relationship with other cytochromes c_6. The term cytochrome c_{6A} is now widely accepted, and, as discussed below and elsewhere in this volume, has been used as the basis for designating additional families as cytochrome c_{6B} and cytochrome c_{6C}.

II. The Protein

A. Evidence Against Cytochrome c$_{6A}$ as a Substitute for Plastocyanin

Notwithstanding the evidence provided by Gupta et al. (2002a) that cytochrome c_{6A} is a functional substitute for plastocyanin, a number of lines of evidence accumulated to the contrary. Molina-Heredia et al. (2003) showed that the redox midpoint potential of *Arabidopsis* cytochrome c_{6A} was over 200 mV lower than that of *Arabidopsis* plastocyanin or a conventional cytochrome c_6, meaning that it would be very unlikely to be able to oxidize cytochrome *f*, whose redox midpoint potential is similar to plastocyanin. Molina-Heredia et al. also showed that *Arabidopsis* cytochrome c_{6A} reduced *Arabidopsis* PSI preparations in vitro much more slowly than *Arabidopsis* plastocyanin or cytochrome c_6 from the eukaryotic alga *Monoraphidium*. Modelling of *Arabidopsis* cytochrome c_{6A} indicated an overall predominantly positively charged surface, whereas both *Arabidopsis* plastocyanin and *Monoraphidium* cytochrome c_6 would have a predominantly negatively charged surface, again suggesting that cytochrome c_{6A} and plastocyanin had different targets. Increasing ionic concentration (and thus screening charge repulsion) did not lead to increased rate of reaction between cytochrome c_{6A} and PS I, indicating that the failure of the two to react was not simply due to electrostatic effects.

Weigel et al. ([2003a](#)) provided compelling genetic evidence that cytochrome c_{6A} is not a direct substitute for plastocyanin. They generated stable null alleles of both plastocyanin genes in *Arabidopsis*, and showed that, although the double mutant was viable when propagated on medium containing sucrose, it was unable to grow photosynthetically, with fluorescence data indicating a block in photosynthetic electron transfer somewhere between Photosystem II (PSII) and PSI. Furthermore, overexpression of cytochrome c_{6A} from the powerful 35S promoter failed to alleviate the mutant phenotype, indicating that cytochrome c_{6A} was unable to compensate for the lack of plastocyanin. This study raises the question of why the plastocyanin knockdown lines of *Arabidopsis* studied by Gupta et al. ([2002a](#)) remained viable, even though plastocyanin was not detectable by immunoblot analysis. Given that these were knockdown lines, it seems likely that some plastocyanin expression remained, even though this was below the level of detection in immunoblots. The major reduction in plastocyanin levels was presumably responsible for the impaired growth of the plants. Why the loss of cytochrome c_{6A} was lethal in a background with low levels of plastocyanin is discussed below.

Weigel et al. ([2003b](#)) also argued for a different function for cytochrome c_6 from plastocyanin on the basis of RNA profiles. They showed that in *Arabidopsis* lines mutant for cytochrome c_{6A} transcript profiles for more than 3000 genes in microarrays, most of which encoded chloroplast proteins, were significantly different from profiles for single or double plastocyanin mutants.

B. Structure

Structures of cytochrome c_{6A} were reported independently by two groups in 2006. Marcaida et al. ([2006](#)) reported structures at 1.2 Å and 1.4 Å resolution for protein with the heme in the ferric and ferrous states respectively (Fig. 33.1), and Chida et al. ([2006](#)) reported the structure of the ferric form at 1.5 Å resolution. The structure was
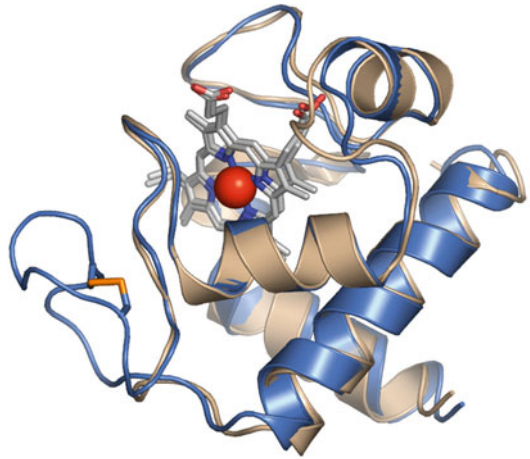
Fig. 33.1. Superposition of the structure of *Arabidopsis* ferri-cytochrome c_{6A} on that of ferro-cytochrome c_6 from the green alga *Monoraphidium braunii* (pdb code 1ctj). *Blue*, cytochrome c_{6A}; *pink*, cytochrome c_6. Reprinted from Journal of Molecular Biology, Vol 360, Marcaida MJ et al. 'Structure of cytochrome c_{6A}, a novel dithio-cytochrome of *Arabidopsis thaliana*, and its reactivity with plastocyanin: implications for function.' pp 968–977, Copyright (2006), with permission from Elsevier.

dominated by four alpha-helices, and was similar to predictions from modelling based on homology with cytochrome c_6 (Molina-Heredia et al. [2003](#)), with heme iron coordinated by the sulfur of a methionine residue and the nitrogen of a histidine. There was little difference between the structures of ferrous and ferric forms, a point discussed below. The 12 amino-acid insertion diagnostic of cytochrome c_{6A} was located on the outside of the molecule and was well-structured, with the two cysteines forming a disulfide bridge. The structures formed the basis for hypotheses to explain the big change in redox midpoint potential of cytochrome c_{6A} compared to cytochrome c_6, and this is discussed in more detail below.

C. Loop Insertion Peptide

As previously described, the cytochrome c_{6A} proteins all showed an insertion of 12 amino acids in a loop connecting two alpha helices (Fig. 33.1), and this has been designated the

Loop Insertion Peptide, abbreviated to LIP (Howe et al. 2006). Wastl et al. (2004b) compared the biochemical properties of the wild type *Arabidopsis* protein and forms carrying site-directed mutations in the LIP that resulted in deletion of the LIP or replacement of the cysteine residues with serines. Deletion of the LIP led to a slight drop in the p*I* from 5.1 to 4.6, but not enough to bring it to the p*I* of *Arabidopsis* plastocyanin (4.1). The redox midpoint potentials were unchanged in the mutants, and the second order rate constants for reaction with PS I were essentially unchanged (or slightly reduced) and some 70-fold lower than the rate constants for reaction of plastocyanin with PS I. The authors concluded that the LIP was not responsible for the low redox midpoint potential, or the low rate of reaction with PS I, including the p*I* unsuitable for the reaction (Wastl et al. 2004b).

Chida et al. (2006) analysed the denaturation of wild type cytochrome c_{6A} and mutants with the cysteines in the LIP mutated to serine, or with the LIP deleted, and concluded that the disulfide bridge contributed to the stability of the protein. Worrall et al. (2008) analysed the disulfide bond angles, with reference to the work of Schmidt et al. (2006) on relating disulfide bond angles to their function. Worrall et al. concluded that the LIP disulfide bond was structural, rather than regulatory or catalytic, and that the LIP might be involved in interaction with other proteins. Mason et al. (2012) analysed the folding of wild type cytochrome c_{6A}, the mutant with the LIP cysteines replaced by serines, and a cytochrome c_6 from a cyanobacterium, and also concluded that the disulfide makes a significant contribution to stability, with the formation of the structure of the region containing it occurring late in the folding pathway.

Overall, therefore, it seems that the LIP is not directly responsible for the failure to react with PS I. However, it may be involved in interaction with other substrates, and the disulfide bridge contained within it probably has a structural rather than a regulatory or catalytic role.

D. Redox Midpoint Potential

The low redox midpoint potential is one of the most striking differences between cytochrome c_{6A} and cytochrome c_6 or plastocyanin. As summarized above, Wastl et al. (2004b) showed that this was not due to the LIP of cytochrome c_{6A}. Consideration of the three-dimensional structures of cytochrome c_{6A}, together with sequence comparisons with cytochrome c_6, led to the identification of a number of residues in the vicinity of the heme that might affect the redox midpoint potential (Marcaida et al. 2006; Chida et al. 2006). Worrall et al. (2007) tested the contribution of position 52 (*Arabidopsis* cytochrome c_{6A} numbering), which is consistently a glutamine in conventional cytochrome c_6, but a hydrophobic residue in cytochrome c_{6A} (valine in the *Arabidopsis* protein). The glutamine is within hydrogen-bonding distance of a water molecule that, in turn, is putatively able to interact with the propionate sidechains of the heme. Worrall et al. (2007) also tested the contribution of an alanine (A31 in *Arabidopsis* cytochrome c_{6A}) whose corresponding position in cytochrome c_6 is taken by a residue with an electropositive side chain that is again likely to interact with a heme propionate side chain. Substitution of V52 with glutamine in the *Arabidopsis* protein resulted in an increase in the redox midpoint potential of +109 mV, a remarkable change for a single residue. Consistent with this, replacement of the equivalent glutamine in cytochrome c_6 from the cyanobacterium *Phormidium laminosum* with valine lowered the midpoint potential by 100 mV. Substitution of A31 with lysine in the *Arabidopsis* cytochrome c_{6A} protein raised the midpoint potential by +31 mV. Thus, the V52Q change alone is sufficient to account for a large part of the difference in midpoint potential between cytochrome c_{6A} and cytochrome c_6, which Worrall et al. estimated as approximately 250 mV, larger than previously indicated (Molina-Heredia et al. 2003). However, the detailed basis for the effect of the residue at position 52 on the midpoint potential is not fully clear

(Rajagopal et al. 2011). The low midpoint potential of cytochrome c_{6A} contrasts with the general situation with cytochrome c proteins, where evolution has acted to stabilize the reduced state (Worrall et al. 2008).

E. Interaction Partners

A limited number of studies have considered possible interaction partners of cytochrome c_{6A}. The first of these was the study by Gupta et al. (2002a) that was one of the first to report the existence of cytochrome c_{6A}. In this study, a cDNA encoding the *Arabidopsis* cytochrome c_{6A} was identified through the ability of its product to interact with the immunophilin FKBP13 in a yeast two-hybrid assay. FKBP13 is one of a family of proteins predicted to be located in the thylakoid lumen with peptidyl-prolyl *cis-trans* isomerase (PPI) activity. The precursor of the FKBP13 protein had also been shown on the basis of a two-hybrid screen and assays in vitro to interact with the Rieske Fe-S protein component of the cytochrome $b_6 f$ complex (Gupta et al. 2002b). Surprisingly, although the precursor form of FKBP13 (including the chloroplast transit sequence) and the intermediate form (lacking the transit sequence but retaining the thylakoid targeting sequence) interacted with the Rieske protein, the mature form of FKBP13 did not (Gupta et al. 2002b). This was interpreted as indicating that interaction occurs at some stage during the import pathway. Downregulation of expression of FKBP13 by RNAi led to an increase in the level of Rieske protein. Structural analysis of FKBP13 (Gopalan et al. 2004) showed the presence of two disulfide bridges in the protein. The cysteines were shown to be redox-active with thioredoxin, with reduction leading to a loss of PPI activity. Similarly, loss of one or other disulfide bridge was also shown to lead to reduction in PPI activity, and it was proposed that reduction of the disulfides was a basis for redox regulation. Whether cytochrome c_{6A}, with its pair of cysteines in the LIP, was involved in this system was not known.

Marcaida et al. (2006) examined the possibility that cytochrome c_{6A} reacts with plastocyanin (see below), which would be thermodynamically feasible in terms of the midpoint potentials of the two proteins. They found that *Arabidopsis* cytochrome c_{6A} (with the heme reduced) could reduce pea plastocyanin in vitro, and obtained a value for the second order rate constant for the reaction of 1.2×10^7 $M^{-1}s^{-1}$ for the reaction at 300 K and pH 6.0. Although this was significantly lower than the value of 8.7×10^7 $M^{-1}s^{-1}$ for the reaction of plastocyanin with PS I in *Arabidopsis* (Wastl et al. 2004b), they argued that the value for the reaction between cytochrome c_{6A} and plastocyanin was potentially physiologically significant, especially if the reaction were involved in regulation or biogenesis.

Pesaresi et al. (2009) examined further the possible interaction between cytochrome c_{6A} and plastocyanin in yeast two-hybrid screens. Using this assay they were unable to detect interaction between *Arabidopsis* cytochrome c_{6A} and either of the plastocyanin isoforms. It should be noted, though, that interactions between redox proteins are typically very weak. For example, the K_D for the interaction between turnip cytochrome f and spinach plastocyanin is approximately 0.14 mM (Kannt et al. 1996). It is therefore possible that an interaction between cytochrome c_{6A} and plastocyanin would not have been detected in this assay. Unexpectedly, Pesaresi et al. (2009) found that cytochrome c_{6A} appeared to form a homodimer, based on the two hybrid screen, and this interaction was seen both with the wild type protein and with the mutant form with the LIP cysteines mutated to serine, indicating that the dimerization did not depend on the formation of disulfide bridges involving the LIP.

III. 'Omics Studies

Apart from providing the genetic evidence for the existence of the protein, 'omics studies have provided few additional insights.

The protein is encoded on chromosome 5 of *Arabidopsis* (gene model AT5G45040.1) and contains 5 introns in the coding region. Curiously, the coding region overlaps with, but is on the opposite strand, to a region corresponding to the 3' untranslated region for a gene AT5G45030.1, encoding a trypsin-like protein (http://www.arabidopsis.org/servlets/TairObject?name=AT5G45040&type=locus, accessed 2 Feb 2014). The significance, if any, of this is unclear, as the arrangement is not conserved. The transcript has been recorded from a wide range of tissue types, from sperm cells to embryos and leaves. There is little evidence of strong regulation of transcript levels in response to environmental changes. Interestingly, though, transcript levels were markedly lower in *Arabidopsis* lines with the *vtc1* (vitamin C defective) mutation which results in loss of GDP-mannose pyrophosphorylase activity, and lowered ascorbate levels (Kerchev et al. 2011). However, the significance of the low transcript level is again difficult to assess, as a similar effect was not seen in the *vtc2* mutant, which lacks the GDP-L-galactose phosphorylase, also leading to lowered ascorbate levels. The apparent contradiction between *vtc1* and *vtc2* mutants may reflect the fact that the GDP-mannose pyrophosphorylase mutation (*vtc1*) is pleiotropic, influencing, for example, protein glycosylation and ammonium sensitivity, as well as ascorbate biosynthesis (Qin et al. 2008). Other genes showing a similar pattern to that for cytochrome c_{6A} in *vtc1* mutants included *psbA*, *petG* and genes for a small number of components of the NADH dehydrogenase complex of the thylakoid membrane (Queval and Foyer 2012).

Gupta et al. (2002a) reported that immunoblot analysis indicated the presence of the protein in wild type *Arabidopsis* leaves, with much lower levels in roots. They also reported studies using isolated chloroplasts indicating a thylakoid lumen location in overexpression lines. However, proteomic studies have, to our knowledge, failed to detect cytochrome c_{6A} directly in spite of the fact that transcripts for the protein have been detected in a wide range of tissues. It should be noted that some studies have included the protein in chloroplast thylakoid luminal proteome databases on the basis of the literature or studies in silico (e.g. Zybailov et al. 2008; Karpowicz et al. 2011; Huang et al. 2012). The use of the literature appears to lead ultimately to the initial report of Gupta et al. and it is therefore important for this to be independently confirmed. Howe et al. (2006) discussed the reasons for the failure to detect the protein in proteomic studies, and concluded it was unlikely to be due to extremes of size or isoelectric points, as plastocyanin is smaller than cytochrome c_{6A} (10.5 kDa compared to 11.8 kDa) and has a more extreme isoelectric point (4.1 for *Arabidopsis* plastocyanin as opposed to 5.1), yet is readily detected. This suggests that cytochrome c_{6A} is at very low abundance under all the conditions used so far in proteomic studies.

IV. Distribution Outside the Green Plant Lineage

We consider here the distribution of cytochrome c_{6A} proteins, as this may shed some light on their function. The evolutionary interpretation of their distribution is discussed in more detail below. Database searches indicate the widespread distribution of sequences encoding cytochrome c_{6A} among green plants and algae (e.g. Howe et al. 2006). However, if the presence of the LIP with its two cysteines and a hydrophobic residue rather than a glutamine at the equivalent of position 52 (see above) are taken as diagnostic features in addition to sequence similarity, searches (as at March 2014) failed to identify any sequences encoding cytochrome c_{6A} (as opposed to cytochrome c_6) in organisms with secondary green chloroplasts, such as *Euglena* or *Chlorarachnion*. [See Walker et al. (2011) and Dorrell and Howe (2012) for detailed descriptions of the evolutionary positions of these organisms and a summary of the secondary and tertiary plastid endosymbioses that have taken place in eukaryote evolution].

On the criteria of LIP and a hydrophobic residue at position 52, cytochrome c_{6A} also appears to be absent from other photosynthetic eukaryotes, including those with primary chloroplasts but outside the green lineage (i.e. red algae and glaucophytes). However, there are a limited number of examples outside the green lineage of sequences encoding apparently similar polypeptides that lack the LIP, but which may have a hydrophobic residue at the equivalent of position 52, as in cytochrome c_{6A} (although this may depend on the details of the alignment). These include sequences from the red alga *Galdieria sulphuraria* (e.g. XM_005704567.1), the haptophyte alga *Emiliania huxleyii* (e.g. XM_005765899.1), the cryptophyte *Guillardia theta* (e.g. XM_005827306.1), and the dinoflagellate *Amphidinium carterae* (e.g. CF065358.1). Whether these genuinely are homologues of cytochrome c_{6A}, including having a low redox midpoint potential, remains to be seen, but would repay further study.

Understanding the distribution of cytochrome c_{6A} became more complex with the recognition that proteins of the low potential cytochrome c_6 family do in fact occur in cyanobacteria, typified by the PetJ2 protein of *Synechococcus* sp. PCC 7002 (Bialek et al. 2008). Although this protein also lacks the LIP with two cysteines, it has a hydrophobic residue (leucine) at the equivalent of position 52 for cytochrome c_{6A}, and a redox midpoint potential of 148 ± 1.7 mV. This is much lower than for the conventional cytochrome c_6 found in the same organism and, as for cytochrome c_{6A}, would make it unsuitable to act as an electron acceptor from cytochrome f (Bialek et al. 2008). On the basis of a phylogenetic analysis, Bialek et al. proposed the existence of two groups of these proteins in cyanobacteria, and designated them cytochrome c_{6B} and cytochrome c_{6C}. There is something of an irony here. Cytochrome c_{6A} was first described as a homologue of the cyanobacterial cytochrome c_6 found, contrary to the understanding at the time, in plants. Cytochromes c_{6B} and c_{6C} were then described as cyanobacterial homologues

of the plant protein. The cyanobacterial cytochromes c_{6B} and c_{6C} are described in more detail elsewhere in this volume. Bialek et al. commented that the cyanobacteria containing the cytochrome c_{6C} family are largely nitrogen-fixing species and that 'the *Nostoc punctiformae NpF1886* gene for a c_6-like cytochrome is expressed at low but significant levels in ammonia-grown and nitrogen-starved cells and in hormogonia and akinetes'. Reyes-Sosa et al. (2011) reported that the Nostoc sp. PCC 7119 cytochrome c_{6C} protein was able to reduce PSI at a rate that could be physiologically significant, and proposed a function for the cytochrome in a different but unidentified electron transfer pathway from the conventional one between the cytochrome b_6f complex and PSI.

V. Possible Functions

We now speculate on possible functions for cytochrome c_{6A}. Evidence presented above implies that it does not function as a simple substitute for plastocyanin in transfer of electrons from cytochrome f to PSI. A role in apoptosis has been suggested (Weigel et al. 2003b), although Howe et al. (2006) argued against this on the grounds that proteomic analysis of programmed cell death in plants had not indicated that cytochrome c_{6A} was upregulated under those conditions (Swidzinski et al. 2004). Howe et al. (2006) suggested the possibility of a regulatory role involving structural effects on some or all of the LIP, the heme, and interaction with FKBP13. They suggested that, given its midpoint potential, the protein might be involved in sensing the redox state of the plastoquinone pool. However, the fact that no significant difference in structure was later seen between the forms of the protein with the heme oxidized or reduced (Marcaida et al. 2006) makes the proposal of a regulatory role associated with conformational change less attractive.

In the light of the ability of cytochrome c_{6A} to react with plastocyanin (Marcaida et al. 2006), Schlarb-Ridley et al. (2006)

suggested the former protein might assist the formation of disulfide bridges in proteins of the thylakoid lumen, with electrons being transferred from two cysteines of the target protein to the disulfide in the bond in the LIP. This would generate a disulfide bond in the target protein, reducing the disulfide bond in the LIP. This might be re-formed by electrons being transferred from the cysteines of the LIP to plastocyanin directly, and also to the heme group of the protein and then to plastocyanin. Schlarb-Ridley et al. suggested that plastocyanin might also be able to form disulfides in target proteins directly, but that cytochrome c_{6A} took over this role under low plastocyanin concentrations. This would explain why the combination of knocked down levels of plastocyanin and knockout of cytochrome c_{6A} was lethal in the experiments of Gupta et al. (2002a). However, there is as yet no direct evidence in support of a role in disulfide bond formation, and the analysis suggesting a structural rather than a catalytic role for the disulfide (Worrall et al. 2008) argues against this.

It may be that we should look for an explanation involving an electron transfer process that is important under stress conditions that have not yet been properly identified. It is increasingly recognized that alternative electron transfer pathways exist in addition to the 'standard' ones, often to allow protection against light stress. For example, the cyanobacterial Flv1 and Flv3 flavodiiron proteins allow direct transfer of electrons generated by PSII activity to oxygen, to protect PSI against photodamage under conditions of rapidly pulsing light (Tikkanen et al. 2012; Allahverdiyeva et al. 2013). Similarly, the respiratory terminal oxidases located in the cyanobacterial thylakoid membrane are necessary for survival under rapidly changing light intensities, protecting against formation of reactive oxygen species and the occurrence of photoinhibition, probably by allowing electrons to be diverted from the plastoquinone pool or the cytochrome b_6f complex to oxygen (Lea-Smith et al. 2013). Given that its redox midpoint potential would be suitable for it to accept electrons from PQH_2 it is tempting to suggest that cytochrome c_{6A} functions as a 'safety valve' to bypass the cytochrome b_6f complex and transfer electrons directly from PQH_2 to PSI (presumably via plastocyanin) when the plastoquinone pool is highly reduced. Although this would require the lipid soluble plastoquinone to interact directly with the soluble cytochrome, there is precedent for interaction between plastoquinone and extrinsic proteins, such as the chloroplast sensor kinase CSK (Puthiyaveetil et al. 2013), and respiratory complex I, where the quinol headgroup appears to protrude some distance from the membrane to participate in the redox reaction (Baradaran et al. 2013).

VI. Evolution of Cytochrome c_{6A}

Before the existence of the cytochrome c_{6B} and cytochrome c_{6C} proteins was recognized in cyanobacteria, it was possible to propose a relatively simple model for the evolution of cytochrome c_{6A} (Howe et al. 2006). According to this, the cyanobacterium that entered endosymbiosis to give primary chloroplasts would have contained cytochrome c_6 and plastocyanin. Duplication and divergence of the cytochrome c_6 gene in the green chloroplast lineage led to the situation in algae such as *Chlamydomonas*, which contain both cytochrome c_6 and cytochrome c_{6A} (Wastl et al. 2004a) as well as plastocyanin. In the land plant lineage, the cytochrome c_6 gene was lost, and in the ancestor to the red algae and glaucophyte algae the plastocyanin gene was lost. In organisms with serial endosymbiosis involving a red algal symbiont, such as diatoms, there was no plastocyanin. (Limited instances of the existence of plastocyanin in such organisms, for example *Thalassiosira oceanica* (Peers and Price 2006) probably reflect horizontal gene transfer).

However, with the identification of the cytochrome c_{6B} and cytochrome c_{6C} proteins in cyanobacteria (Bialek et al. 2008) the situation became more complex. Only a limited number of phylogenetic analyses have been performed, and the shortness of
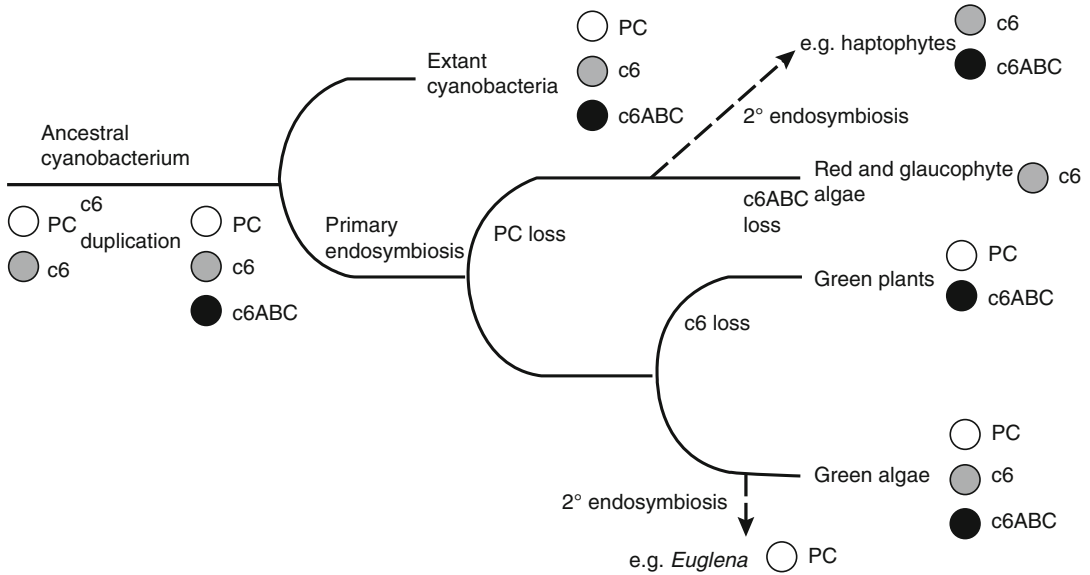
Fig. 33.2. Evolutionary history of cytochrome c_6, cytochromes $c_{6A,B,C}$, and plastocyanin. Secondary endosymbioses are shown by *dashed lines*. The figure assumes that cytochromes $c_{6A,B,C}$ share a common ancestry to the exclusion of cytochrome c_6. It does not show horizontal gene transfer events.

the cytochrome c protein sequences makes phylogenetic analysis difficult. However, if we assume the low redox midpoint potential (demonstrated or inferred) to be indicative of common ancestry, this implies that the cytochrome c_{6A} found in green chloroplasts originated in the cyanobacterial ancestor of primary chloroplasts. Thus the ancestral chloroplast would have had the full complement of cytochrome c_6, low potential cytochrome c_6, and plastocyanin (as in *Chlamydomonas*). All three were retained in the green algal lineage, with the acquisition of the LIP by the low potential cytochrome c_6. Cytochrome c_6 was lost in the land plant lineage. Plastocyanin was lost in the ancestor of red algae and glaucophytes (as there is no evidence for it in those organisms), and the low potential cytochrome c_6 was also lost in a number of these algae. Secondary endosymbiosis involving the green lineage (as in e.g. *Euglena*) seems to have failed to transfer the low potential cytochrome c_6. Secondary endosymbiosis of the red lineage is likely to have involved a line that retained the low potential cytochrome c_6, as it is present

(assuming the features referred to in Sect. IV are diagnostic) in a number of the groups with secondary chloroplasts of red algal origin. This complex evolutionary history is summarized in Fig. 33.2. It will be instructive to test in more detail the phylogenetic relationships among the cyanobacterial cytochrome c_{6B} and cytochrome c_{6C}, cytochrome c_{6A} of the green lineage, and the putatively low potential cytochrome c_6 proteins of lineages with secondary plastids. Might cytochrome c_{6A} instead represent an independent evolution of a low potential form of cytochrome c_6?

VII. General Conclusions

We now have a good knowledge of the structure of cytochrome c_{6A}, and the features responsible for its low redox midpoint potential compared to conventional cytochrome c_6. We recognize that it is not a simple substitute for plastocyanin, and it seems likely that the LIP, with its conserved pair of cysteine residues, is structural rather than catalytic. Beyond that, we have little more concrete information

about the function of this mysterious, but well conserved, protein than when it was first discovered over 10 years ago.

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Cryptic c_6-Like and c_M Cytochromes of Cyanobacteria

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Summary

Cytochromes c_6 are widely-distributed monoheme proteins that transfer electrons from the cytochrome b_6f complex to photosystem I in cyanobacteria and certain eukaryotic algae. Cytochrome c_6 has been replaced by plastocyanin in all known plant chloroplasts and in many cyanobacteria and algae, and was thought to be absent from plants until the discovery of chloroplast cytochrome c_6-like proteins, now named cytochromes c_{6A}. These have midpoint potentials substantially lower than those of typical cytochrome c_6 or plastocyanin indicating that they could not effectively transfer electrons from the cytochrome b_6f complex to photosystem I. Additional c_6-like cytochromes have been discovered in cyanobacteria. The first of these was cytochrome c_M, discovered in *Synechocystis* sp. PCC 6803 followed by discovery of a c_6-like cytochrome encoded by the *petJ2* gene in *Synechococcus* sp. PCC 7002. Subsequent phylogenetic analysis revealed that most, but not all, cyanobacteria carry several genes for putative c_6-like cytochromes. Two deeply-branching clusters, designated

cytochromes c_{6B} and c_{6C}, are distinct from either the classical c_6 cytochromes, chloroplast cytochromes c_{6A}, cyanobacterial cytochromes c_M or c_{550}, or bacterial cytochromes c_{555}. Like cytochrome c_{6A}, representative cytochromes c_{6C}, c_{6B}, and c_M have been analyzed and found to have low midpoint potentials unfavorable for electron transfer from the cytochrome b_6f complex. High-resolution structures have been solved for c_{6C} and c_{6B} cytochromes and key residues identified that govern their low midpoint potentials. However, despite these advances and the widespread distribution of these cryptic cytochromes among cyanobacteria, their functions have remained largely mysterious. Here we review the discovery of cyanobacterial c_6-like cytochromes and current understanding of their properties and possible functions.

I. Introduction

In oxygenic photosynthesis, sunlight catalyzes electron flow from water through localized, membrane-bound protein complexes. Mobile carriers transfer electrons between these complexes. One of these is cytochrome (Cyt) c_6, a water-soluble, high-potential, monoheme protein found in the thylakoid lumen of cyanobacteria and some algal chloroplasts. Cyt c_6 transfers electrons from the Cyt b_6f complex to photosystem I (PSI) and, in cyanobacteria, to a respiratory cytochrome oxidase (Schmetterer 1994; Kerfeld and Krogmann 1998). Crystal structures of Cyt c_6 are available from several sources (Frazao et al. 1995; Kerfeld et al. 1995; Yamada et al. 2000; Sawaya et al. 2001; Bialek et al. 2009), and many additional sequences have been deposited in databases. These proteins are characterized by their low molecular masses ($\sim$80–90 amino acids in the mature protein), covalently bound, c-type heme, and high midpoint potentials of approximately $+310$ to $+390$ mV. Apart from these well characterized cytochromes, the genomes of many cyanobacteria carry sequences for cytochromes similar to cytochrome c_6. These are the so-called, c_6-like cytochromes that are widely distributed among cyanobacteria and include cytochromes c_M (Malakhov et al. 1994), c_{6B}, and c_{6C} (Bialek et al.

2008). They are characterized by midpoint potentials substantially lower than those of typical c_6 cytochromes and surface charge properties that make them poorly suited for electron transfer from the Cyt b_6f complex to PSI. These proteins have been difficult to detect, their transcript levels are typically low although elevated under certain stresses, and despite detailed structural and biophysical analysis of some of these proteins expressed in bacteria, their functions have remained largely obscure. In this chapter we review current understanding of these cryptic c_6-like cytochromes of cyanobacteria, their distinguishing properties, structures, and possible functions.

II. Discovery of the c_6-Like and c_M Cytochromes

Cytochrome c_M was initially described as Cyt c_{552} (Holton and Myers 1967). The c_M designation originates from Malakhov et al. (1994) who described a water-soluble, moderately basic protein with a redox potential of about $+150$ mV isolated from *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis* 6803). To test the function of Cyt c_M, its gene was deleted but this mutation had no perceptible impact on photosynthetic growth, respiration, or oxygen evolution. However, Cyt c_M could not be inactivated in a PSI deletion strain of *Synechocystis* (Manna and Vermaas 1997), implying that at least under some conditions, Cyt c_M is indispensable. However, the same authors could not obtain a double deletion mutant of plastocyanin and cytochrome c_6 in the same PSI-

minus background indicating that Cyt c_M could not substitute as an electron carrier for these proteins. Manna and Vermaas (1997) observed a sequence similarity between Cyt c_M and the Cyt c-binding region of the aa_3 cytochrome oxidase (COX) from *Bacillus* sp. and *Thermus thermophilus*. They suggested that Cyt c_M may serve as a soluble or membrane-bound component of cytochrome oxidase. In that case the Cyt c_M N-terminal domain might function as a membrane anchor. Manna and Vermaas (1997) further proposed that plastocyanin or Cyt c_6 may transfer electrons from the Cyt b_6f complex to COX through Cyt c_M. Indeed, Bernroitner et al. (2009) showed that Cyt c_M can donate electrons to cytochrome oxidase as efficiently as Cyt c_6 and more efficiently than PC. However, it has also been shown that no additional component is needed for electron transfer from Cyt c_6 or PC to COX (Paumann et al. 2004a, b) (see also Chap. 31 by Diaz-Moreno et al.). Thus a role for Cyt c_M in electron transfer to PSI or COX remains uncertain, at least under typical laboratory growth conditions.

A c_6-like cytochrome was subsequently discovered in plant chloroplasts (Gupta et al. 2002; Wastl et al. 2002). Because land plants, and some algae, exclusively use the blue-copper plastocyanin protein as an electron carrier between the Cyt b_6f complex and PSI, Cyt c_6 was thought to be absent from chloroplasts prior to this discovery. However, genome and expressed sequence tag (EST) analyses of higher plants (Gupta et al. 2002; Wastl et al. 2002), as well as the green alga *Chlamydomonas reinhardtii* (Wastl et al. 2004), revealed sequences that encode the characteristic, CXXCH c-heme-binding motif (where XX represents any of a number of amino acids but is often AA or AG), and are strikingly similar to Cyt c_6 sequences. Indeed the initial report by Gupta et al. (2002) presented evidence suggesting that these c_6-like cytochromes (then designated Cyt c_x) could, at least to an extent, replace plastocyanin as an electron carrier. However, subsequent studies concluded that these novel cytochromes are unlikely substitutes for plas-

tocyanin because they have midpoint potentials 200 mV lower than those of typical chloroplast PC or cyanobacterial and algal Cyt c_6, and positive surface charges in contrast to the negative chloroplast PC (Molina-Heredia et al. 2003; Howe et al. 2006). In addition, Weigel et al. (2003) showed convincingly that an *Arabidopsis* double mutant, with both of its plastocyanin genes inactivated, could not grow photosynthetically. Wastl et al. (2004) showed by phylogenetic analysis that the novel c_6 cytochromes are monophyletic, and although similar to Cyt c_6, are distinct from them, and thus named these 'cytochrome c_{6A}.' These are discussed in detail in Chap. 33 by Howe et al.

Nomura (2001) first discovered a gene for a second, putative c_6 cytochrome in the cyanobacterium *Synechococcus* sp. PCC 7002 (hereafter *Synechococcus* 7002). The *petJ* gene encodes Cyt c_6 and accordingly, the second gene for a c_6-like cytochrome was named *petJ2*. In a subsequent phylogenetic analysis of 40 some cyanobacterial genome sequences, Bialek et al. (2008) discovered that most of these carry genes for c_6-like cytochromes that are similar to the PetJ2, c_6-like cytochrome of *Synechococcus* 7002. Their analysis revealed two deeply-branching clusters, designated cytochromes c_{6B} and c_{6C}, that have features distinct from cytochrome c_6, c_M, or other c-type cytochrome proteins. Bialek et al. (2008) found that the PetJ2, c_{6C} cytochrome of *Synechococcus* 7002 has a midpoint potential ($E_{m,7}$) of $+148$ mV and a positive, overall surface charge, indicating that it, like cytochromes c_M and c_{6A}, would be poorly suited for electron transfer from the Cyt b_6f complex to PSI.

III. Cytochromes c_{6C} and c_{6B}

A. Genome Analyses

Figure 34.1 illustrates the phylogenetic relationships of cyanobacterial cytochrome c_M, c_{6C}, and c_{6B} proteins relative to cyanobacterial c_{550}, bacterial c_{555}, and chloroplast
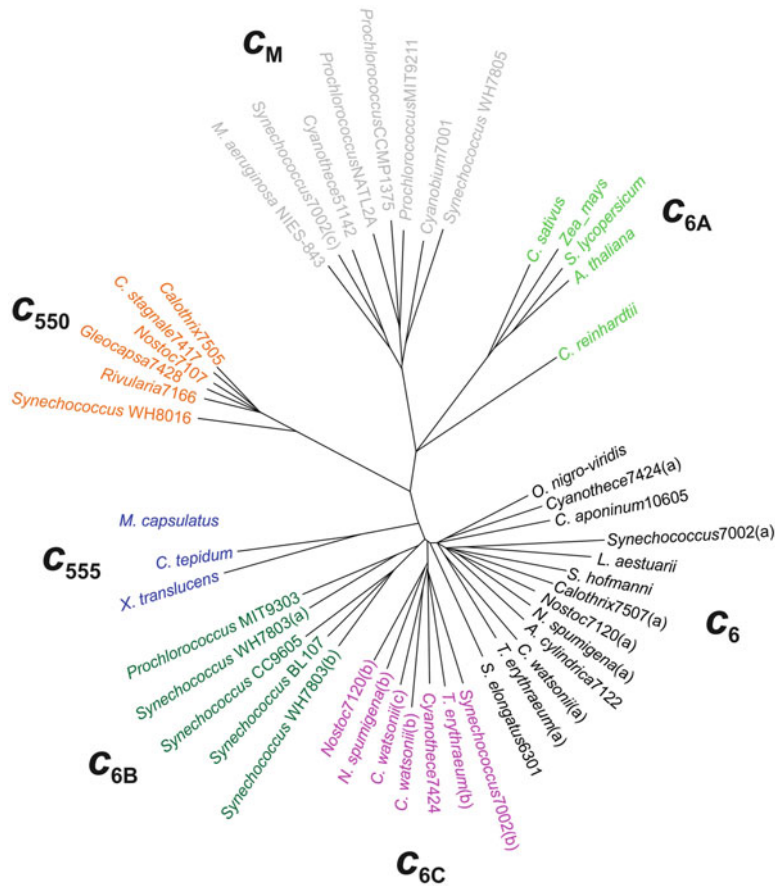
Fig. 34.1. Unrooted tree of *c*-type cytochrome proteins from cyanobacteria, algae, and plants. Cytochrome c_6 sequences are: *Oscillatoria nigro-viridis* (gi504991236), *Cyanothece* sp. PCC 7424 (gi506436589), *Cyanobacterium aponinum* PCC 10605 (gi428685443), *Synechococcus* sp. PCC 7002 (gi2522460), *Lyngbya aestuarii* BL J (gi550814042), *Scytonema hofmanni* (gi516352295), *Calothrix* sp. PCC 7507 (gi427350415), *Nostoc* sp. PCC 7120 (gi17133386), *Nodularia spumigena* (gi493209471), *Anabaena cylindrica* PCC 7122 (gi428677279), *Crocosphaera watsonii* (gi494517037), *Trichodesmium erythraeum* (gi499931389), *Synechococcus elongatus* PCC 6301 (gi56751283). Cytochrome c_{6A} sequences are: *Cucumis sativus* (gi449433475), *Zea mays* (gi293334331), *Solanum lycopersicum* (gi460374873), *Arabidopsis thaliana* (gi332007810), *Chlamydomonas reinhardtii* (gi158273740). Cytochrome c_{6B} sequences are: *Prochlorococcus marinus str*. MIT 9303 (gi124023458), *Synechococcus* sp. WH 7803 (gi148239320), *Synechococcus* sp. CC9605 (gi499683262), *Synechococcus* sp. BL107 (gi497475850), *Synechococcus* sp. WH 7803 (gi148239088). Cytochrome c_{6C} sequences are: *Synechococcus* sp. PCC 7002 (gi22652035), *Trichodesmium erythraeum* (gi499933174), *Cyanothece* sp. PCC 7424 (gi501597452), *Crocosphaera watsonii* (gi494515064 and gi494518195), *Nodularia spumigena* (gi493209475), *Nostoc* sp. PCC 7120 (gi17135139). Cytochrome c_{550} sequences are: *Calothrix* sp. PCC 7507 (gi427353344), *Cylindrospermum stagnale* PCC 7417 (gi428259159), *Nostoc* sp. PCC 7107 (gi427710663), *Gloeocapsa* sp. PCC 7428 (gi428265205), *Rivularia* sp. PCC 7116 (gi427374782), *Synechococcus* sp. WH 8016 (gi351677206). Cytochrome c_{555} sequences are: *Methylococcus capsulatus str. Bath* (gi53756998), *Chlorobium tepidum* TLS (gi21672916), *Xanthomonas translucens* (gi497260364). Cytochrome c_M sequences are: *Microcystis aeruginosa* NIES-843 (gi166363520), *Synechococcus* sp. PCC 7002 (gi169884672), *Cyanothece* sp. ATCC 51142 (gi171699631), *Prochlorococcus marinus str*. NATL2A (gi72382534), *Prochlorococcus marinus subsp. marinus str*. CCMP1375 (gi33240028), *Prochlorococcus marinus str*. MIT 9211 (gi159888850), *Cyanobium* sp. PCC 7001 (gi197624664), *Synechococcus* sp. WH 7805 (gi88786922). Sequence similarities were calculated with Neighbor Joining using BLOSUM62.

c_{6A}. The diversity of cyanobacteria in which c_6-like cytochrome genes are found (single-celled, filamentous, and nitrogen-fixing) suggests that these cytochromes appeared early in cyanobacterial evolution. Distinguishing features of the c_{6B} and c_{6C} cytochromes include (i) absence of the 12-residue, cysteine-containing, 'Loop Insertion Sequence' that characterizes the c_{6A} family (see Wastl et al. (2004) and Bialek et al. (2008) for alignments), (ii) a hydrophobic residue (leucine, isoleucine, valine) adjacent to the heme edge (position 50 in the *Synechococcus* 7002 PetJ2, c_{6C} protein) relative to a conserved glutamine in the corresponding location in c_6 cytochromes, and (iii) a conserved tyrosine (position 61 in the *Synechococcus* 7002 protein) relative to a phenylalanine or tryptophan at this location in cytochromes c_6 and c_{6A}. The hydrophobic residue at position 50 in the $c_{6A,B,C}$ cytochromes and the resulting more limited hydrogen-bonding network around the heme appear to play major roles in lowering the redox midpoint potential of these c_6-like cytochromes relative the classical, c_6 cytochromes (Worrall et al. 2007; Rajagopal et al. 2011; Bialek et al. 2014; Zatwarnicki et al. 2014). Genes for the c_{6B} cytochromes are found mostly among the non-nitrogen-fixing, *Procholorococcus* and *Synechococcus* strains, which are widely distributed in diverse marine environments (Ting et al. 2002). The *petJ2*, cytochrome c_{6C} gene in the marine, coastal cyanobacterium *Synechococcus* 7002 seems to be rather exceptional because these genes are found mainly in heterocyst-forming or unicellular, nitrogen-fixing strains (Bialek et al. 2008).

Among related cytochromes, c_{550} is named after its α-band absorbance maximum at 550 nm and is an extrinsic component of PSII in cyanobacteria and some eukaryotic algae such as red algae, dinoflagellates, and stramenopiles such as diatoms and brown algae (reviewed in Roncel et al. (2012)). Cytochromes c_{550} help stabilize the Mn cluster and oxygen-evolving complex of PSII, but are not essential for PSII activity, at least under standard growth conditions

(Shen et al. 1995; Li et al. 2004). The c_{550} heme has a unique, bis-histidine axial ligation, and a role in dissipating over-reduction of PSII has been proposed, but its function remains unresolved. Cytochrome c_{550} is also found in non-photosynthetic organisms, such as *Bacillus subtilis* where it fulfills a regulatory role in initiation of sporulation (Shin et al. 2005). Cytochrome c_{555}-encoding genes were initially found in *Chlorobium* genomes but further sequencing projects have identified increasing numbers of their homologs. Members of this family, for example from the hyperthermophilic bacterium, *Aquifex aeolicus*, have representatives that include soluble as well as membrane-anchored variants (Baymann et al. 2001b). Cytochrome c_M proteins are discussed in detail in Sect. IV.

B. Biophysical Features

All c-type cytochromes, including c_6, c_6-like and Cyt f proteins exhibit characteristic absorbance features in the visible wavelength range (see Holton and Myers (1967); Ho and Krogmann (1980); Bialek et al. (2008); Zatwarnicki et al. (2014) for representative spectra). Spectra of the oxidized forms have two absorbance peaks—an intense, narrow peak with a maximum at 410 nm (the Soret or γ band) and a broad flattened peak with a maximum at 530 nm. Upon heme reduction, the Soret band shifts slightly to higher wavelengths and two new peaks appear– the β peak at 520 nm and the α peak at 550 nm–with simultaneous disappearance of the 530 nm peak. Table 34.1 shows the spectral properties, midpoint potentials ($E_{m,7}$), and isoelectric points of three, representative c_6-like cytochromes purified from *Synechococcus* sp. WH 8102 (Cyt c_{6B} Zatwarnicki et al. (2014)), *Synechococcus* 7002 (Cyt c_{6C}, Bialek et al. (2008, 2014)), and *Nostoc* sp. PCC 7119 (a Cyt c_6-like protein that falls within the c_{6C} group, Reyes-Sosa et al. (2011)). For example, the *Nostoc* sp. PCC 7119 (hereafter *Nostoc* 7119) c_{6C} cytochrome, when reduced, shows absorbance maxima at 417 (Soret band),

Table 34.1. Biophysical properties of cyanobacterial c_6-like cytochromes[a].

Protein	Isoelectric point	E_{m7} (mV)	Absorbance maxima (nm)		
			Band γ	Band β	Band α
Cyt c_{6B}	8.4	113	418.3	523.5	557.0
Cyt c_{6C}	9.7	155	416.5	523.6	556.1
c_6-like Cyt	8.0	199	417	523	553

[a]Data for cytochrome c_{6B} are from *Synechococcus* sp. WH 8102 (Zatwarnicki et al. 2014), for c_{6C} from *Synechococcus* sp. PCC 7002 (Bialek et al. 2008, 2014), and for a c_6-like (c_{6C}) cytochrome from *Nostoc* sp. PCC 7119 (Reyes-Sosa et al. 2011). The peak absorbance bands are from the reduced cytochromes

523 (β-band) and 553 nm (α-band), whereas the oxidized protein has peaks at 409 and 528 nm. The spectral features of the reduced forms of cytochrome c_{6B} from *Synechococcus* sp. WH 8102 and c_{6C} from *Synechococcus* 7002 are very similar to those of the *Nostoc* 7119 protein, with the exception of their α-bands, which are red-shifted (to 556 and 557 nm, respectively) relative to the *Nostoc* Cyt c_{6C} and the α-bands of typical c_6 cytochromes.

In contrast to their similar spectral properties, the c_6-like cytochromes have significantly lower midpoint potentials than their cytochrome c_6 counterparts (Table 34.1). Cytochromes c_6 from *Nostoc* 7119 and *Synechococcus* 7002 have midpoint potentials ($E_{m,7}$) of +337 mV (Molina-Heredia et al. 1998) and +319 mV (Bialek et al. 2008), respectively. Potentials in this range enable these proteins to efficiently transfer electrons from Cyt f in the b_6f complex (which has midpoint potentials in the +270 to +340 mV range in cyanobacteria (Ho and Krogmann 1980; Ponamarev et al. 2000; Baymann et al. 2001a) to PSI (with most estimates of cyanobacterial P700+/P700 midpoint potentials in the +420 to +450 mV range, Nakamura et al. (2005); Bailleul et al. (2008)). For detailed discussion of the protein-protein interactions and mechanism of Cyt c_6-mediated electron transfer from Cyt f to PSI, see Chap. 31 by Diaz-Moreno et al.

The low midpoint potentials of +113 to +199 mV for representative c_6-like cytochromes (Table 34.1) exclude the possibility that these proteins could readily oxidize cytochrome f. The same argument applies to chloroplast c_{6A} cytochromes that have midpoint potentials in the +100 to +140 mV range (Molina-Heredia et al. 2003; Marcaida et al. 2006). Nonetheless, it remains possible that under some conditions the c-like cytochromes may conduct electrons from Cyt f to PSI. Despite the unfavorable equilibrium for Cyt f oxidation by Cyt c_{6A} or Cyt $c_{6B,C}$, the overall transfer to PSI would be favorable. There are several examples of 'roller coaster' electron transfer pathways with unfavorable, 'uphill' intermediate steps. These include transfer from soluble Cyt c_2 (E_m +300 mV) to Cyt c_{554} (E_m −50 mV), the first heme in the tetraheme donor chain in reactions centers of purple photosynthetic bacteria such as *Rhodobacter viridis* and *Blastochloris viridis*. A second uphill transfer in the overall, downhill pathway from Cyt c_2 to the reaction center is at the step from Cyt c_{556} (E_m +320 mV) to Cyt c_{552} (E_m +30 mV) (see Baymann and Rappaport (1998) and Alric et al. (2006) for further discussion). Although electron flow from the Cyt b_6f complex to PSI via a 'roller coaster' pathway involving a $c_{6B,C,A}$ or c_M cytochrome remains theoretically possible, no evidence has been found to support this. In *Synechocystis* 6803 (Manna and Vermaas 1997) and *Arabidopsis* (Weigel et al. 2003) Cyt c_6 and PC double mutants, and in a *Synechococcus* 7002 PetJ1 Cyt c_6 mutant (Nomura and Bryant 1999), the respective c_M, c_{6A}, or c_{6C} cytochromes were unable to support phototrophic growth under the conditions tested.

Electron transfer from the *Arabidopsis* Cyt c_{6A} to PSI as well as cytochrome oxidase has

been demonstrated in vitro, although at significantly lower rates than from *Arabidopsis* PC or algal Cyt c_6 (Molina-Heredia et al. 2003). Moreover, a significant electron transfer rate has been shown in vitro from the *Nostoc* 7119 Cyt c_{6C} to PSI at rates only 3.8 and 2.4-fold lower, respectively, than for the native Cyt c_6 and PC carriers (Reyes-Sosa et al. 2011). In *Nostoc* 7119, Cyt c_{6C} has a basic isoelectric point or 8.0, which is close to those of the Cyt c_6 and PC proteins from this cyanobacterium. Reyes-Sosa et al. (2011) proposed that the c_{6C} cytochrome could serve as an alternative electron donor to PSI, perhaps under nitrogen-fixing conditions from a pathway that does not include the Cyt b_6f complex. Electron transfer to a cytochrome oxidase remains possible as well, and has been shown in vitro for Cyt c_M (Bernroitner et al. 2009). To our knowledge this has not been tested for the c_{6B} or c_{6C} cytochromes.

C. Structures and Heme Environment

Numerous Cyt c_6 structures have been deposited into databases (e.g. Frazao et al. 1995; Kerfeld et al. 1995; Yamada et al. 2000; Sawaya et al. 2001; Bialek et al. 2009). High-resolution structures of *Arabidopsis* c_{6A} have been solved by Marcaida et al. (2006) both with the heme oxidized (ferric state, at 1.2 Å) and reduced (ferrous state, 1.4 Å) and by Chida et al. (2006) with the heme oxidized. Oxidation or reduction of the heme had little impact on the protein structure. See Chap. 33 by Howe et al. for further discussion and comparison of the Cyt c_{6A} structure relative to Cyt c_6 from *Monoraphidium braunii*. Among related cytochromes of cyanobacteria, structures have been solved for Cyt c_{550} both in isolated form and in association with PSII (reviewed by Roncel et al. (2012)). The structure of a cyanobacterial Cyt c_{6B} from *Synechococcus* sp. WH 8102 has been determined at 1.4 Å (Zatwarnicki et al. (2014), PDB accession number 4KMG) and that of Cyt c_{6C} from *Synechococcus* 7002 at 1.03 Å (Bialek et al. (2014), 4EIE). Despite efforts toward this, no structures are yet available for Cyt c_M. Figure 34.2a, b show the overall structures of the cyanobacterial c_{6C} and c_{6B} cytochromes.
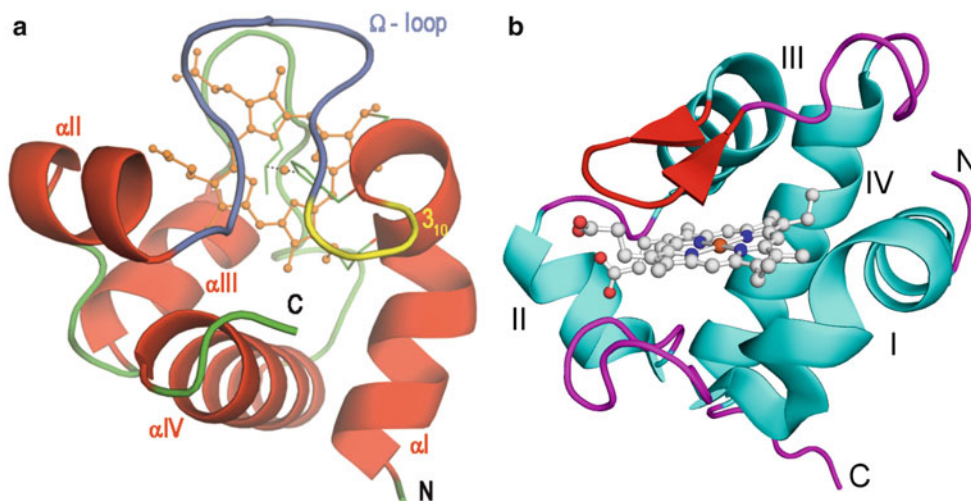
Fig. 34.2. Overall architecture of Cyt c_{6C} from *Synechococcus* sp PCC 7002 and Cyt c_{6B} from *Synechococcus* WH 8102. (**a**) Cyt c_{6C}. Secondary structures include α-helices (*red*), 3_{10}-helix (*yellow*) and several loops (*green*). The Ω-loop characteristic of cytochromes c_{6C} is indicated in *cyan*. The heme group is shown as *orange balls*-and-*sticks*. (**b**) Cyt c_{6B}. The heme group is represented by *balls* and *sticks*. Secondary structures include α-helices (*cyan*), loops (*magenta*), and a β-hairpin (*red*) (Figures **a** and **b** are reproduced from Bialek et al. (2014) and Zatwarnicki et al. (2014), respectively, with permission from the publishers).

The structures of classical c_6 cytochromes as well as the c_6-like cytochromes share an overall architecture typical of class I cytochromes (Bialek et al. 2014). Their tertiary structure is determined by a four α-helix bundle (with an additional 3_{10} helix following the first α-helix) wrapped around a single heme moiety. The first of these helices contains a characteristic kink at Cys14 belonging to the CXXCH heme-binding motif, which is involved in covalent attachment of the heme through a double, thioether linkage. The helices are separated by loops of varying lengths, with the characteristic, so-called Ω-loop located between helices I and II. The central heme iron is axially coordinated by the imidazole ring of a conserved histidine (His18, in Cyt c_{6C} of *Synechococcus* 7002) and the sulfur atom of a specific methionine side chain (Met58 in Cyt c_{6C} of Synechococcus 7002). Whereas the overall fold of Cyt c_{6C} is not different from Cyt c_6 proteins, the c_{6A} and c_{6B} proteins exhibit unique structural features. The c_{6A} proteins contain an additional, 12-residue, surface-exposed loop, designated the 'Loop Insertion Sequence' or 'LIP' (Howe et al. 2006). Because this loop contains two cysteines it was thought to have a role in redox-dependent regulation by oxidation of dithiol/disulfide groups via reduction of the LIP disulfide and electron transfer to the c_{6A} heme (Marcaida et al. 2006). However, a more recent study, in which the two cysteines were converted to serines, shows that the LIP disulfide has an important role in on-pathway folding and stability of the c_{6A} protein (Mason et al. 2012). Thus the postulated catalytic role of the LIP disulfide has fallen out of favor. In contrast to other known c_6 and c_6-like protein structures, Cyt c_{6B} from *Synechococcus* sp. WH 8102 contains a β-hairpin located in the loop between helices III and IV (Fig. 34.2b). The significance of this feature is unclear at this time.

Key biophysical differences in the c_6-like cytochromes relative to c_6 arise from the amino acids that surround the heme pocket and in surface charge distributions. In Cyt c_6, two separate surface domains have been distinguished. The so called 'north face' consists of primarily hydrophobic residues surrounding the heme crevice, whereas the 'east face' has charged amino acids (Molina-Heredia et al. 1999). Both are considered important for rapid electron transfer from Cyt f to Cyt c_6 and Cyt c_6 to PSI. According to current models, efficient electron transfer and turnover first requires long-range electrostatic attractions via the Cyt c_6 east face to establish initial, weak contacts. This is followed by hydrophobic interactions with the north face to bring donor-acceptor centers into proximity, followed by electron transfer and then rapid dissociation of the weakly bound encounter complex (Diaz-Moreno et al. 2014; Diaz-Moreno et al., Chap. 31). In cyanobacteria, cytochrome c_6 may be acidic (as in *Synechococcus* 7002) or basic (as in *Nostoc* 7119) and the interacting Cyt f and PSI surfaces typically carry complementary charges (Hervas et al. 2005). Notably, in contrast to the acidic pI and surface charge of Cyt c_6 from *Synechococcus* 7002, its Cyt c_{6C} has a basic pI and positively charged east face (Bialek et al. 2008, 2014). This further suggests that Cyt c_{6C} would not be an effective electron carrier from the Cyt b_6f complex to PSI or cytochrome oxidase and rather implies that it may interact with different partners or by a different mechanism. However, it has also been shown that PSI, at least from *Nostoc* 7119, can be relatively indiscriminate with respect to the surface charge of the Cyt c_6 donor (Hervas et al. 2005). Table 34.2 shows the calculated molecular weights and pI values of c_{6A}, c_{6B}, and c_{6C} cytochromes from a number of sources. As in *Synechococcus* 7002, the predicted pI values of c_{6C} cytochromes are basic, whereas those for the chloroplast c_{6A} cytochromes are slightly acidic. The cyanobacterial c_{6B} cytochromes cover a wide range of pI values from acidic to basic.

All cytochrome c_6 and c_6-like proteins carry a covalently bound heme. Thioether bonds between the heme and polypeptide chain are established between the vinyl groups of the porphyrin ring and sulfhydryl groups of conserved cysteines (Cys14 and

Table 34.2.   Calculated pI values and molecular weights of cytochrome c_6-like proteins[a].

Cyt c_{6A}			Cyt c_{6B}			Cyt c_{6C}		
Species	pI	MW (Da)	Species	pI	MW (Da)	Species	pI	MW (Da)
Glycine max	5.3	10,986.4	*S.* RCC307	4.9	8884.0	*S.* 7002	8.7	9400.5
Medicago	5.1	11,948.4	*S.* WH7803	5.5	9045.1	*A. marina*	8.0	9448.7
Solano	5.1	11,387.7	*S.* WH7805	5.5	9235.3	*A. maxima*	8.0	9718.9
Chlamydomonas	5.8	10,585.0	*P.* NAT1	5.6	9662.7	*Cr. watsonii*	9.6	9709.2
Arabidopsis	5.1	11,703.1	*S.* CC9311	5.7	9325.4	*Cr. watsonii*	9.7	9779.2
Triticum	5.2	11,452.8	*S.* RS9916	6.0	9004.1	*S.* 7942	8.7	9134.4
Aegilops	6.5	10,883.3	*S.* BL107	6.0	9129.3	*S.* 6301	8.7	9134.4
Hordeum	6.5	12,412.0	*S.* CC9902	6.1	9290.5	*C.* 51142	9.0	10,006.4
Oriza	5.7	11,173.7	*S.* CC9605	6.1	9608.8	*C.* CCY0110	9.2	10,065.5
			P. NAT2A	6.1	9691.8	*C.* 7424	8.0	10,123.4
			P. 9313	6.9	9491.6	*C.* 7425	8.1	9251.4
			P. 9303	6.9	9519.6	*C.* 7822	9.6	9631.0
			P. 9211	7.0	9889.3	*C.* 8801	8.0	9618.9
			S. WH8102	8.0	9223.4	*C.* 8802	8.0	9618.9
			P. 1375	8.0	9733.9	*A. variabilis*	9.0	9494.8
			Cy. 7001	8.1	9494.7	*Nostoc* 7120	8.1	9668.9
			S. RS9917	8.1	9400.5	*N. punctiforme*	8.7	9597.9
			S. WH5701	8.1	9323.6	*N. spumigena*	8.7	9515.7
			P. 9301	9.0	9505.9	*T. erythraeum*	9.6	9848.3
			P. 9312	9.0	9550.0	*Lyngbya* 8106	9.5	9647.9

[a]Isoelectric points and predicted molecular weights were calculated with ProtParam software (Gasteiger et al. 2005)

Cys17) in the canonical CXXCH heme-binding motif located within a hydrophobic pocket of the protein (Fig. 34.3a). The residue numbers are those of the *Synechococcus* 7002 proteins. A typical feature of these proteins as well as other *c*-type cytochromes is a slight, saddle-shape bend of this ligand and coordination of its central iron ion by a nitrogen from a histidine (His18) imidazole ring and a sulfur atom from a conserved methionine (Met58). Despite the differences in redox properties between the Cyt c_6 and c_6-like families, the distances from their central heme irons to their His-Met, axial coordination partners as well as their N-Fe-S angles remain virtually the same among the characterized c_6 and c_6-like proteins (Marcaida et al. 2006; Bialek et al. 2014; Zatwarnicki et al. 2014).

Key structural features that govern the midpoint potential and presumably the function of c_6-like cytochromes include the amino acids that occupy positions adjacent to the heme. The most important of these is a glutamine at position 50 (numbered according to the *Synechococcus* 7002 Cyt c_{6C}) that is conserved among c_6 cytochromes (Bialek et al. 2008). Its replacement by a hydrophobic residue such as valine, leucine or isoleucine in c_6-like cytochromes results in a significant decrease in the midpoint potential. The role of the amino acid at position 50 has been confirmed by directed mutagenesis. Re-introduction of glutamine at this site in cytochromes c_{6A} (Worrall et al. 2007) and c_{6C} (Bialek et al. 2014) led to a 50–100 mV increase in midpoint potential. Conversely, substitution of valine for Gln50 in the Cyt c_6 heme crevice decreased the E_m by   100 mV in these proteins from *Synechococcus* 7002 (Bialek et al. 2014) and *Phormidium laminosum*
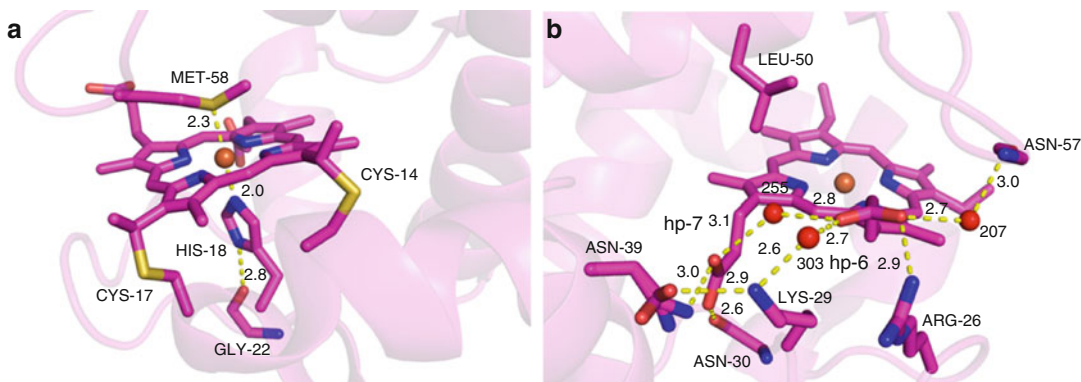
Fig. 34.3. Heme-binding pocket of Cyt c_{6C} from *Synechococcus* sp. PCC 7002. (**a**) Cyt c_{6C} heme pocket showing thioether linkages to Cys-14 and -17, and the His-18 and Met-58 axial ligands. The heme iron is depicted as an *orange sphere*. (**b**) Cyt c_{6C} heme environment showing three bound water molecules (*large red spheres*) and polar residues that participate in a hydrogen bonding network surrounding the heme. The heme iron is shown as an *orange sphere*. The H-bonding network does not extend above the heme plane as it does in Cyt c_6. The hydrophobic Leu-50 lies in proximity to the heme edge in a position occupied by the polar Gln-50 residue in Cyt c_6 proteins. *Dashed lines* in both panels represent hydrogen bonds and distances are given in Å.

(Rajagopal et al. 2011). The structures of the c_6 and c_6-like cytochromes indicate that in addition to the amino acid at position 50, an equally important determinant of redox properties may be the extent and distribution of structural water molecules and the hydrogen-bonding network around the heme group (Fig. 34.3b). Whereas all of these water molecules are located above the heme propionate-7 plane in cytochromes c_6, replacement of the conserved Gln50 in the heme cavity by a hydrophobic residue completely alters the pattern of the hydrogen bonding among amino acid side chains and structural water molecules around the heme (Bialek et al. 2014; Zatwarnicki et al. 2014). Thus the polar Gln residue in the heme pocket of Cyt c_6, as well as the H-bonding network around the heme, stabilize the reduced form of the heme resulting in the characteristically high midpoint potentials of c_6 cytochromes. In contrast, a more hydrophobic environment and diminished H-bonding network around the $c_{6A,B,C}$ hemes decreases the stability of an electron on the reduced heme resulting in a lower midpoint potential (Worrall et al. 2007; Rajagopal et al. 2011; Bialek et al. 2014; Zatwarnicki et al. 2014).

IV. Cytochrome c_M

A. Genome Analyses

The sequence alignments in Fig. 34.4 show the conserved heme-binding motif CXYCH (residues 16–20) near the predicted N-terminus of the mature Cyt c_M protein, where X is predominantly alanine and Y almost exclusively a glycine residue. Note, however, that it remains unclear whether the mature Cyt c_M protein is as shown in Fig. 34.4 or whether the N-terminal domain (not shown in this figure) is retained and functions as a membrane anchor. At least two studies (Cho et al. 2000; Bernroitner et al. 2009) support the latter hypothesis as discussed further below. The heme-binding motif is directly preceded by an asparagine in all of the aligned sequences (Fig. 34.4). His20 in the heme-binding motif provides the fifth ligand (an axial ligand) for the heme, but because of limited data, the sixth ligand (second axial ligand) of Cyt c_M remains unresolved. Based on sequence alignments, two methionine residues, Met56 and Met66 (as numbered in Fig. 34.4) are likely candidates. The former is a part of an absolutely conserved GXTPPMPXF motif,
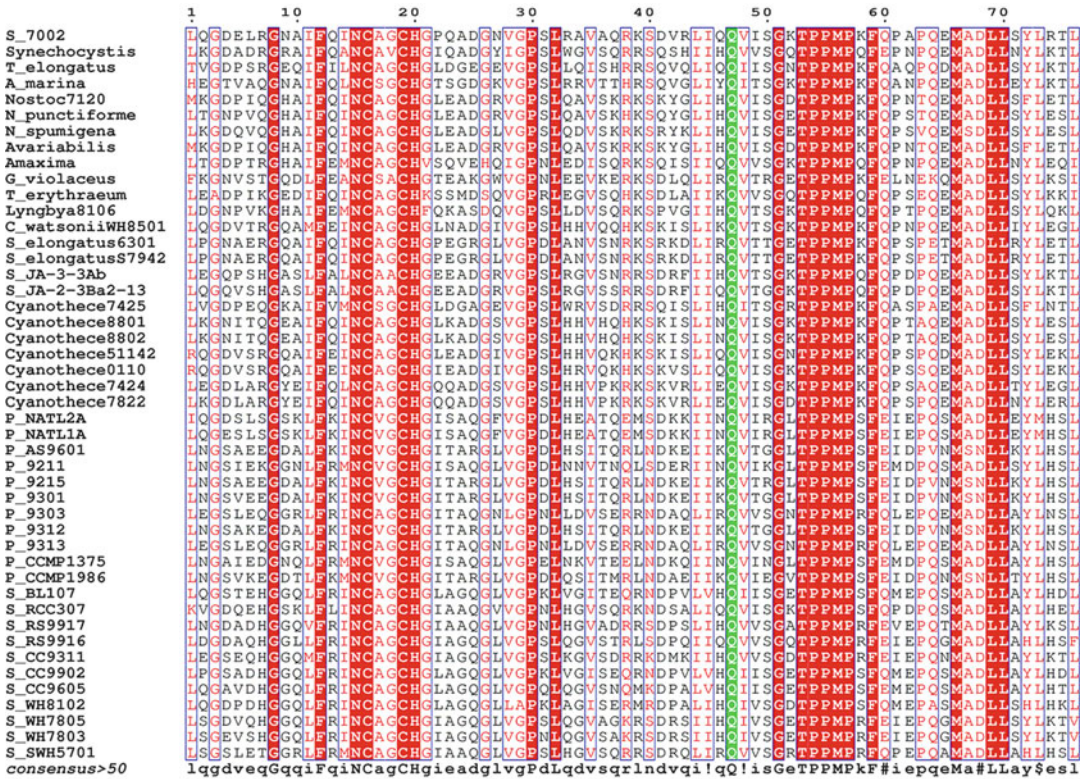
Fig. 34.4. Sequence alignments of the putative 'mature,' processed region of Cyt c_M proteins where the N-terminal targeting domain has been removed. It remains controversial whether this N-terminal domain is a targeting sequence or a membrane anchor. The glutamine (Q-47) residue in Cyt c_M that corresponds to an absolutely conserved glutamine in Cyt c_6 and a small hydrophobic residue (I, L or V) in Cyt c_{6B} and Cyt c_{6C} is highlighted in green. In the strain designations, *P* represents *Prochlorococcus* and *S* represents *Synechococcus* strains (Figure prepared using ESPript (Gouet et al. 1999)).

and in our opinion is most likely to act as a heme ligand as Met66 may be too close to the C-terminus. Homology modeling of Cyt c_M from *A. maxima* using horse-heart Cyt c as a template did not clarify which methionine acts as the sixth ligand (Ho et al. 2011). In contrast to cytochromes c_6, $c_{6A,B,C}$ and c_{550}, Cyt c_M has yet to be crystallized. Surprisingly, all attempts at crystallization from *Synechocystis* 6803 (Ho et al. 2011) and *Synechococcus* 7002 (Bialek, unpublished results) have failed.

Analysis of currently available cyanobacterial genomes reveals a single copy gene, *cytM*, encoding the Cyt c_M protein, which occurs in almost all cyanobacterial species, with the exception of one known strain (*Prochlorococcus marinus* MIT9515). The

cytM gene is more common than either the Cyt c_{6B} or c_{6C} genes. Cyt c_M seems to be evenly distributed among species harboring Cyt c_{6B} or c_{6C} sequences (Fig. 34.5). As in Cyt c_6, but in contrast to Cyt c_{6A}, c_{6B} and c_{6C}, all predicted Cyt c_M proteins harbor an absolutely conserved glutamine residue, Q47 (residue numbering as in Fig. 34.4). The amino acid at this position modulates redox potential, as shown for Cyt c_6, c_{6A} and c_{6C} (Worrall et al. 2007; Bialek et al. 2014). On the one hand, Cyt c_M seems more similar to Cyt c_6 because of the key, conserved glutamine residue at a homologous position. On the other hand, the measured redox midpoint potential ($E_{m,7}$) of Cyt c_M is only +151 mV (Cho et al. 2000), which is less than half the typical value for Cyt c_6,
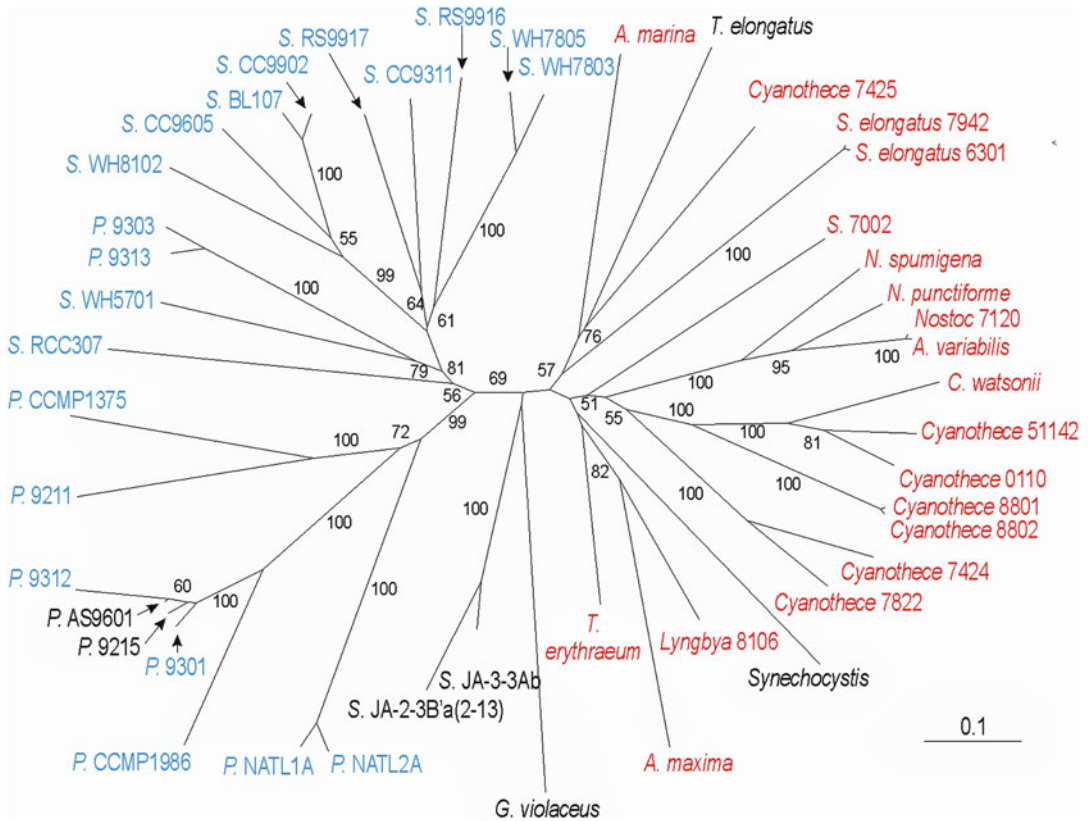
Fig. 34.5. Unrooted phylogenetic tree of Cyt c_M proteins. Cyanobacterial species harboring genes for Cyt c_{6B} or c_{6C} are shown in *blue* and *red*, respectively. Species harboring the Cyt c_M gene but lacking genes for c_{6B} or c_{6C} are shown in *black*. The *bar* represents evolutionary distance in substitutions per residue.

virtually the same as that of Cyt c_{6C} from *Synechococcus* 7002 (+148 mV, Bialek et al. (2008)), and similar to that of Cyt c_{6B} from *Synechococcus* sp. WH 8102 (+113 mV, Zatwarnicki et al. (2014)). In that regard, Cyt c_M appears to be more similar to the other Cyt c_6-like cytochromes in terms of its likely functional properties.

B. Biophysical Features

The nucleotide sequence of Cyt c_M from *Synechocystis* 6803 contains two putative transcription start sites. Given the location of a probable ribosome binding site (RBS), Malakhov et al. (1994) proposed that transcription results in a pre-protein of 11.4 kDa. Analysis of the Cyt c_M N-terminal region suggested that it could be either a

transit peptide for targeting to the thylakoid lumen, the periplasm, or else a membrane anchor. Because this region resembles transit peptides of cytochromes c_6, Molina-Heredia et al. (2002) concluded that it most likely functions as a genuine transit peptide, which, after cleavage, results in a mature protein of 8.3 kDa. The presence of the VLA motif, as in the 18 kDa protein of the PSII oxygen-evolving complex (OEC) from spinach (Malakhov et al. 1994; Seidler 1996; Molina-Heredia et al. 2002), supported the view that the Cyt c_M N-terminus functions in targeting to the lumen. However, it was not clear whether the hydrophobic N-terminal domain is processed *in vivo*. Immunoblotting with antibodies against recombinant Cyt c_M revealed a protein from *Synechocystis* 6803 that is larger than the presumptive, 'mature'

Cyt c_M expressed in *E. coli* (Cho et al. 2000). This suggested that the N-terminal domain functions as a membrane anchor. A subsequent immunoblot study supported this hypothesis. Bernroitner et al. (2009) detected Cyt c_M in both cytoplasmic and thylakoid membrane fractions but not cytosolic extracts, strongly suggesting that Cyt c_M is an integral membrane protein. Further confirmation is lacking but the apparent localization of Cyt c_M in cytoplasmic as well as thylakoid membranes raises intriguing questions about possible roles in specific electron transport chains.

Bernroitner et al. (2009) further showed that the Cyt c_M protein content increases under high salt stress, corroborating earlier findings showing that stresses such as low temperature and high light elevate Cyt c_M (*cytM*) transcripts (Malakhov et al. 1999; Shuvalov et al. 2001). Increased *cytM* transcript levels were subsequently also detected in *Synechocystis* 6803 grown under sulfur deprivation (Zhang et al. 2008). In depth 'RNA Seq,' transcriptomic studies of *Synechococcus* 7002 further revealed that low temperature, heat shock, oxidative stress, mixotrophic growth, high salt, and high light intensity increased, whereas low salt and phosphorus deprivation modestly decreased *cytM* (SynPCC7002_A0375) transcripts (Ludwig and Bryant 2011, 2012a, b). The same authors showed that CO_2, nitrogen, sulfur, and iron limitations also increased *cytM* transcripts. However, it should be noted that under standard growth conditions for *Synechococcus* 7002 (250 µmol photons m^{-2} s^{-1}, 5 % CO_2), the *petJ1* (SynPCC7002_A0167) transcript for Cyt c_6 represents 1 % of the total mRNA whereas the *cytM* transcript is less than 0.002 %, or >500-fold lower than that for *petJ1*. Under high salt, low nitrogen, and low sulfur, *cytM* transcripts increase by 12, 14, and 24-fold, respectively; whereas *petJ1* transcripts decline by 2–3-fold. Under these conditions, *cytM* transcripts comprise 0.025–0.05 % of the total mRNA, relative to *petJ1* at 0.35–0.6 %, but are still 10–20-fold lower than the *petJ1* mRNA. We are unaware of any quantitative study of Cyt c_M specific protein content and turnover relative to Cyt c_6. Assuming that the mRNA transcript levels reflect protein abundance, these data suggest that even under stress conditions, Cyt c_M may not be present in quantities sufficient to play a large role in a major, photosynthetic electron transport pathway. However, these gene expression profiles may be compatible with a regulatory role for Cyt c_M or one that alleviates over reduction of electron carriers under certain stress conditions and the damaging consequences of this.

V. Possible Functions of c_6-Like and c_M Cytochromes

The functions of c_6-like cytochromes remain obscure but interesting clues have begun to emerge. The most likely roles for Cyt c_{6C}, c_{6B}, and c_M cytochromes are: (1) in alternative electron transfer pathways to alleviate sudden over-reduction of electron carriers and generation of damaging oxygen radicals, (2) in the cytoplasmic membrane in electron transfer pathways that energize that membrane for transport processes, or (3) in regulation, for example of electron transfer, metabolic pathways, or gene expression.

With respect to stress responses and redox homeostasis, it is well established that photosynthetic and respiratory electron transfer chains in cyanobacteria intersect through the plastoquinone pool, Cyt $b_6 f$ complex, and soluble carriers (Cyt c_6 or PC) that conduct electrons to PSI or a terminal oxidase (see Kallas (2012); Mullineaux (2014) for reviews). The respiratory electron transfer pathways are much less active than photosynthetic pathways but may operate concurrently to maintain redox balance, mitigate photoinhibition, and minimize the formation of reactive oxygen species (ROS) (Helman et al. 2003, 2005). In addition, cyanobacteria have evolved a further suite of mechanisms to alleviate the sudden and dangerous over-reduction of electron carriers. These include photoprotective, non-photochemical quenching (qE) mediated by

the orange carotenoid protein (OCP), that when activated by blue-green light dissipates excitation energy as heat and thereby effectively decreases the phycobilisome antenna (reviewed by Kirilovsky and Kerfeld (2012)).

At the level of electron transfer, cyanobacteria have evolved a number of branched pathways to maintain redox balance and this is where the c_6-like cytochromes may have roles. Almost all cyanobacteria encode the Flv1/Flv3 flavodiiron proteins and a more limited number among the β-cyanobacteria have Flv2/Flv4 (reviewed by Allahverdiyeva et al. (2015)). Flv1/3 catalyze electron transfer from PSI to molecular oxygen in a harmless 'Mehler-like' reaction that does not generate the superoxide as in the classical Mehler reaction in chloroplasts (Mehler 1951). Interestingly, △*flv1/3* knockout mutants have no noticeable impact on growth under either continuous light or light–dark cycles with steady illumination phases. However, the △*flv1/3* mutation severely limits growth or even result in cell death in cultures grown under fluctuating light regimes such as those encountered in natural environments (Helman et al. 2003; Allahverdiyeva et al. 2011, 2013). Indeed it has now become evident that such fluctuating conditions with rapid shifts from very low to high light intensities are extremely stressful because they result in rapid over-reduction of electron carriers and production of damaging ROS (Lea-Smith et al. 2013). Thus the Flv1/3 flavodiiron proteins have been shown to provide crucial protection for PSI against acceptor side over-reduction and ROS production. In the cyanobacteria that have them, the Flv2/4 proteins protect PSII against damage from over excitation at high light intensities and low (ambient) CO_2 (Zhang et al. 2009, 2012; Bersanini et al. 2014). Part of this mechanism involves a not yet characterized electron transfer route that funnels electrons from the vicinity of the Q_B, acceptor site of PSII into an unknown electron transfer pathway. Might this pathway involve the c_{6B}, c_{6C}, or c_M cytochromes?

In *Synechocystis* 6803, *flv2/4* genes are upregulated in response to high light, oxidative stress, and particularly under CO_2 limitation (Allahverdiyeva et al. 2015). *Cyt* c_M transcripts are upregulated, at least in *Synechococcus* 7002, under the same conditions. Note, however, that *Synechococcus* 7002 does not have *flv2/4* genes and *Synechocystis* 6803 is one of the few cyanobacteria that does not carry genes for Cyt c_{6B} or c_{6C}. Thus a $Q_B > $ Flv2/4 route involving Cyt c_M to mitigate acceptor side (PQ pool) over-reduction in cyanobacteria such as *Synechococcus* 7002 remains possible, but at present no evidence exists to support this. Given the emergent appreciation of mechanisms that have evolved to alleviate the hazards of fluctuating light exposures, it may be interesting to test knockout mutant of the c_{6B}, c_{6C}, and c_M cytochromes under such conditions. To our knowledge, such experiments have not been conducted.

Other branched pathways and mechanisms that cyanobacteria may use to alleviate over-reduction and ROS production include photorespiration (Eisenhut et al. 2008; Allahverdiyeva et al. 2011), terminal oxidases (Lea-Smith et al. 2013), quinol (Cyd) oxidases (Berry et al. (2002); see also Chap. 19, Young et al.), alternative respiratory terminal (ARTO or Cta-II) oxidases (Howitt and Vermaas 1998; Nomura et al. 2006b), 'plastid' terminal oxidases (PTOX) (Bailey et al. 2008), and nitrate reductases (Flores et al. 2005). All cyanobacteria appear to have at least one terminal oxidase and may have different combinations or none of the *bd*-type quinol, ARTO, and PTOX oxidases that either take electrons from the PQ pool or from water soluble carriers (see Chap. 17 by Schmetterer). The c_6-like cytochromes may function in some of these pathways, perhaps again as safety-valves at specific electron transfer steps to help relieve over-reduction induced by sudden upswings in light intensity. PSI or terminal oxidases could be available as electron acceptors under some of these conditions. Electron transfer has been demonstrated at reasonable rates from Cyt c_{6C} to PSI (Reyes-Sosa et al.

2011) and from Cyt c_M to the CtaI, aa_3 cytochrome oxidase (Bernroitner et al. 2009) and might be performed as well, at least in principle, by any of the c_6-like cytochromes. An intriguing, possible, 'overflow' pathway may be one from PQH_2 in the PQ pool to PSI or a terminal oxidase via the Rieske iron-sulfur protein (ISP) and one of the c_6-like cytochromes. This may confer an important selective advantage because PQH_2 oxidation by the Cyt b_6f complex via the Rieske ISP and Cyt f is the usual rate-limiting step in electron transport (Kallas (2012) and references therein). The high-resolution, 2.0 Å structure of the PetC Rieske ISP of *Thermosynechococcus elongatus* reveals a deep hydrophobic pocket facing the membrane in the soluble 2Fe-2S cluster-bearing domain of the protein (Veit et al. 2012). This pocket appears to be conserved in PetC1 Rieske ISPs. As suggested by Veit et al. (2012) and Bernat et al. (Chap. 13), this pocket might serve as the binding site for a lipid, plastoquinone, chlorophyll, or carotenoid molecule, or perhaps the hydrophobic domain of a protein such as plastocyanin. A cytochrome c_{6B}, c_{6C}, or c_M might similarly either bind here or accept electrons from plastoquinol bound to this site to provide an electron pathway that circumvents Cyt f en route to PSI or a terminal oxidase. This could provide an alternative, 'safety-valve' route to alleviate PQ pool over-reduction. Hydrophobic contacts are important in forming encounter complexes between both PC and Cyt c_6 with Cyt f (see Chap. 30, Bendall and Howe, and Chap. 31, Diaz-Moreno et al.) and would likely be important as well in interactions involving the c_6-like cytochromes. Some experimental data suggest an electron pathway through the Cyt b_6f complex that circumvents Cyt f (Fernandez-Velasco et al. 2001), but to our knowledge this has not been corroborated by further studies.

A possible problem with roles for the c_{6B}, c_{6C}, or c_M cytochromes in 'safety-valve' electron flow pathways is that available transcription data, as discussed above, suggests a relatively low abundance of these proteins. For example, in *Synechococcus* 7002 grown under standard photoautotrophic conditions, the *petJ1* transcript for Cyt c_6 represents 1 % of total transcript counts (normalized per kb); whereas the Cyt c_{6C} and c_M counts under these conditions are 0.01 % and 0.002 % of the total, respectively (Ludwig and Bryant 2011, 2012a, b). Cyt c_{6C} transcripts increase up to 3–4-fold under stresses such as high salt or sulfur deprivation. Cyt c_M transcripts increase more dramatically up to 12–24-fold under stresses such as high salt or nitrogen and sulfur deprivation, where they may represent up to 0.05 % of total mRNA counts (Ludwig and Bryant 2011, 2012a, b). By comparison, the *flv1* (_A1321) transcript for Flv1, with an established role in alleviating PSI acceptor-side over-reduction (Allahverdiyeva et al. 2013), represents 0.015 % of total transcripts under standard conditions in *Synechococcus* 7002. *Flv1* transcripts increase 19-fold up to 0.3 % of the total under limiting CO_2 but only 2–3-fold up to 0.02 % to 0.04 % of the total under high salt or limiting nitrogen or sulfur. The *flv1* transcript level is similar to that of *petJ2* for Cyt c_{6C} under standard conditions but 30-times higher under low CO_2. However, under high salt or limiting N or S, the *flv1* transcript is similar within a factor or twofold or even lower that the transcript levels for *petJ2* or *cytM* (for Cyt cM) (Ludwig and Bryant 2011, 2012a, b). This does not answer the question of whether the Cyt c_{6C}, c_{6B}, or c_M proteins may be present in sufficient quantity for roles in 'overflow' electron pathways, but does suggest that they could be significant under certain stress conditions.

Immunological data show that Cyt c_M is localized to both thylakoid and cytoplasmic membranes of *Synechocystis* sp. PCC 6803 (Bernroitner et al. 2009). The same authors showed that Cyt c_M transfers electrons to the Cu_A center of cytochrome oxidase in vitro as efficiently as Cyt c_6. These findings raise interesting possibilities for participation of Cyt c_M (and possibly c_{6B} or c_{6C}) in a cytoplasmic membrane electron pathway, possibly leading to a terminal oxidase. However, at this stage, it is not known what that pathway might be. Indeed the composition of cyanobacterial

cytoplasmic membranes remains unresolved but important differences exist among ecotypes. According to the model of Schultze et al. (2009), the cytoplasmic membrane of *Synechocystis* 6803 carries an electron transfer chain comprised of succinate (SDH) and NDH-2 NADH dehydrogenases, quinol oxidase (Cyd), a CtaII (or ARTO) terminal oxidase, and an ATP synthase. The dehydrogenases and oxidases are linked via a plastoquinone pool and a pH gradient across the membrane is established either by the quinone reduction-oxidation reactions or the ATP synthase operating in reverse. A similar model is presented by Lea-Smith et al. (2013) and supported by proteomics data (Huang et al. 2002; Agarwal et al. 2010). The thylakoid membrane of PCC 6803 also contains the Cyd quinol oxidase in addition to the CtaI cytochrome oxidase, succinate dehydrogenase, and NDH-I, NDH-2 NAD(P)H dehydrogenases. The importance of the CtaI (Howitt and Vermaas 1998) and Cyd (Berry et al. 2002) oxidases for redox balance is well established, and essential under some conditions as illustrated by the lethality of a CtaI mutant of *Synechocystis* sp. PCC 6803 grown at low light intensity (Kufryk and Vermaas 2006). Lea-Smith et al. (2013) have now demonstrated convincingly that one or the other of these oxidases is essential for survival under fluctuating light conditions. *Synechococcus* 7002 possesses only the CtaI and CtaII (ARTO) terminal oxidases and CtaII has been shown to have an important role in regulation of oxidative stress responses (Nomura et al. 2006a, b). See also Chap. 17 by Schmetterer for further discussion.

With respect to cytoplasmic membranes and c_6-like cytochromes, the immunological analysis of *Synechocystis* 6803 by Schultze et al. (2009) shows that these membranes exclusively contain the PetC3 Rieske iron-sulfur protein but not the PetC1 or PetC2 ISPs or other subunits of the Cyt b_6f complex. The role of the PetC3 ISP in the cytoplasmic membrane is not resolved but its midpoint potential of +135 mV

(Schneider et al. 2002) may be compatible with electron transfer to a Cyt c_{6C} or c_M with midpoint potentials of +150 to 200 mV or perhaps with reverse flow from some of these cytochromes or c_{6B} which has a lower potential (Table 34.1). In Chap. 13, Bernat et al. show that the PetC3 ISP shares similarity with Rieske-type subunits of periplasmic, membrane-bound arsenite oxidases (Lebrun et al. 2003) and propose that PetC3 may be a component of such an enzyme. Co-transcribed genes for sulfide:quinone oxidoreductase (*suoS*) and a putative arsenite importer (*suoT*) or arsenite:quinone oxidoreductase are extensively upregulated in the presence of sulfide or arsenite (Nagy et al. 2014). The involvement, if any, of c_{6C}, c_{6B}, or c_M cytochromes in such pathways is not clear although their midpoint potentials would make them suitable as electron acceptors for arsenite ($E_{m,7}$ +60 mV, van Lis et al. (2013)) or plastoquinol ($E_{m,7}$ + 80 mV, Schuurmans et al. (2014)). The terminal electron acceptor for a cytoplasmic membrane, c_6-like cytochrome is also unclear because these membranes may be limited to CtaII (ARTO) oxidases that accept electrons from quinol, thus apparently excluding electron transfer from Cyt c_{6C}, c_{6B}, or c_M.

With respect to possible roles in regulation, Hiraide et al. (2015) uncovered an interesting phenotype for Cyt c_M relative to dark, heterotrophic growth. They show that Cyt c_M mutants exhibit faster dark, heterotrophic growth on glucose than the wild type *Leptolyngbya boryana* and enable genuine, dark heterotrophic growth (in contrast to 'light activated heterotrophic growth'—LAHG, Anderson and McIntosh (1991)) in *Synechocystis* 6803. They suggest that this curious phenotype may reflect an adaptation that spares respiratory, carbohydrate reserves under extended stress conditions that induce Cyt c_M expression. The basis for this may be related to the finding of Manna and Vermaas (1997) of sequence similarity between Cyt c_M and Cyt *c*-binding domains of CtaI, aa_3-type cytochrome oxidases and their proposal that Cyt c_M is subunit of this oxidase in cyanobacteria. Cyt c_M associated with the

CtaI oxidase may exert a suppressive effect as suggested by Hiraide et al. (2015). Because the CtaI oxidase is apparently not located in cytoplasmic membranes of typical cyanobacteria and because only a small fraction of known cyanobacteria can grow heterotrophically in darkness, Cyt c_M must have functions that extend beyond the modulation of heterotrophic growth.

Several heme-binding proteins, including c-type cytochromes, have known or postulated regulatory roles. The heme iron interacts with molecules such as O_2, CO, and nitric oxide (NO) and is thus well adapted as a sensor of such molecules as well as redox changes. For recent reviews of heme proteins in regulation, see Green et al. (2014) for a review of transcriptional regulation of bacterial virulence in response to O_2 and NO, and Arora et al. (2015) for NO regulation of bacterial biofilms. Several regulators of tetrapyrrole synthesis such as HbrL and PpsR/CrtJ are heme-binding proteins. See Yin and Bauer (2013) and Chap. 25 by Zappa and Bauer for reviews. C-type cytochrome regulators include Cyt c_{550} of *Bacillus subtilis*, which has a midpoint potential of $+190$ mV (David et al. 1999) and a role in initiation of sporulation based on knockout and overexpression studies (Shin et al. 2005). Oxygen binding to the heme of the FixL sensor-kinase modulates nitrogen-fixation gene expression (Bauer et al. 1999). Other bacterial cytochrome sensors include the *Clostridium botulinum* b-heme SONO (Nioche et al. 2004) and the *Rhodobacter capsulatus* (Cross et al. 2001) and *Alcaligenes xylosoxidans* (Kruglik et al. 2007) periplasmic, pentacoordinate c' cytochromes for sensing and/or reductive detoxification of nitric oxide. NO production also occurs in nondenitrifying bacteria and some cyanobacteria possess quinol-nitric oxide reductases as a line of defense (Busch et al. 2002). Based on such precedents, roles for cyanobacterial c_6-like cytochromes in sensing-signaling pathways are conceivable but entirely speculative at this time.

VI. Concluding Remarks

Cytochromes c_{6C}, c_{6B}, or c_M are small, c_6-like cytochrome proteins of cyanobacteria. Despite the widespread distribution of genes for these cytochromes among cyanobacterial lineages, their functions remain largely unknown. Cytochrome c_M was the first of these to be discovered (Malakhov et al. 1994). Genes for cytochromes c_{6C} and c_{6B} were discovered from phylogenetic analysis (Bialek et al. 2008) and so named in relation to the previous, unexpected finding of genes for a c_6-like cytochrome, designated c_{6A}, in plants and some eukaryotic algae. Cytochrome c_M is found in almost all known cyanobacteria and cytochromes c_{6C} or c_{6B} are found in most cyanobacteria. One exception is the widely studied *Synechocystis* 6803, which carries a gene for cytochrome c_M but not for c_{6C} and c_{6B}. Representative c_M, c_{6C}, and c_{6B} cytochromes have midpoint potentials in the $+110$ to $+190$ mV range, too low to effectively substitute for cytochromes c_6 or plastocyanin in electron transfer from the cytochrome b_6f complex to photosystem I (Cho et al. 2000; Bialek et al. 2008; Zatwarnicki et al. 2014). Immunological data show that cytochrome c_M is located in both thylakoid and cytoplasmic membranes of *Synechocystis* 6803 (Bernroitner et al. 2009). The localization of cytochromes c_{6C} and c_{6B} is unknown. The *petJ2* gene for cytochrome c_{6C} is transcribed at consistently low levels under most conditions tested in *Synechococcus* 7002 but increases up to 4-fold under certain stresses. Transcripts for cytochrome c_M in the same strain are still lower under standard conditions but increase as much as 24-fold, up to 0.05 % of total mRNA counts, under stresses such as high salt or nitrogen or sulfur limitation (Ludwig and Bryant 2012a, b). These findings suggest possible roles for these c_6-like cytochromes in overflow electron transfer pathways to alleviate over-reduction of electron carriers and the production of reactive oxygen species. Heme groups are well adapted for interactions with ligands such as molecular oxy-

gen, nitric oxide, or with electrons, and thus the c_6-like cytochromes may well serve in yet to be defined regulatory or signal transduction roles. A recently discovered regulatory role for cytochrome c_M in *Synechocystis* 6803 is in suppression of dark heterotrophic growth, possibly through interaction with the CtaI, aa_3 cytochrome oxidase (Hiraide et al. 2015). Most likely, these widespread c_6-like cytochromes of cyanobacteria are not simply evolutionary relics and further research will reveal additional functions.

Acknowledgements

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