Chapter 21

Electron Transport in the Mitochondrial Respiratory Chain

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Summary

The metabolic capacity of the eukaryotic cell to convert free energy contained in nutrients into ATP is a process accomplished by a multi-step system: the mitochondrial respiratory chain. This chain involves a series of electron-transferring enzymes and redox co-factors, whose biochemical characterization is the collective result of more than 50 years of scientists' endeavors. The current knowledge describes in detail the structure and function of the individual proton-translocating "core" complexes of the respiratory chain (Complex I, III, IV). However, a holistic approach to the study of electrons transport from NAD-dependent substrates to oxygen has recently directed our attention to the existence of specific albeit dynamic interactions between the respiratory complexes. In this context, the respiratory complexes are envisaged to be either in form of highly ordered assemblies (i.e. supercomplexes) or as individual enzymes randomly distributed in the mitochondrial membrane. Either model of organization has functional consequences, which can be discussed in terms of the structural stability of the protein complexes and the kinetic efficiency of inter-complex electron transfer. Available experimental evidence suggests that Complex I and Complex III behave as assembled supercomplexes (ubiquinone-channeling) or as individual enzymes (ubiquinone-pool), depending on the lipid environment of the membrane. On the contrary, a

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strict association of Complexes III and Complex IV is not required for electron transfer via cytochrome *c*, although there are supercomplexes in bovine heart mitochondria, known as the respirasomes, that also include some molecules of Complex IV. Our recent experimental results demonstrate that the disruption of the supercomplex I_1–III_2 enhances the propensity of Complex I to generate the superoxide anion; we propose that any primary source of oxidative stress in mitochondria may perpetuate generation of reactive oxygen species by a vicious cycle involving supercomplex dissociation as a major determinant.

I. Introduction

The recent findings in the study of electron transfer and energy transformation in mitochondria are the logical consequence, as we observe in many other sciences, of a continuous development of knowledge, through more than 50 years. In this view, the reader may appreciate an historical introduction that emphasizes some retrospective landmark studies on the respiratory chain prior to a description of more recent scientific endeavors leading to advances in understanding of mitochondrial respiration.

Following this line of thought, we will first point out that it was recognized early on that the metabolic capacity of the cell to convert free energy from nutrients into ATP is a process accomplished by a multi-step system involving molecular oxygen. Researchers soon realized the very complex nature of respiration and fundamental differences of this process from fermentation and glycolysis, which were also investigated at the time.

In the first edition of his textbook about bioenergetics, Albert L. Lehninger (1965) describes the molecular basis of energy-conversion in mitochondria by schematically drawing a flow chart (Fig. 21.1 top) that consists of "a series of cytochromes, which are electron-transferring enzyme molecules containing deeply colored active groups… comprised of porphyrin and iron". In this scheme, electrons can enter via one of two flavoproteins (FP) that accepts electrons either from reduced nicotinamide adenine dinucleotide (now conventionally referred to as NADH, formerly known by the name "reduced diphosphopyridine nucleotide" and abbreviated as DPNH), or from succinate.

The electron donors are arranged in a thermodynamic series of decreasing "electron pressure" where only the last cytochrome, called the "respiratory enzyme" or cytochrome oxidase, gives up its electron directly to molecular oxygen. Four such electron transfers are required to form water (Chance and Williams 1956). Since it was known that the components of the respiratory chain are located in mitochondria in simple ratios to each other (Criddle et al. 1962; Green and Wharton 1963) and cytochromes can carry only one electron at a time whereas FP can carry two at a time, the flow of electrons down the chain was written out allowing each cytochrome to react twice in a series of five sequential oxidation-reduction reactions connected by a common substrate intermediate. However, Lehninger's chart (Lehninger 1965) did not show an important detail of the respiratory chain that a number of investigators had already suggested (Racker 1965): electrons donated from NAD- and FAD-dependent dehydrogenases funnel into a common acceptor, ubiquinone (Morton 1958; Crane et al. 1957), which in turn feeds into the cytochrome system (Lester and Fleischer 1961; Hatefi et al. 1962b; Green and Tzagoloff 1966; Lenaz et al. 1968).

Abbreviations: BN-PAGE – Blue native polyacrylamide gel electrophoresis; $C_{I–IV}$ – Metabolic flux control coefficient of the corresponding respiratory complex; CoQ – Coenzyme Q ubiquinone; EPR – Electron paramagnetic resonance; ETF – Electron transfer flavoprotein; FP – Flavoprotein; $O_2^{-}\cdot$ – Superoxide anion; OXPHOS – Oxidative phosphorylation system; PL – Phospholipids; ROS – Reactive oxygen species; SDS – Sodium dodecyl sulfate

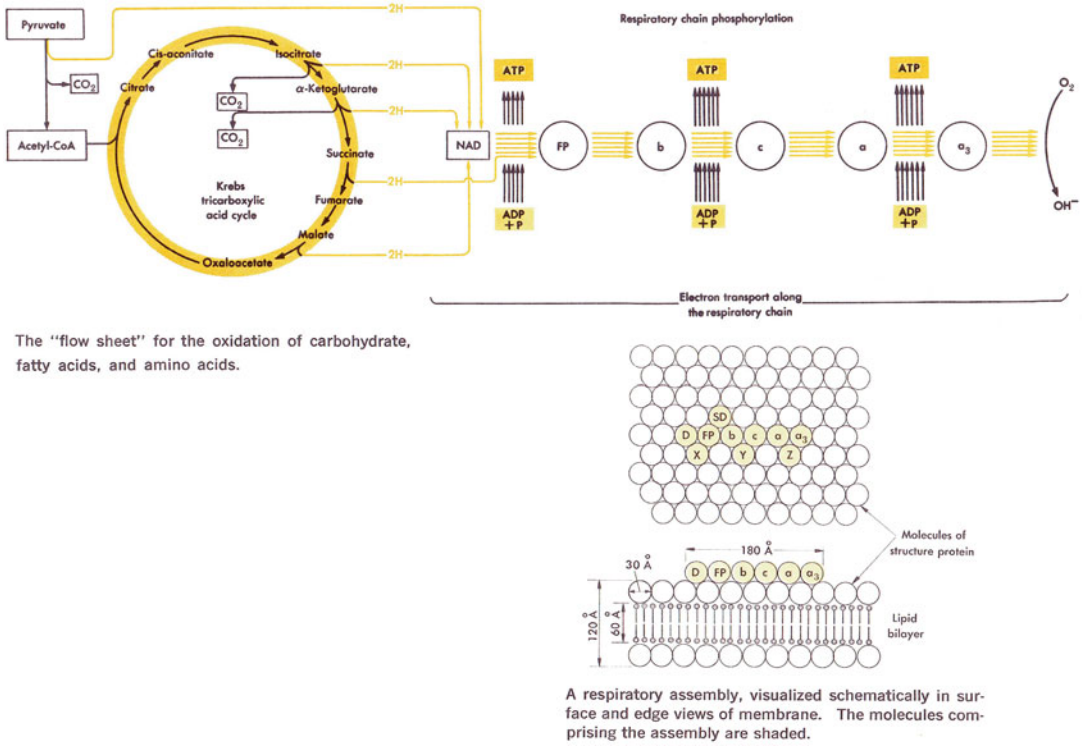

Fig. 21.1. Scheme of the respiratory chain as originally drawn by Lehninger (1965). (*Top*) Flow chart of electron transfer; (*Bottom*) Respiratory assembly. *D* reduced diphosphopyridine nucleotide, *FP* flavoprotein, *SD* succinate dehydrogenase, *b, c, a, a_3* designate the cytochromes, *X, Y, Z* designate hypothetical high-energy intermediates (see text for details).

It is worth noting that those early investigators presented the electron carriers of the respiratory chain as geometrically fixed assemblies, located next to each other in the exact sequence in which they interact and intimately connected with the lipid components of the mitochondrial membrane (see also Sect. III). These assignments were derived from available staining methods using electron microscopy, which demonstrated the existence of clusters of cytochromes and flavoproteins studded in the inner membrane at regular intervals about 200 Å apart (Fig. 21.1 bottom). These studies also revealed for the first time that the high molecular weight enzymes responsible for the formation of ATP project from the inner surface of the inner mitochondrial membrane (Fernandez-Moran 1963).

In Lehninger's scheme, three segments of the chain contribute a relative large amount of free energy that was suggested to serve for donating high-energy phosphate groups to ATP generation (Chance and Williams 1955). Although the sequence of the cytochromes in the respiratory chain was known in principle, Lehninger (1965) had stated that "many important details remain to be established with more certainty" for a clear description of the bioenergetic role of respiration. Indeed, a lot of effort in the following years was spent in the vain search of the hypothesized high-energy intermediate compounds that could convey energy and phosphate to the ADP molecule (chemical coupling hypothesis).

By the time that the second edition of Lehninger's textbook was published (Lehninger 1971), interesting progress was made in understanding the molecular organization of the respiratory chain. The kinetic properties of the respiratory protein complexes

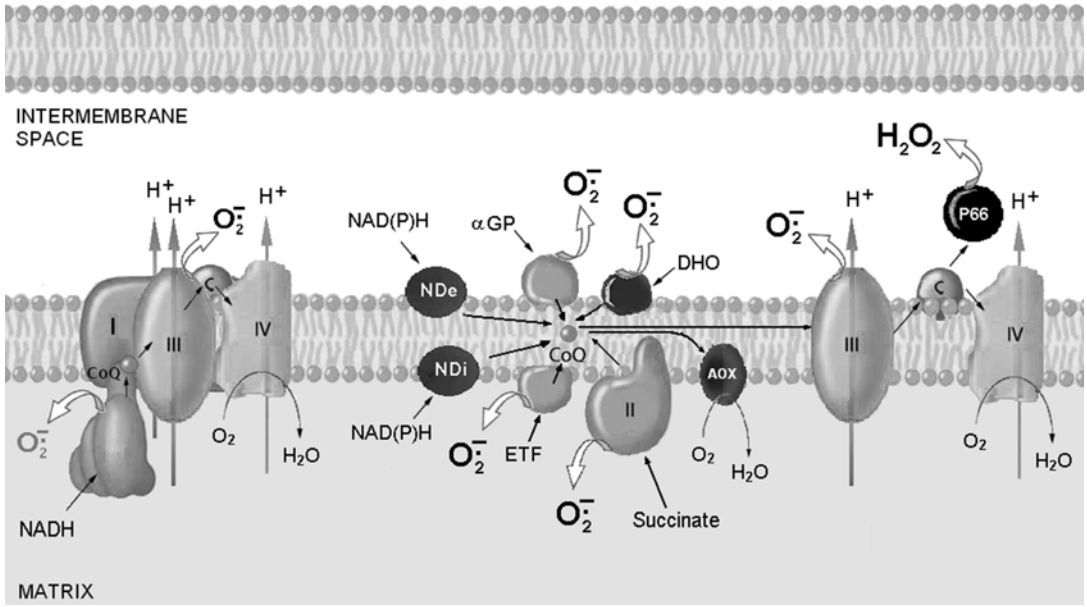

Fig. 21.2. A schematic drawing of the respiratory chain depicting the protein complexes and their substrates in relation to the inner mitochondrial membrane. Complex I is depicted as a component of the $I_1III_2IV_1$ supercomplex; whereas Complex III and Complex IV are also shown in their free form. The *white arrows* represent the major sites of ROS generation. *I* NADH-ubiquinone oxidoreductase, *II* succinate-ubiquinone oxidoreductase, *III* ubiquinol-cytochrome c oxidoreductase, *IV* cytochrome oxidase, *ND_i and ND_e* internal and external alternative NAD(P)H dehydrogenases, *AOX* alternative oxidase, *αGP* glycerol-3-phosphate, *ETF* electron transfer flavoprotein, *DHO* dihydroorotate, *CoQ* Coenzyme Q ubiquinone, *C* cytochrome *c*, *P66* cytochrome p_{66} (see text for details).

were unraveled by systematic studies in purified segments of the respiratory chain (Griffiths and Wharton 1961; Hatefi et al. 1962a; Baum et al. 1967). However, despite years of hard work, the exact mechanism, by which ATP is generated during electron transport, was still debated. It became clearer and clearer, however, that the intactness of the inner membrane is an essential element in the process of oxidative phosphorylation of ADP. Furthermore, it was emphasized that H^+ ions may be pumped out of the mitochondrial matrix during electron transport (Mitchell and Moyle 1967). Those observations suggested possible mechanisms for the conversion of redox energy into phosphate-bond energy (Hinkle et al. 1972; Ragan and Hinkle 1975; Leung and Hinkle 1975) other than the previous 'chemical coupling model': alternative thinking culminated in the 'chemiosmotic hypothesis' proposed by Peter Mitchell (1975) and the demonstration that the proton gradient produced across the inner mitochondrial membrane concomitantly with the electron transport along the respiratory chain is the immediate driving force for ATP formation.

Since then, the characteristics of the respiratory chain from a wide variety of sources have been enriched by growing evidence describing in detail the structure and function of the proton-translocating "core" complexes (Complex I, III, IV). Several auxiliary enzymes that reduce ubiquinone (Coenzyme Q, CoQ), bypassing Complex I, or that deliver electrons directly from reduced ubiquinone (ubiquinol) to oxygen bypassing Complex III and IV were also described (Fig. 21.2). All those auxiliary enzymes are characterized by the lack of energy-conserving mechanisms of proton translocation (see Chap. 9) Lenaz and Genova 2010 for a review).

The present chapter will discuss available evidence concerning the supramolecular organization of the respiratory enzymes that are envisaged to be either in the form of highly ordered assemblies (i.e. supercomplexes), or as individual enzyme units that are randomly distributed in the mitochondrial membrane. We will characterize the functional consequences of inter-complex electron transfer in both of these models of organization.

II. Electron Transport and Proton Translocation in Mitochondrial Membrane Systems

A. Redox Centers

Several redox centers that are associated with enzyme complexes allow electron transfer in the respiratory chain that follows a gradient in midpoint potential (E_m). The electrons donated by NADH at $E_m = -320$ mV are accepted by FMN in Complex I and then transferred to a series of iron-sulphur clusters (Fe-S centers) (Ohnishi et al. 1998). NADH-ubiquinone oxidoreductases from different sources have different numbers of Fe-S centers, most of which share the same midpoint potential (Dutton et al. 1998). Recently, the arrangement of the prosthetic groups in the hydrophilic domain of Complex I from *Thermus thermophilus* has been determined by x-ray crystallography, showing a linear chain of seven conserved clusters (i.e. N3, N1b, N4, N5, N6a, N6b, N2), whereas two additional clusters do not participate in the main pathway and have been proposed to represent an evolutionary remnant (cluster N7) and a possible anti-oxidant center (cluster N1a) (Hinchliffe and Sazanov 2005; Sazanov and Hinchliffe 2006). N2, which is a Fe_4-S_4-type center, has the most positive E_m (between −50 and −150 mV) and is considered to be the direct electron donor to ubiquinone, being most likely located along the interface between the peripheral and the membrane arm of Complex I where the ubiquinone head group could be located, as suggested by Brandt et al. (2003). The mechanism of ubiquinone reduction by Complex I is particularly intriguing, because more than one bound quinone species has been assigned to the enzyme (Magnitsky et al. 2002); a tentative two-step scheme was drawn by Fato et al. (2009) where a Fe-S cluster located upstream of N2 may act as a switch for electron delivery.

Most certainly, ubiquinone molecules bound to Complex I coexist with a mobile pool of molecules in the inner mitochondrial membrane (ubiquinone-pool). This pool is of crucial importance for various ubiquinone-reducing dehydrogenases to connect with Complex III (see also Sect. III). As schematically depicted in Fig. 21.2, among those dehydrogenases is Complex II, which conveys electrons to ubiquinone through one covalently linked FAD, three Fe-S centers acting in sequence, and a single *b*-type heme with a very positive E_m (Yankovskaya et al. 2003; Horsefield et al. 2006). Moreover, some rotenone-insensitive NADH dehydrogenases (Yagi et al. 2001) and other auxiliary enzymes involved in different metabolic pathways (i.e. glycerol-3-phosphate dehydrogenase, ETF-ubiquinone oxidoreductase, choline dehydrogenase, dihydroorotate dehydrogenase, malate dehydrogenase) can donate electrons through their prosthetic groups into the ubiquinone-pool at an E_m around zero. Electrons move from ubiquinol via the Fe-S center and to cytochrome c_1 and involvement of *b*-cytochromes in Complex III (Covian and Trumpower 2008; Cramer et al. 2011; and Chap. 8) to cytochrome *c* ($E_m = +230$ mV).

Electrons transported by cytochrome *c* are donated to Complex IV, where electrons transfered to oxygen via the Cu_A center (which acts as a single-electron receptor), heme a, heme a_3/Cu_B center, and heme a3 (cf. Belevich and Verkhovsky 2008 for extensive review). However, in some mitochondrial systems, like plant mitochondria, alternative oxidases (see Chap. 9) are able to reduce molecular oxygen by directly accepting electrons from ubiquinol (Krab 1995).

B. Protonic Coupling

Electron transport along the respiratory chain is directly coupled to the translocation of protons from the mitochondrial matrix (H^+_{in}) to the intermembrane space. This H^+-pumping is operated by Complex I, III and IV. The change in redox energy mediated by the three sites is far from equal, with the smallest at Complex III (250 mV), the next larger at Complex I (360 mV) and the largest at Complex IV (470 mV).

The coupling of oxidation-reduction reactions to the deprotonation-protonation of ubiquinol/ubiquinone within the cytochrome bc_1 complex is central to the mechanism of proton translocation at site-2, as described in the ubiquinone-cycle model proposed by Mitchell (1975) and in subsequent discussion of his model (for a review, see Cramer et al. 2011). Different energy coupling mechanisms have been proposed for the other two sites. In particular, the x-ray crystallographic structure of bovine cytochrome *c* oxidase shows possible pathways extending across the enzyme where cooperative linkage between the heme a/Cu_A redox center and nearby acid/base protolytic residues is envisaged to result in the uptake and vectorial translocation of protons from the matrix surface (N space) toward the cytosolic surface (P space) in association with the oxygen reduction chemistry at the binuclear center (heme a_3/Cu_B) (Brzezinski and Gennis 2008; Papa et al. 2006a; Belevich et al. 2007). Based on the emerging structures of the bacterial (Efremov et al. 2010) and mitochondrial (Hunte et al. 2010) Complex I, a hypothetical mechanism for its redox-driven proton pumping proposed that two conformational strokes, generated during turnover by the stabilization of semiquinone/quinol molecules in the peripheral arm of the enzyme, can drive two membrane integral pump modules connected by a long helical "transmission element". The overall proposed pumping cycle is reminiscent to the function of a coupling rod of a stem engine (Efremov and Sazanov 2011). However, the actual mechanism how redox chemistry and proton pumping are coupled remains elusive. Very recent insights into the molecular architecture of Complex I from *Thermus thermophilus* (Baradaran et al. 2013) provide a complete picture of all the components of this giant proton pump and suggest that a long chain of charged residues, which pass through the middle of the membrane integral P-module of the enzyme, connects the ubiquinone-binding pocket to four putative pump sites consisting of separate proton-input and -output channels (Brandt 2013). This corroborates an earlier proposal that energy transmission within Complex I may occur through electrostatic coupling (Euro et al. 2008). Future functional testing of the predictions in the models proposed by Brandt and coworkers and by Efremov and coworkers will be of critical importance.

Protons from the intermembrane space (H^+_{out}) are mainly utilized to drive the synthesis of ATP molecules from ADP and inorganic phosphate by the F_0F_1-ATP synthase of the mitochondrial inner membrane. Proton transport stoichiometries have had a turbulent history, but $H^+_{in}/2e^-$ ratios of 4; 2 and 4, respectively for the three proton-translocating complexes, and values of $H^+_{out}/ATP = 4.3$ (i.e. including energy consumption for nucleotide- and Pi-transporters) are generally accepted today. As a consequence, the overall P/O ratio of oxidative phosphorylation is about 2.5 when NADH-linked substrates are oxidized through Complex I whereas the electron donation from the flavin dehydrogenases, which reduce ubiquinone without pumping protons result in P/O values close to 1.5 (Hinkle 2005).

The controlled dissipation of the protonmotive force of the H^+ gradient through reverse proton transloaction mediated by the uncoupling proteins of the inner mitochondrial membrane (Ricquier 2005), and slips in the proton pumping of respiratory complexes (Kadenbach 2003; Papa et al. 2006b) can contribute to preventing excessive and harmful metabolic production of reactive oxygen species (ROS) under conditions of high reducing power (see Sect. IV).

III. Overall Organization of Classic and Alternative Redox Complexes

A. Working in the Chain Gang or Free?

The supramolecular organization of the respiratory chain was a major research subject in the 1970s through the 1980s, culminating in the 'random collision model', of electron transfer (Hackenbrock et al. 1986) accepted by the majority of the investigators at the time. Evidence in favor of the random collision model derived from direct observation that: (i) the integral proteins of the inner mitochondrial membrane are randomly distributed in the bilayer (Höchli and Hackenbrock 1976; Sowers and Hackenbrock 1981) and phospholipid dilution of the respiratory proteins slows electron transfer (Schneider et al. 1982); (ii) according to the analysis of Kröger and Klingenberg (1973), both ubiquinone and cytochrome *c* behave kinetically as homogeneous pools, which follow saturation kinetics.

On the other hand, circumstantial evidence for the existence of highly aggregated protein assemblies in the native membrane was already present in early studies on the isolation of the respiratory enzymes. Studies that obtained purified enzyme fractions comprising both Complex I and Complex III (Hatefi et al. 1961) or Complex II plus Complex III (Yu and Yu 1980) are indicative of higher level aggregation. We believe that the investigators who first discovered the mitochondrial respiratory complexes already had the key for demonstrating the supercomplex organization in their hands, but were unaware of it. In addition, a close inspection of the fractured faces in the freeze-fracture electron micrographs (originally taken as a demonstration of long-range random distribution of the mitochondrial particles (Höchli and Hackenbrock 1976)) reveals the apposition of small clusters, and also hints at a possible association of the integral membrane proteins.

A clear demonstration of such protein associations came during the past decade from investigations that reported multi-complex units in yeast and mammalian mitochondria. A distinct structure was observed for all these supercomplexes, supporting the idea of highly ordered associations of respiratory supercomplexes and discarding most doubts that the postulated structures arose through artificial interactions. In particular, polyacrylamide gel electrophoresis under non-denaturating conditions (BN-PAGE (see Chap. 12) in digitonin-solubilized mitochondria of *Saccharomyces cerevisiae*, which possesses no Complex I, revealed two bands with apparent masses of ~750 and 1,000 kDa containing the subunits of complexes III and IV. Similar interactions of supercomplexes were investigated in bovine heart mitochondria: Complex I–III interactions were apparent from the presence of Complex I in the form of the I_1III_2 supercomplex that was also found further assembled into larger supercomplexes (respirasomes) comprising different copy numbers of Complex IV ($I_1III_2IV_{1-4}$). Only 14–16 % of total Complex I was found in free form in the presence of digitonin (Schägger and Pfeiffer 2001). Therefore it seems likely that all Complex I is bound to Complex III in physiological conditions (i.e. in the absence of detergents).

Current literature indicates an average ratio of oxidative phosphorylation complexes I:III:IV equal to 1.1:3:6.7 (Lenaz and Genova 2010). Therefore it appears plausible that approximately one-third of total Complex III in bovine mitochondria is not bound to monomeric Complex I. The fraction of Complex IV in free form represents >85 % of total cytochrome oxidase in mitochondria. Strong evidence has been accumulating that complexes I, III, and IV are organized as stoichiometric supercomplexes also in plants and in fungi (Eubel et al. 2004; Krause et al. 2004a, b; Marques et al. 2007). Interestingly, in contrast to the situation in mammals, complexes III and IV are not essential for assembly/stability of Complex I in the ascomycote fungus *Podospora anserina*. The function of the arrangement of the respiratory complexes in *Podospora anserina* is rather an open question, since electrons can be released

from dimeric Complex I to the so-called alternative oxidase, while Complex III is mostly kinetically inactive in this fungus (Krause et al. 2006; Maas et al. 2009).

It is worth noting that, in the prevailing opinion, Complex II acts as an independent enzyme with no specific association with other OXPHOS complexes, although the work of Acín-Pérez et al. (2008) showed Complex II-containing supercomplexes in mouse liver mitochondria. Moreover, neither the alternative NAD(P)H dehydrogenases nor the extra terminal oxidases were found associated with the major respiratory complexes (Sunderhaus et al. 2010), except in *Yarrowia lipolytica* (Ascomycote fungus) where the dehydrogenase NDH2e is reported to form part of a large supercomplex (Guerrero-Castillo et al. 2009).

A precise docking of the existing atomic x-ray structures of individual OXPHOS complexes into the cryo-electron microscopy map of supercomplexes from a variety of sources has resulted in pseudo-atomic models of their three-dimensional structure (Schäfer et al. 2007; Dudkina et al. 2011; Althoff et al. 2011; Heinemeyer et al. 2007; Mileykovskaya et al. 2012). It has been also proposed (Bultema et al. 2009; Strauss et al. 2008) that the OXPHOS complexes may assemble into even higher types of organization, forming row-like megacomplexes composed by supercomplexes as building blocks, with important implication for the morphology of the inner mitochondrial membrane. The characterization of the supercomplexes by biochemical functional analysis is incomplete, so a functional role of the supramolecular assemblies cannot be assigned with certainty.

Most evidence for a structural coordination of OXPHOS complexes has been obtained by indirect observations indicating deviations from "pool behavior" of electron transfer reactions involving ubiquinone, and from studies directly aimed to demonstrate substrate channeling by metabolic flux control analysis of the electron transfer in mitochondrial membranes, whereas the study of the kinetic properties of isolated supercomplexes is still in its infancy. In fact, the first functional demonstration of the existence of supercomplexes was provided by kinetic analysis of the pool function of ubiquinone and cytochrome *c* in mitochondria from *Saccharomyces cerevisiae* (Boumans et al. 1998). The finding that those mitochondria did not follow pool behavior unless treated with chaotropic agents was considered a peculiarity of this organism, because pool behavior was still widely accepted at that time. Later on, our studies by Metabolic Control Analysis (Bianchi et al. 2004) indicated the existence of functional supercomplexes in bovine heart submitochondrial particles. A strong kinetic evidence of a functionally relevant association between Complex I and Complex III is provided by the high metabolic control of both complexes over NADH oxidation (control coefficients equal 1.06 and 0.99, respectively), whereas Complex IV appears to be randomly distributed, as indicated by its low control coefficient ($C_{IV}=0.26$). Moreover, Complex II is fully rate-limiting for succinate oxidation ($C_{II}=0.88$, $C_{III}=0.34$, $C_{IV}=0.20$), clearly indicating the absence of substrate channeling toward Complex III and Complex IV. In permeabilised mitochondria from freshly harvested potato tubers, analogous inhibitor titration experiments indicate that Complexes III and IV are both involved in the formation of a supercomplex assembly that also comprises Complex I, whereas the alternative dehydrogenases, as well as the molecules of Complex II, act as independent structures within the inner mitochondrial membrane (Genova et al. 2008).

B. Solid-State or Fluid-State Model: Kinetic Advantages

Today, a solid-state model of the respiratory chain, based on specific, albeit dynamic interactions between individual respiratory components, is well consolidated (Wittig and Schägger 2009; Lenaz and Genova 2007, 2009a, b, 2010), although its possible relation with a random diffusion model of electron transfer is not completely clarified.

Acin-Perez and coworkers (2008) propose a "Plasticity model" where both types of organization are possible and functional, depending on different mitochondrial systems and on particular physiological states. The dynamic character of the supercomplexes is compatible with the factors affecting the association of their subunit components: some factors may act in a short or medium time scale, such as the membrane potential (Piccoli et al. 2006; Dalmonte et al. 2009) or protein phosphorylation/dephosphorylation (Rosca et al. 2011), whereas other factors may be operative on longer time scales, such as changes in phospholipid composition (for a review, see Lenaz and Genova 2012). However, a recent study (Muster et al. 2010) suggests that supercomplex dissociation, at least in a random fashion, may not be a fast event.

Supercomplex association confers several new properties to the respiratory chain with respect to the non-associated respiratory complexes. From a kinetic point of view, the most obvious is substrate channeling, specifically addressing ubiquinone and cytochrome *c* to interact directly with the partner enzymes, thereby replacing the random diffusion characteristics expected for non-associated components. In that case, inter-complex electron transfer becomes indistinguishable 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 (Hackenbrock et al. 1986), would rather be buried at the interface between two consecutive complexes. Intra-protein electron transfer is typically limited by tunneling through the insulating protein medium between the interacting redox centers. This electron tunneling is reasonably well described by a simple exponential decay depending on distance, so that the maximal distances that allow for physiological electron transfer should not exceed 13–14 Å (Moser et al. 2005). Inter-protein electron tunneling obeys the same exponential rate dependence on distance as intra-protein electron transfer; however, in addition small-scale constrained diffusive motions are sometimes necessary to bring redox centers within the 14 Å tunneling limit.

Ideally, in order to describe the mechanism of electron transfer within supercomplexes formed by apposition of individual respiratory complexes connected by potentially mobile cofactors, we should have a detailed knowledge of the molecular structure of the interacting sites. The recent work by Althoff and colleagues (2011) demonstrates that only few points of direct contact are allowed between the three complexes in the mammalian supercomplex $I_1III_2IV_1$, because the average distances exceed 2 nm. Moreover, the same authors indicate that at 19 Å resolution the membrane-embedded part of the supercomplex shows intermediate values of density between that of soluble protein and the hydrophobic membrane interior, thus suggesting that the supercomplex is held together at least partly by lipid–protein interactions. Likely, in Althoff's model, a gap of about 13 nm between Complex I and the proximal Complex III monomer is filled with membrane lipid that would facilitate restricted diffusion (microdiffusion) of ubiquinol between the ubiquinone-binding sites of the two enzyme partners.

Data obtained by us in proteoliposomes enriched with mitochondrial Complex I and Complex III in the presence of ubiquinone 10 (Genova et al. 2008) demonstrate that the respiratory complexes behave as individual enzymes (ubiquinone-pool behavior) or as assembled supercomplexes, depending on the distance induced between the intramembrane particles by phospholipid dilution. In particular, pool behavior is not effective in proteoliposomes at 1:1 protein:lipid ratio, where Complex I and Complex III are within a short range (<50 nm) resembling the mean nearest neighbor distance between respiratory complexes in mitochondria. In this experimental condition, NADH-cytochrome *c* reductase activity is indeed higher than predicted by the pool equation (Table 21.1) and BN-PAGE analysis confirms that Complex I and Complex III are mostly

Table 21.1. Experimental and calculated values of NADH-cytochrome *c* reductase in a mitochondrial protein fraction diluted with different levels of phospholipids.

Protein/PL[a] (w:w)	Distance[b] (nm)	NADH-cytochrome *c* (μmol/min/mg protein)	V_{obs} (calculated)[c]
1:1	18	0.472±0.091 (7)	0.210
1:30	97	0.273±0.028 (4)	0.236

Values in brackets indicate multiple experiments

[a]A protein fraction enriched in Complex I and Complex III from bovine heart mitochondria was fused with phospholipids (PL) and Coenzyme Q_{10} by cholate dilution (Lenaz et al. 1999)

[b]The theoretical distances between Complex I and Complex III were calculated according to Hackenbrock et al. (1986)

[c]NADH-cytochrome *c* reductase activity as calculated from the pool equation of Kröger and Klingenberg (1973) using experimental values of NADH-CoQ reductase and ubiquinol-cytochrome *c* reductase activity as Vred and Vox, respectively (Data reprinted from Genova et al., Copyright (2008), with permission from Elsevier)

assembled in the form of supercomplex I_1III_2 (Lenaz et al. 2010).

Therefore, the function of the mobile pool of ubiquinone molecules that most certainly exist in the inner mitochondrial membrane has to be reconsidered in view of the presence of supercomplexes. As discussed in detail by Lenaz and colleagues (Lenaz 2001; Lenaz and Genova 2009a), although electron transfer between Complex I and Complex III may not be best described by the pool equation of Kröger and Klingenberg (1973) and it is likely that substrate channeling of ubiquinone takes place, the pool equation still seems to represent the best description of the mechanism of electron transfer from Complex II and other flavin-linked dehydrogenases to Complex III (or from any dehydrogenase, including Complex I, to the alternative oxidase when present). In addition, the ubiquinone pool participates in a reverse electron transfer from Complex II to Complex I (energy-dependent reduction of NAD by succinate).

Moreover, the concept of a ubiquinone pool remains crucial in evaluating ubiquinone channeling from Complex I to Complex III. In fact, we may conclude that this pool is in equilibrium with protein-bound ubiquinone molecules. In addition the ubiquinone molecules that facilitate electron transfer within the supercomplex I–III may well be in dissociation equilibrium with the ubiquinone pool. Therefore, at steady state, the amount of the supercomplex-associated quinone would be dependent on the size of the pool. This proposition conforms with the above-mentioned experimental observations of the kinetic behavior in the presence of respiratory supercomplexes, provided that the dissociation rate constants of bound CoQ is considerably slower than the rate constants of inter-complex electron transfer via the same bound quinone molecules; however, our hypothesis should be explored by further experimental analysis.

Concerning cytochrome *c*, the model of the supercomplex $I_1III_2IV_1$ by Althoff and colleagues (2011) suggests that a shallow cavity lined by negative charges on the exterior membrane surface near the Cu atoms in Complex IV directly faces the similarly shaped binding sites of cytochrome *c* on Complex III. This arrangement would make it possible for the small, globular, partly positive cytochrome *c* molecules to pass efficiently from one complex to the other along a trajectory of approximately 11 nm. Indeed, residual amounts of cytochrome *c* remaining bound throughout the purification procedure of this supercomplex suggest at least partial occupancy of specific binding sites in the supercomplex (Althoff et al. 2011). This is in accordance with some observations from our laboratory also showing the presence of bound cytochrome *c* by two dimensional BN/SDS-PAGE analysis and Western blot immuno-detection in digitonin-solubilised respirasomes from potato tuber mitochondria (Lenaz et al. 2010). However, despite the presence of some molecules of Complex IV in the respirasome

of bovine heart mitochondria, the kinetic evaluation of NADH oxidase activity by Metabolic Control Analysis (Bianchi et al. 2004) suggests that cytochrome *c* obeys pool behavior. We hypothesize that such behavior may be due to rapid exchange of bound cytochrome *c* and freely diffusing-molecules of cytochrome *c*.

The presence of a large excess of free cytochrome oxidase, together with the loss of cytochrome *c* bound to supercomplexes, as assessed by 2D BN/SDS-PAGE in isolated bovine submitochondrial particles (Lenaz et al. 2010), are in line with our hypothesis. This strongly implies that a strict association of Complexes III and IV is not required for efficient electron transfer by cytochrome *c*.

Very recently, Trouillard et al. (2011) have developed another kinetic approach to address the issue of the functional relevance of the supercomplex III–IV in intact cells of *Saccharomyces cerevisiae*; they also conclude that cytochrome *c* is neither trapped within supercomplexes nor does it encounter particular restriction to diffusion, and that oxidation of cytochrome *c* by a given cytochrome oxidase is a random process.

Likewise, we cannot exclude that the presence of Complex IV in the supercomplex assembly may provide kinetic advantages other than substrate channeling of cytochrome *c*, by modifying the conformation of the partner complexes in order to enhance their catalytic activity, as suggested by the study of Schäfer et al. (2006). This study indicated higher Complex I and Complex III activities in the bovine supercomplex comprising cytochrome oxidase ($I_1III_2IV_1$) compared to the supercomplex devoid of the terminal oxidase (I_1III_2).

IV. By-Products of Aerobiosis: Generation of Reactive Oxygen Species by the Respiratory Chain

Generation of ROS (see also Chap. 24) within mitochondria is closely associated to the primary function of these organelles; namely the supply of electrons to the enzymes of the respiratory chain in order to drive the vectorial transfer of protons into the mitochondrial intermembrane space. This process inadvertently results in leaking of electrons from reduced cofactors onto molecular oxygen and hence ROS generation under normal circumstances. Murphy (2009) has carefully analyzed the thermodynamics of the production of the superoxide anion ($O_2^{-}\cdot$) in mitochondria. Oxygen can theoretically undergo one-electron reduction to generate superoxide at every enzymes of the respiratory chain (see Fig. 21.2). Indeed, Complex I and Complex III have been identified as robust sources of superoxide anion radicals, whereas cytochrome oxidase, which catalyzes the tetravalent reduction of dioxygen without the release of partially reduced molecular species, represents an exception (Wikström 2012).

In Complex III, $O_2^{-}\cdot$ is thought to arise from the reaction of oxygen with a component located at the Q_o site, the quinone binding site on the outer or cytosolic face of the protein (Boveris and Cadenas 1975; Turrens et al. 1985; Muller et al. 2002). Conditions that favor the formation of a semiquinone in the Q_o site should yield the highest rates of superoxide production. Consequently, one might suppose that a fully reduced ubiquinone pool in the presence of antimycin A, an inhibitor that blocks the normal egress of electrons from the system through Q_i site (van den Berg et al. 1979), would lead to the production of superoxide. Remarkably, however, conditions of substrate limitation that allow a partial oxidation rather than a full reduction of the ubiquinone pool (e.g. when oxidation of the substrate succinate is experimentally restricted by the presence of the competitive inhibitor malonate) result in significantly higher rates of superoxide production by Complex III. Mechanistic studies (Dröse and Brandt 2008; Quinlan et al. 2011) of the cytochrome bc_1 complex have clarified that the electron is transferred onto oxygen in a reverse reaction from reduced cytochrome b_{566} via ubiquinone, rather than during the forward ubiquinone-cycle. It was also observed that the production of $O_2^{-}\cdot$ in the antimycin-inhibited enzyme can be suppressed by the protonmotive force generated by the

hydrolytic activity of the F_1F_0-ATPase. In this condition a more equal distribution of electrons between cytochrome b_{566} (on the cytosolic side of the mitochondrial inner membrane) and cytochrome b_{562} (on the matrix side) is achieved. It is noteworthy that the controlled state of respiration (state 4) might ensure a relatively reduced ubiquinone-pool and limit the possibility of electron backflow from cytochrome b_{566} to ubiquinone and the generation of the semiquinone. The observation that the $O_2^{-}\cdot$ production by Complex III is negligible in the absence of respiratory chain inhibitors has lead some authors to argue that this phenomenon is likely to be of limited physiological relevance (Adam-Vizi and Chinopoulos 2006). However, it is worth to note that the modulation of the ROS production by modulation of the redox state of the ubiquinone-pool may contribute significantly to the control of mitochondrial redox signaling.

By contrast, the production of $O_2^{-}\cdot$ by Complex I is certainly more relevant from a metabolic point of view (Lenaz 2012), although the precise mechanism that promotes the one-electron reduction of oxygen at Complex I still requires further clarification. The identification of the oxygen-reducing site has been the subject of extensive investigation and several prosthetic groups in the enzyme (FMN, ubisemiquinone, and iron sulphur cluster N2) have been suggested to be the electron donors to oxygen (Lenaz et al. 2006; Fato et al. 2009). In isolated Complex I, fully reduced FMN is considered the major electron donor to oxygen (Galkin and Brandt 2005; Kussmaul and Hirst 2006; Esterházy et al. 2008). However, the effect of specific inhibitors acting downstream of the iron sulfur clusters in Complex I (Fato et al. 2009) suggests the possibility of a second site for oxygen reduction in the enzyme. In fact, EPR studies in bovine heart submitochondrial particles in the presence of NADH (Fato et al. 2009) show that rotenone but not stigmatellin enhances ROS production and quenches the semiquinone radical signal, whereas the FeS cluster N2 is kept reduced in presence of rotenone but is oxidized in presence of stigmatellin. Overall these results are compatible with the idea that N2 can be the source of enhanced production of $O_2^{-}\cdot$ in submitochondrial particles. Ohnishi et al. (2010) presented a new hypothesis that the generation of superoxide in a purified preparation of Complex I reflects a dynamic balance between the flavosemiquinone and the ubiquinone semiquinone. Superoxide generation from the flavosemiquinone would be increased, if electron transfer was inhibited under pathological conditions. The identification of the ubiquinone semiquinone rather than N2 as the electron donor to oxygen is, however, in contrast with the findings reported above, that no superoxide is produced in the presence of stigmatellin (Fato et al. 2009). Moreover, studies in ubiquinone-depleted and reconstituted mitochondria indicate that endogenous ubiquinone is not required for superoxide generation (Genova et al. 2001). The site of oxygen reduction is even less well defined in reverse electron transfer when $O_2^{-}\cdot$ production by Complex I is maximal (Murphy 2009).

The hypothesis that the tight organization of Complex I into supramolecular structures may hide autooxidable prosthetic groups, thereby preventing the reaction with oxygen, suggests that a dynamic assembly of the respiratory supercomplexes can also provide a regulation mechanism for $O_2^{-}\cdot$ production (Fig. 21.3). Very recently, we have directly addressed this issue and produced experimental evidence under conditions in which Complex I is either arranged as a component of the supercomplex I_1III_2 or dissociated as an individual enzyme (Maranzana et al. 2013). Our results show that disruption of the supercomplex I_1III_2 strongly enhances the ability of Complex I to generate ROS both in reconstituted proteoliposomes and in bovine heart mitochondrial membranes. It is easy to predict the implications of these findings in human diseases and in aging, where oxidative stress plays a major etiologic and pathogenic role. We propose that oxidative stress in mitochondria, generated by any primary source, may perpetuate ROS generation by a vicious

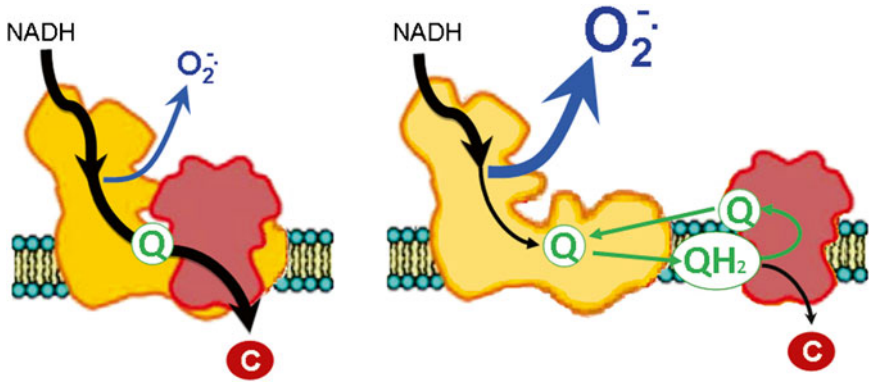

Fig. 21.3. Scheme of electron flux in two models of integrated NADH-cytochrome *c* reductase activity in the respiratory chain. (*Left*) In a supercomplex I_1III_2, any step in the obligatory pathway is regarded as a component of a single enzyme unit where the overall transfer of electrons to the final acceptor is optimized by channeling of the intermediate substrates. Protein-protein interactions modulate the reactivity of the intra-complex redox centers with dioxygen thereby preventing excessive ROS generation from Complex I (*yellow*). (*Right*) In a linear pathway composed of individual Complex I (*yellow*) and Complex III (*red*), a result of supercomplex disaggregation, the overall electron transfer to cytochrome *c* is mediated by the pool behavior of ubiquinone. The efficiency of electron transport is modified by the absence of protein-protein interactions, and $O_2^{\cdot-}$ production by Complex I is strongly enhanced.

cycle involving supercomplex dissociation as a major determinant.

Finally, it is worth mentioning that mitochondria from different tissues may vary conspicuously in their capacity to produce ROS depending on the utilized substrates (Kwong and Sohal 1998), and depending on the animal species and age. As a matter of fact, in addition to the major sites of superoxide formation in the respiratory chain, further sites in the inner mitochondrial membrane may have importance and physiological relevance (see Fig. 21.2). For example, a significant proportion of ROS may be released from several other ubiquinone-reducing enzymes (e.g. Complex II, glycerol-3-phosphate dehydrogenase, dihydroorotate dehydrogenase, ETF-dehydrogenase), whose contribution is still poorly characterized (see Lenaz and Genova 2010; Lenaz 2012, for extended review).

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