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Reduction of Chemically Stable Multibonds: Nitrogenase-Like Biosynthesis of Tetrapyrroles

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Abstract

The sophisticated biochemistry of nitrogenase plays a fundamental role for the biosynthesis of tetrapyrrole molecules, acting as key components of photosynthesis and methanogenesis. Three nitrogenase-like metalloenzymes have been characterized to date. Synthesis of chlorophylls and bacteriochlorophylls involves the reduction of the C17-C18 double bond of the conjugated ring system of protochlorophyllide which is catalyzed by the multi-subunit enzyme dark operative protochlorophyllide oxidoreductase (DPOR). Subsequently, biosynthesis of all bacteriochlorophylls requires the reduction of the C7-C8 double bond by a second nitrogenase-like enzyme termed chlorophyllide oxidoreductase (COR). Mechanistically, DPOR and COR make use of a reductase component which links ATP hydrolysis to conformational changes. This dynamic switch protein is triggering the transient association between the reductase and the core catalytic protein complex, thereby facilitating the transduction of electrons via two [4Fe4S] clusters. X-ray crystallographic structural investigations in combination with biochemical experiments revealed the molecular basis of the underlying energy transduction mechanism. The unique nickel-containing tetrapyrrole cofactor F_{430} is located in the active site of methyl-coenzyme M reductase, which is catalyzing the final step of methane formation in methanogenic archaea. The nitrogenase-like protein NflH/NflD has been proposed to catalyze one or more ring reduction steps during the biosynthesis of F_{430}. The present working hypothesis mirrors a DPOR and COR related enzyme mechanism of NflH/NflD. Furthermore, *nfl*-encoded

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proteins were suggested as "simplified" ancestors lying basal in the phylogenetic tree between nitrogenase and DPOR/COR.

Keywords
Dark operative protochlorophyllide oxidoreductase • Chlorophyllide oxidoreductase • Nitrogenase-like enzyme • Chlorophyll biosynthesis • Cofactor F_{430} biosynthesis

Abbreviations

DPOR	dark operative protochlorophyllide oxidoreductase
COR	chlorophyllide oxidoreductase
$(NifH)_2$	iron protein
Molybdenum-iron protein	MoFe protein or $(NifD/NifK)_2$
MCR	methyl-coenzyme M reductase

1 Introduction

The complex metalloenzyme nitrogenase is responsible for biological dinitrogen fixation, a process playing a central role in the global biogeochemical nitrogen cycle. This enzymatic conversion requires considerable energy input for the addition of electrons and protons to yield two molecules of ammonia (Fig. 1a). For more than 50 years, intense studies have been focusing on the understanding of the underlying enzyme mechanism, which allows for the sophisticated cleavage of the dinitrogen triple bond under ambient conditions. This is of special relevance, since the fueling of the related industrial dinitrogen fixing process (Haber-Bosch process at ~450 °C and >200 atm pressure) accounts for the consumption of approximately 1.4 % of the global energy demand (Lancaster et al. 2011).

The extensively characterized nitrogenase from *Azotobacter vinelandii* is composed of two subcomplexes, the iron protein $(NifH)_2$ and the molybdenum-iron protein (MoFe protein). Homodimeric $(NifH)_2$ is bridged by a single intersubunit [4Fe4S] cluster and contains one ATP-binding site per polypeptide. The heterotetrameric MoFe protein $(NifD/NifK)_2$ is carrying two unique metal clusters per (NifD/NifK)-dimer: One [8Fe7S] cluster (termed P-cluster) located at the NifD/NifK-subunit interface and the [Mo7Fe9SC-homocitrate] cluster (termed FeMoco or M-cluster) which is buried within subunit NifD (Einsle et al. 2002; Kim and Rees 1992) (Fig. 1a). For catalytic dinitrogen reduction, the iron protein transiently forms a complex with the MoFe protein. This octameric complex $(NifH)_2(NifD/NifK)_2(NifH)_2$ then allows for the transfer of electrons from the [4Fe4S] cluster of $(NifH)_2$ to the P-cluster of $(NifD/NifK)_2$ in a strictly ATP-dependent process. Electrons are then further translocated and eventually accumulated on the FeMoco which is described as the substrate reduction site (Hoffman et al. 2014). These electron transfer processes are schematically depicted in Fig. 1a. Significant progress in understanding biological dinitrogen fixation was made in the last years: The interstitial carbide "holding together" the FeMoco was discovered, which is thought to be relevant for the developing of improved synthetic dinitrogen reducing catalysts (Magistrato et al. 2007; Kirchner et al. 2007; Bjornsson et al. 2015; Wiig et al. 2013). Furthermore, nitrogenase derived hydrocarbon formation from CO in analogy to the technical Fischer Tropsch synthesis was discovered and proposed as a potential strategy for fuel production in the future (Yang et al. 2011; Lee et al. 2012, 2015; Hu et al. 2011).

Photosynthesis performed by plants and bacteria is also a crucial biological process that

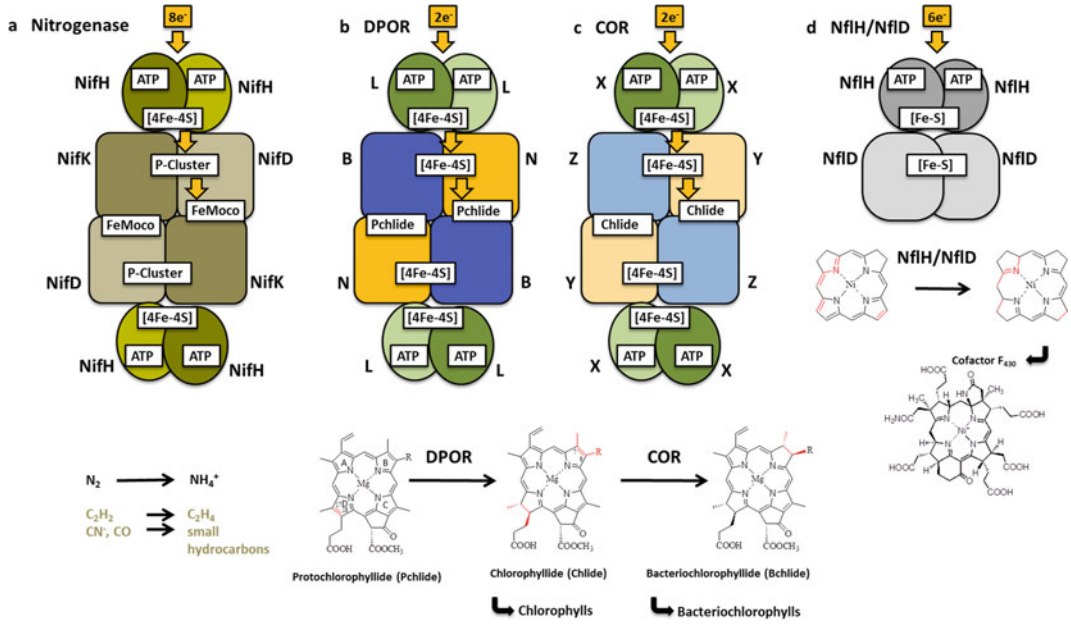

Fig. 1 Schematic comparison of transient octameric complexes involved in nitrogenase, DPOR and COR catalysis, hypothetical structural layout and catalysis of NflH/NflD. (**a–c**) Reductases $(NifH)_2$, L_2 and X_2 carrying an intersubunit [4Fe4S] cluster are shaded green. Core catalytic complexes $(NifD/NifK)_2$ of nitrogenase, $(NB)_2$ of nitrogenase-like DPOR and $(YZ)_2$ of nitrogenase-like COR are colored light brown/brown, yellow/blue and light yellow/light blue, respectively. ATP-driven electron transfer processes of nitrogenase (via [4Fe4S] cluster → P-cluster → FeMoco) and of DPOR or COR (via two [4Fe4S] clusters → Pchlide or Chlide) are schematically indicated. P-cluster [8Fe7S] cluster, FeMoco iron-molybdenum cofactor [Mo7Fe9SC-homocitrate] cluster also termed M-cluster. Reactions catalyzed by the respective systems are shown (*bottom*). (**d**) Hypothetical arrangement of subunits NflH and NflD, involved in cofactor F_{430} biosynthesis. The NflH/NflD substrate is to be determined; therefore tetrapyrrole ring substituents have been omitted. R is either ethyl or vinyl

converts visible light into chemical energy to drive most ecosystems on earth (Hohmann-Marriott and Blankenship 2011). It is responsible for the fixation of $123 \cdot 10^9$ tons of carbon annually and the accumulation of vast quantities of organic deposits from which our current fossil fuels derive. Tetrapyrroles like chlorophylls and bacteriochlorophylls mainly contribute three distinct functions to photosynthesis: They are responsible for light capturing, the subsequent transfer of the obtained excitation energy, and they constitute the special pair involved in primary charge separation – a process which generates chemical energy in the form of a reduction potential (Muh et al. 2012). Primarily for the purpose of light capturing, huge quantities of (bacterio)chlorophylls are synthesized in photosynthetic organisms annually. Accordingly, these pigments belong to the most abundant molecules on earth.

Chlorophyllide is a central hub metabolite for the biosynthesis of all chlorophylls and bacteriochlorophylls. For the synthesis of this molecule, the conjugated tetrapyrrole macrocycle of the pheoporphyrin protochlorophyllide must be stereospecifically reduced at the C17-C18 double bond on ring D (Fig. 1b). This chemically difficult two-electron trans-hydrogenation results in a significant change of the absorption characteristics. Protochlorophyllide reduction is either catalyzed by a single subunit enzyme that requires light for catalysis (light-dependent protochlorophyllide oxidoreductase; found in cyanobacteria, algae, gymnosperms and angiosperms) or alternatively by a dark operating system composed of three different polypeptides

termed dark operative protochlorophyllide oxidoreductase (DPOR; found in cyanobacteria, algae, gymnosperms and anoxic photosynthetic bacteria) (Fujita 1996; Reinbothe et al. 2010). DPOR performs an ATP-dependent catalysis and is composed of polypeptides L, N and B. The respective genes are denoted *chlL*, *chlN* and *chlB* in chlorophyll synthesizing organisms and *bchL*, *bchN* and *bchB* in bacteriochlorophyll synthesizing organisms. The metalloprotein DPOR is organized as a two-component system consisting of an ATP-dependent reductase L_2 and a catalytic subcomplex $(NB)_2$ both sharing a high degree of sequence and structural homology to the related nitrogenase proteins (Fig. 1b).

Bacteriochlorophylls characteristically differ from chlorophylls with respect to the redox state of the conjugated tetrapyrrole ring system (Burke et al. 1993) (Fig. 1c). Chlorophyllide oxidoreductase (COR) catalyzes the reaction step following DPOR catalysis in the bacteriochlorophyll biosynthesis pathway. COR enables for the chemically difficult reduction of the C7-C8 double bond of chlorophyllide. The resulting bacteriochlorophyllide molecule then is the central precursor for the biosynthesis of all bacteriochlorophylls (Nomata et al. 2006a). Mechanistically, DPOR and COR perform closely related two-electron reductions. However, accurate substrate recognition and adequate redox characteristics for the discrimination of the individual substrates are required. The ATP-dependent catalysis of COR requires polypeptides X, Y and Z, the respective genes have been denoted as *bchX*, *bchY* and *bchZ*. The subcomplex X_2 acts as an ATP-dependent reductase that interacts with the catalytic component $(YZ)_2$ of COR. The individual subunits of COR share an amino acid sequence identity of 31–35 % for subunit X and 15–22 % for subunits Y or Z when compared to the corresponding DPOR subunits (L, N or B). This homology might indicate a catalytic mechanism of COR which is closely related to DPOR catalysis.

Biological methane formation is a globally important process that is conducted exclusively by prokaryotes belonging to the domain of the archaea (Thauer et al. 2008). About one billion tons of methane are produced by methanogenic archaea annually. Almost half of the formed methane escapes into the atmosphere, where most of it is photochemically oxidized to CO_2 thus contributing to the greenhouse effect and global warming (Thauer 2011). Moreover, methane itself also acts as a very effective greenhouse gas. Methanogenic archaea also play a crucial role for the production of biogas with the formed methane representing the most important product.

During methane formation by methanogenic archaea the enzyme methyl-coenzyme M reductase (MCR) catalyzes the key step of the process, namely the reduction of the coenzyme M bound methyl group to gaseous methane (Ermler et al. 1997). In order to catalyze this reaction, MCR depends on the unique nickel-containing tetrapyrrole cofactor F_{430} as an essential prosthetic group. Cofactor F_{430} is an unusual tetrapyrrole with regard to the central metal ion (nickel), the presence of two additional rings attached to the central tetrapyrrole core (lactam ring E and cyclohexanone ring F) and the redox state of the ring system, which represents the most reduced tetrapyrrole found in nature (Fig. 1) (Friedmann et al. 1990). Although the biosynthesis route of cofactor F_{430} is currently largely unknown, it is obvious that an appropriate reductase is required for the reduction of the tetrapyrrole macrocycle (Pfaltz et al. 1987). Accordingly, it was proposed that unusual nitrogenase-like catalysis could be involved in the chemically difficult reduction of either two or three double bonds depending on the redox state of the actual substrate (Raymond et al. 2004). Assuming that the initial redox state before the reductase step corresponds to that of an isobacteriochlorin, the reductase has to catalyze the stereospecific two-electron reductions of the C12-C13 and the C18-C19 double bonds as well as the two electron-reduction of the C20-C4 diene to a single double bond as shown in Fig. 1d. Interestingly, all methanogenic archaea contain genes encoding a nitrogenase-like reductase system of unknown function consisting of a

reductase component NflH and a catalytic unit NflD (Nfl = Nif-like). Whereas NflH shows ~23 % amino acid sequence identity to NifH, NflD exhibits only ~6 % and ~5 % amino acid sequence identity to NifD and NifK, respectively. It was proposed that these enzymes might be responsible for the ring reduction steps during cofactor F_{430} biosynthesis (Bröcker et al. 2010a; Hu and Ribbe 2015).

2 Dark Operative Protochlorophyllide Oxidoreductase (DPOR)

2.1 Overview

DPOR is a highly oxygen sensitive two-component metalloprotein which performs the chemically difficult reduction of the conjugated ring system of protochlorophyllide in a strictly ATP-dependent reaction. Interdisciplinary approaches combining genetic, biochemical, spectroscopic, chemical and x-ray crystallographic methods contributed to the detailed understanding of the catalytic mechanism of DPOR. The enzyme is composed of two distinct subcomplexes: The homodimeric reductase L_2 is responsible for the initial uptake of an electron from a plant-type ferredoxin and for the subsequent transfer of this electron onto the heterotetrameric $(NB)_2$ subcomplex, where protochlorophyllide reduction takes place (Bröcker et al. 2008a). According to this oligomeric architecture, $(NB)_2$ contains two symmetry-related active site cavities for the binding of protochlorophyllide substrate molecules. At the same time, each NB half-tetramer has the ability to interact with one L_2 dimer, which gives rise to a theoretical $L_2(NB)_2L_2$ heterooctamer (Fig. 1b). Overall, the DPOR catalyzed two-electron reduction can be described as a reductive protonation of the C17-C18 double bond of protochlorophyllide which results in stereospecific chlorophyllide formation.

2.2 L_2 Is an ATP-Dependent Reductase

By analogy to $(NifH)_2$ of nitrogenase, L_2 forms a homodimer (M_r ~ 60 kDa) that is bridged via a symmetric [4Fe4S] cluster. This redox active metallocenter is coordinated by two conserved cysteine residues from each subunit (Bröcker et al. 2008a). The solved three-dimensional structure of the L_2 protein from *Rhodobacter sphaeroides* revealed this symmetrical dimer with one Mg^{2+}-coordinated ADP molecule bound to each subunit (Sarma et al. 2008). On the basis of mutational experiments, functional relevance of the highly conserved ATP cofactor binding motif called P-loop sequence (YGKGGIGK) and of the so called switch II region (LGDVVCGGF) was demonstrated (Bröcker et al. 2008a). In $(NifH)_2$ of nitrogenase, this switch II sequence acts as a conformational relay that communicates the binding of ATP to the [4Fe4S] cluster. Based on biochemical investigations and the overall structural similarity of L_2 and $(NifH)_2$ parallelism for the initial electron transfer steps of DPOR and nitrogenase was concluded.

2.3 The Catalytic $(NB)_2$ Complex: Substrate Recognition and Electron Transfer

The three-dimensional structures of substrate-free $(NB)_2$ (from the cyanobacterium *Thermosynechococcus elongatus*) (Bröcker et al. 2010a) and of $(NB)_2$ in the presence of protochlorophyllide (from the proteobacterium *Rhodobacter capsulatus*) (Muraki et al. 2010) revealed a high degree of structural similarity with respect to the quaternary and ternary structure. In both cases, $(NB)_2$ forms a symmetric heterotetramer which is comprised of two NB half tetramers. Sequences of N and B are paralogous and share an amino acid sequence identity of ~14 %, respectively. The overall fold of these individual subunits is rather similar which might indicate that sequences of N and B have evolved from a

common ancestor. The catalytic $(NB)_2$ complex binds two symmetry related [4Fe4S] centers at the NB interface, respectively. These clusters are asymmetrically coordinated by three cysteinyl ligands from N and one unusual aspartate ligand from B (Bröcker et al. 2010a; Muraki et al. 2010). When this aspartate was replaced by cysteine, the resulting mutant protein still assembled the [4Fe4S] cluster but almost no residual enzymatic activity was determined (Muraki et al. 2010). It was hypothesized, that the original aspartate ligand alters the redox potential of the [4Fe4S] cluster to a value below that of standard four cysteine ligated clusters. Obviously, the aspartate ligand is a prerequisite for protochlorophyllide reduction (Takano et al. 2011).

Each NB half tetramer contains one deeply buried protochlorophyllide binding site which is mainly located within subunit N. A partial unwinding of one helical segment from subunit B is required for the channeling of the substrate into the mainly hydrophobic binding pocket (Muraki et al. 2010; Moser et al. 2013). Furthermore, the C-terminal domain of subunit B is responsible for the closing of the active site cavity thereby preventing the organism from protochlorophyllide-induced photodynamic damage. DPOR substrate recognition has also been investigated using artificial protochlorophyllide derivatives. The related enzyme kinetics indicated that minor modifications on rings A, B, C and E (e.g. reducing the size or polarity of the side chain) were tolerated by the enzyme. However, substrate variants with an increased volume of the ring substituents and also variants with modifications on ring D were not converted. Obviously, the catalytic target on ring D is coordinated with high specificity (Bröcker et al. 2008b).

Each [4Fe4S] cluster is located at a distance of ~11 Å from the protochlorophyllide ring system (edge-to-edge distance), thereby allowing for rapid electron transfer to the substrate (Muraki et al. 2010; Moser et al. 2013; Bröcker et al. 2008b). However, the C17-C18 double bond of the substrate faces away from the [4Fe4S] cluster, arguing for an electron transfer process involving the conjugated ring systems A or B of the substrate (compare Fig. 1b and the ternary DPOR structure depicted in Fig. 2a).

With respect to the regio- and stereo-specific reduction of the substrate, a direct protonation at C17 via a highly conserved aspartate residue was concluded. By contrast, the C18 protonation is mediated by 'substrate assisted catalysis'. A water molecule is well-positioned by combined interaction with a histidine residue and the C18 propionate of protochlorophyllide. This water then facilitates the trans-specific C18 protonation (Moser et al. 2013).

2.4 Trapping the Ternary Complex Reveals the Catalytic Redox Cycle of DPOR

As in nitrogenase, it was demonstrated that subcomplexes L_2 and $(NB)_2$ solely perform a transient protein protein interaction during the time course of DPOR catalysis. The dynamic interplay of these two components is triggered by ATP hydrolysis in a process that facilitates the appropriate timing of the electron transfer step between L_2 and $(NB)_2$. The trapping of the ternary transition state complex allowed for the detailed understanding of DPOR subcomplex interaction and the involved protein dynamics. Biochemical experiments in the presence of the non-hydrolysable ATP analog MgADP-AlF_3 efficiently produced the octameric $L_2(NB)_2L_2$ protein. This stabilized complex is impaired in electron transfer to $(NB)_2$ which confirms the coupling between ATP hydrolysis and electron transfer. It was studied in great detail and subsequently the crystal structure of this ~360 kDa complex was resolved (Moser et al. 2013; Bröcker et al. 2010b) (Fig. 2a, c).

The catalytic cycle of DPOR involves a large number of individual steps. Biochemical, EPR spectroscopic methods and the comparative analysis of the available X-ray crystallographic structures provided a detailed picture of the order of events. The catalytic cycle of DPOR is summarized in Fig. 3, individual states that have been characterized by EPR spectroscopy are

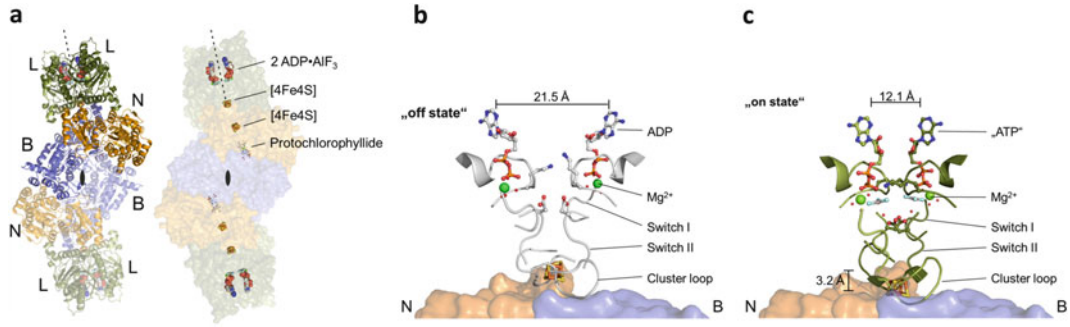

Fig. 2 Three-dimensional structure of the ternary DPOR complex and dynamic switch mechanism of L_2. (**a**) L_2 carrying an intersubunit [4Fe4S] cluster is a dynamic switch protein triggering the transient interaction with $(NB)_2$. This results in the transfer of a single electron onto the substrate protochlorophyllide via a second [4Fe4S] cluster located on $(NB)_2$ (PDB ID code 2YNM). (**b**, **c**) Nucleotide-dependent switch mechanism of DPOR subcomplex L_2, minor residues are omitted for clarity. (**b**) Key secondary structural elements of L_2 in the "off state" conformation (*grey*), observed in the presence of ADP (PDB ID code 3FWY). (**c**) Identical structural elements of L_2 in the "on state" conformation (*green*), observed in the presence of the ATP analog ADP-AlF_3. Bound nucleotide cofactors (with indicated distances), switch region I, switch region II and the cluster loop responsible for the dynamic repositioning of the [4Fe4S] cluster of L_2 are indicated. Movement of the [4Fe4S] cluster of L_2 over a distance of 3.2 Å is indicated. Panel a modified according to (Moser et al. 2013)

highlighted grey (Bröcker et al. 2010b). The catalytic $(NB)_2$ complex was shown to have a high affinity for its protochlorophyllide substrate (Bröcker et al. 2008b; Nomata et al. 2008). Obviously, the initial binding of the substrate might be a critical step in vivo to overcome the well described phototoxicity of the DPOR substrate (Walther et al. 2009). In the laboratory, DPOR experiments are performed in the presence of the artificial electron donor dithionite which results in the reduction of the [4Fe4S] cluster of L_2. This single electron reduction does not require the presence of $(NB)_2$ and/or ATP. It was shown that the reduced and ATP-charged L_2 protein is the only electron donor with the ability to transfer electrons onto $(NB)_2$ during DPOR catalysis (Bröcker et al. 2008a; Nomata et al. 2008).

In vivo, a ferredoxin acts as the natural electron donor and transfers one electron onto the [4Fe4S] cluster of L_2 **(I)** (Bröcker et al. 2008a; Nomata et al. 2006b). L_2 is a dynamic switch protein that links the hydrolysis of ATP to significant conformational rearrangements of the overall ternary protein structure. The L_2 protein can be characterized by two different states: the "off state" in the presence of ADP which is not able to form a complex with $(NB)_2$ and the "on state" which possesses a high affinity for $(NB)_2$ (Bröcker et al. 2008a; Sarma et al. 2008; Nomata et al. 2006b). Accordingly, binding of two ATP molecules to L_2 **(II)** results in conformational alterations (compare protein dynamics depicted in Fig. 2b, c) and the subsequent formation of the ternary DPOR complex **(III)** in the presence of $(NB)_2$ containing two protochlorophyllide molecules (Bröcker et al. 2010b; Watzlich et al. 2009). ATP hydrolysis facilitates the translocation of one electron from the [4Fe4S] cluster of L_2 onto the low redox potential [4Fe4S] cluster located on $(NB)_2$ **(IV)**. The reduced $(NB)_2$ protein then has the ability to transfer a single electron onto the substrate. This process is induced by the dynamic repositioning of the [4Fe4S] cluster of L_2 by 3.2 Å as a result of ternary complex formation (compare the "off state" and the "on state" conformation depicted in Fig. 2b, c) (Moser et al. 2013). In the $L_2(NB)_2L_2$ complex rapid electron transfer is facilitated due to an edge-to-edge distance of 14.1 Å between the [4Fe4S] cluster of L_2 and $(NB)_2$. Presence of ADP then triggers the dissociation of the $L_2(NB)_2L_2$ complex **(V)**. Overall, two consecutive rounds of this redox catalytic cycle are required so that the dynamic switch protein L_2

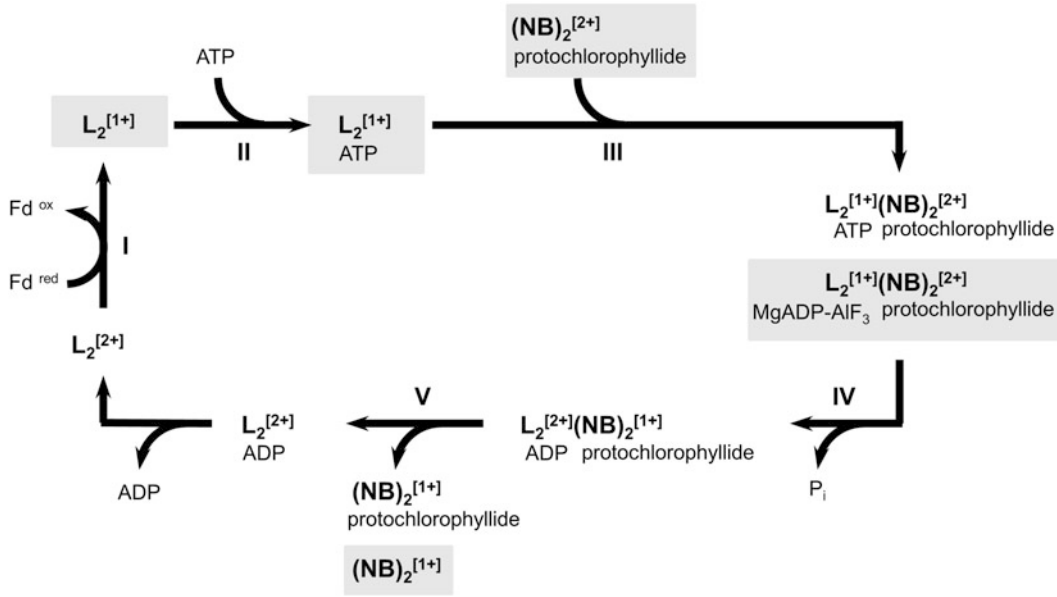

Fig. 3 Redox cycle of DPOR catalysis. Schematic model for the electron transfer processes and dynamic subunit interaction during ATP-driven DPOR catalysis. Five intermediates were confirmed by EPR spectroscopy (*highlighted grey*), the individual redox state is indicated $^{[1+]}$ for reduced and $^{[2+]}$ for oxidized [4Fe4S] clusters. According to this redox cycle two consecutive single electron reductions of $(NB)_2$ are required to provide the two electrons necessary for protochlorophyllide reduction

can supply the two electrons which are required for the reduction of the substrate. These individual electron transfer steps go along with the stereospecific addition of two protons to C17 and C18 of protochlorophyllide in the active site of DPOR (Muraki et al. 2010; Moser et al. 2013). Only recently, formation of the proposed substrate radical after single electron transfer was confirmed by EPR spectroscopy (Nomata et al. 2014).

The spectrum of artificial 'small-molecule substrates' of DPOR was demonstrated in correlation with those of the related nitrogenase system (Fig. 1a). DPOR does not catalyze the conversion of 'complex' nitrogenase substrates like N_2 or CO since these reductions require more than two electrons. By contrast, DPOR enables for the two-electron reduction of N_3^- or N_2H_4 to NH_3 analogously as also determined for nitrogenase (Moser et al. 2013). These results might indicate that DPOR and nitrogenase make use of a closely related energy transduction mechanism. According to this, DPOR might be used as an important tool to gain further insight into the mechanism of dinitrogen fixation.

3 Chlorophyllide Oxidoreductase (COR)

3.1 Overview

Synthesis of all bacteriochlorophylls involves the ATP-driven reduction of the C7-C8 double bond of chlorophyllide by the multi-subunit enzyme COR (Fig. 1c). Structural information for this oxygen sensitive enzyme system is not available to date. However, combined mutational and spectroscopic analyses, the homology to the DPOR system and also chimeric COR/DPOR experiments revealed a detailed picture of COR catalysis. The reductive protonation of chlorophyllide is based on the transient interaction of subcomplex X_2 with the catalytic $(YZ)_2$ protein for the electron transfer via two redox active iron-sulfur centers.

Only recently, it was demonstrated that COR systems from *R. capsulatus*, *R. sphaeroides*, *Rhodopseudomonas palustris*, *Chlorobaculum tepidum* and *Roseiflexus castenholzii* are also capable of the reduction of the $C8^1$-$C8^2$ double bond (8-vinyl group) of chlorophyllide (Tsukatani et al. 2013a; Harada et al. 2014). Besides these bifunctional enzymes, it was also demonstrated that the COR enzyme from *Heliobacterium modesticaldum* is able to catalyze the direct 8-ethylidene group formation during the biosynthesis of bacteriochlorophyll *g* (Tsukatani et al. 2013b). In Fig. 4 these additional COR activities, the reductive 1,2 protonation at $C8^1$-$C8^2$ and the reductive 1,4 protonation at C7-$C8^2$ of chlorophyllide are shown. Mechanistically, these 'additional' enzymatic activities of specific COR enzymes might be closely related to the enzyme catalysis of NflH/NflD described in chapter 4.

3.2 The Reductase X_2 Resembles L_2

Size exclusion experiments for the COR reductase protein from the purple bacterium *Roseobacter denitrificans* indicated a homodimeric quaternary structure (Kiesel et al. 2015). Sequence comparisons for X proteins with the related L sequences of DPOR revealed that both cysteinyl ligands responsible for the formation of the redox-active intersubunit [4Fe4S] cluster of L_2 are fully conserved among all X proteins (Burke et al. 1993). EPR measurements for X_2 displayed the characteristic signal of a [4Fe4S] cluster (Kiesel et al. 2015; Kim et al. 2008). Besides this, the key amino acid residues for the dynamic switch mechanism of L_2 (responsible for ATP binding and hydrolysis, signal transduction) are highly conserved in all sequences of X proteins (Watzlich et al. 2009). According to these findings, parallelism for the electron transfer steps of X_2 and L_2 via an intersubunit [4Fe4S] cluster was concluded. To some extent, this hypothesis was experimentally confirmed. The reductase L_2 of DPOR was substituted with subunit X_2 from COR in a DPOR activity assay in the presence of protochlorophyllide. The resulting chimeric enzyme (e.g. X_2 from *R. denitrificans* / $(NB)_2$ from *Chlorobaculum tepidum*) revealed substantial protochlorophyllide reducing activity (Watzlich et al. 2009). These experiments might indicate that the ATP-driven electron transfer mechanism of X_2 mirrors the well described dynamic switch mechanism of L_2. Furthermore, it was speculated that the docking faces of X_2 and $(YZ)_2$ and the related protein protein interplay of COR might resemble that of the DPOR system.

3.3 The Catalytic $(YZ)_2$ Complex: Substrate Recognition and Electron Transfer

Size exclusion experiments resulted in a native molecular mass of ~ 280 kDa, indicative of a heterotetrameric $(YZ)_2$ complex in analogy to the related DPOR system. Substrate binding experiments for the *R. denitrificans* $(YZ)_2$ protein revealed the tight binding of the photolabile chlorophyllide molecule and suggested the presence of two substrate binding sites (Kiesel et al. 2015). COR activity experiments using a series of chemically modified substrates tentatively revealed the binding of chlorophyllide in a buried active site cavity (Kiesel et al. 2015). The COR enzyme efficiently reduced the C7-C8 double bond of substrates containing modified substituents on ring systems A, C, and E. By contrast, artificial substrates modified at the distantly located propionate side chain of ring D were not a substrate of COR. According to this, a closely related substrate binding mode for chlorophyllide and protochlorophyllide with regard to the ring orientation in the active site of COR and DPOR was hypothesized. It was concluded that both systems make use of an evolutionary conserved electron insertion path via ring systems A or B. Accordingly, the active site of COR would make use of two alternative proton donors (when compared to DPOR) for the regio- and stereospecific reduction of ring B. With respect to the COR-dependent 8-ethylidene group formation in *H. modesticaldum* (Fig. 4), this alternative

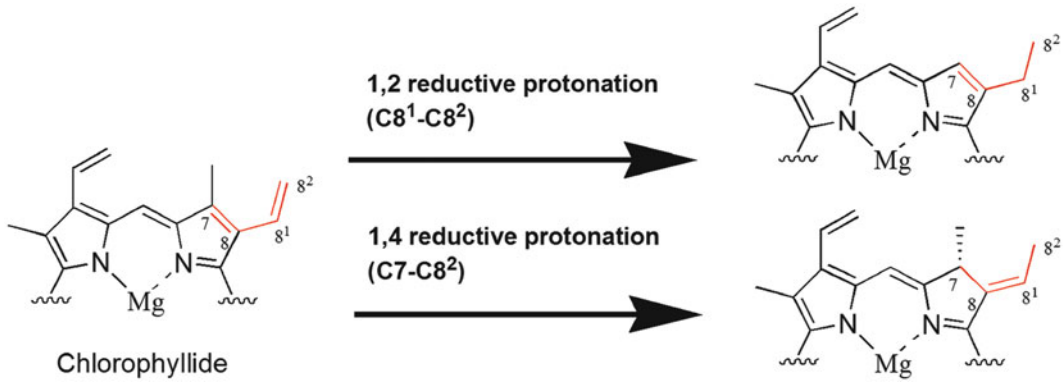

Fig. 4 Additional or alternative enzymatic activities of COR enzymes involved in bacteriochlorophyll *a* or bacteriochlorophyll *g* biosynthesis. COR was also proposed as an alternative 8-vinyl reductase involved in bacteriochlorophyll *a* biosynthesis in *R. capsulatus*, *R. sphaeroides*, *R. palustris*, *C. tepidum* and *R. castenholzii* (*top*). Furthermore, COR catalyzed 8-ethylidene group formation during bacteriochlorophyll *g* biosynthesis was demonstrated for *H. modesticaldum* (*bottom*). These two-electron reduction processes can be described as an 1,2 reductive protonation or as an 1,4 reductive protonation

COR activity is based on the 1,4-hydrogenation of a diene system at C7 and $C8^2$ of chlorophyllide (instead of the well described 1,2-hydrogenation of C7-C8). This catalysis is based on an orthologous COR enzyme and it might only require the spatial rearrangement of a single proton donor ($C8^2$ instead of C8). With respect to the bifunctional COR enzymes, performing the hydrogenation of C7-C8 in parallel with the hydrogenation of the $C8^1$-$C8^2$ double bond, an overall of four specific proton donors would be required for the catalyzed four electron reduction (Kiesel et al. 2015).

EPR experiments for $(YZ)_2$ revealed a characteristic signal for a [4Fe4S] cluster. However, mutational experiments were indicative of four cysteinyl cluster ligands. This was in clear contrast to the related DPOR system where the [4Fe4S] cluster of $(NB)_2$ makes use of three cysteine and one unusual aspartate ligand, which might be relevant for the "tuning" of the respective redox potential (Kondo et al. 2011). For the $(YZ)_2$ protein from *R. denitrificans* a DPOR-like three cysteine/one aspartate ligation pattern for the catalytic [4Fe4S] cluster was implemented by mutagenesis. This mutant protein revealed artificial [4Fe4S] cluster formation as indicated by EPR experiments. However, no enzymatic COR activity was determined in the presence of this cluster variant which was ascribed to an inappropriate redox potential of the assembled [4Fe4S] cluster (Kiesel et al. 2015).

3.4 The Ternary COR Complex Reveals Parallelism for the COR and DPOR Redox Cycle

The ATP-dependent conversion of chlorophyllide strictly requires the presence of X_2 and $(YZ)_2$. In vitro experiments using the artificial reducing agent dithionite clearly indicated that the electron transferring reductase L_2 of DPOR cannot substitute for the COR specific reductase X_2. Biophysical and biochemical investigations suggested [4Fe-4S] cluster-dependent redox catalysis by analogy to the related DPOR system. Accordingly, ternary COR complex formation via the nucleotide-dependent reductase X_2 was investigated in the presence of the substrate and the ATP analog MgADP-AlF_4^-. MgADP in combination with the inorganic compound AlF_4^- is mimicking the ATP molecule in the transition state of nucleotide

hydrolysis. From the stoichiometry of the assembled COR complex an octameric $X_2(YZ)_2X_2$ complex was deduced (Fig. 1c) (Kiesel et al. 2015). A catalytic redox cycle for the COR system was proposed by analogy to the mechanism depicted in Fig. 3.

4 NflH/NflD

4.1 Overview

As mentioned in the introduction, another nitrogenase-like enzyme system besides DPOR and COR was identified on the basis of bioinformatic analysis of prokaryotic genomes and was termed Nif-like (Nfl) due to its homology to nitrogenase (Raymond et al. 2004). The Nfl-system also consists of two components: NflH which was proposed as a homolog of the reductases NifH, L and X and the proposed catalytic component NflD which is related to the paralogous protein sequences NifD/NifK, N/B and Y/Z, respectively. Based on phylogenetic analyses it was proposed that the Nfl-system might represent an ancestor of nitrogenase and DPOR/COR. These enzymes could have evolved from this ancestor through paralogous gene duplication and subsequent divergence (Boyd and Peters 2013). Interestingly, the Nfl proteins occur in all methanogenic archaea. In 2007, the expression pattern of *nflH* and *nflD* in *Methanocaldococcus jannaschii* was investigated. It was found that these genes are expressed constitutively and, therefore, it was concluded that NflH and NflD fulfill some housekeeping function essential for methanogens such as the biosynthesis of cofactor F_{430}. Further, it was shown by co-purification and bacterial two-hybrid studies that NflH and NflD interact with each other *in vivo* (Staples et al. 2007). However, the precise function of NflH/NflD remains to be elucidated. Therefore, the proposed function and mechanism of NflH/NflD described below are based on the homology to nitrogenase, DPOR and COR.

4.2 The Proposed Reductase NflH

Based on amino acid sequence similarity with NifH, L and X proteins it is very likely that NflH also adopts a homodimeric structure carrying a bridging intersubunit [4Fe4S] cluster. The amino acid sequences of NflH proteins contain three highly conserved cysteine residues with two of them corresponding to the iron-sulfur cluster ligands of NifH, L and X. Moreover, NflH sequences also exhibit the characteristic P-loop sequence (YGKGGIGK) and the so called switch II region. However, the switch II region of several NflH is slightly altered. Instead of the highly conserved motif LGDVVCGGF found in NifH, L and X, the amino acid sequences of some NflH proteins carry a PGDIVCGGF motif. The leucine residue within the former motif was shown to be essential for the dynamic switch mechanism (Bröcker et al. 2010b; Lanzilotta et al. 1996). Since this residue is replaced by a proline in these NflH sequences, the mechanism of ATP-dependent conformational changes required for electron transfer from the reductase to the catalytic component might be different in some of the Nfl systems. Nevertheless, it is obvious that NflH and NflD have to interact with each other for electron transfer, and such an interaction was indeed observed to occur in vivo (Staples et al. 2007).

4.3 The Proposed Catalytic $(NflD)_2$ Complex

The amino acid sequences of NflD proteins exhibit similarity to the sequences of NifD/NifK, N/B and Y/Z. Thus, it is assumed that NflD represents the catalytic component of the Nfl system. However, in contrast to nitrogenase, DPOR and COR, the catalytic component of the Nfl system is not a heterotetramer, since there is no second subunit besides NflD, but rather a homodimer or alternatively a homotetramer. Within the amino acid sequences of NflD there are two highly conserved cysteine residues which correspond to two of the iron-sulfur cluster

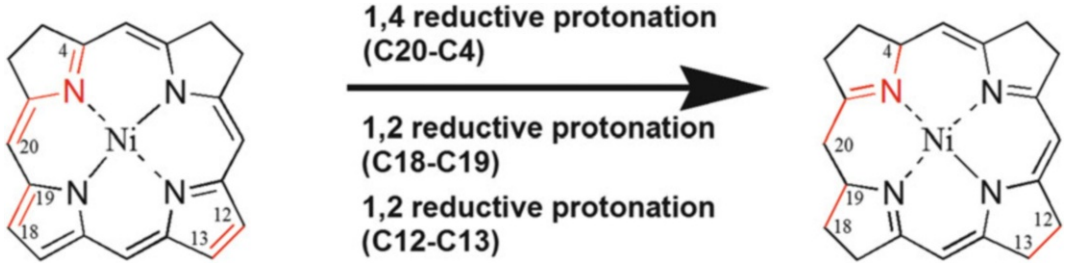

Fig. 5 Proposed NflH/NflD catalysis. The ring reduction steps include the 1,4 reductive protonation of a diene system at C20-C4 and the 1,2 reductive protonation at C18-C19 and at C12-C13. The precise NflH/NflD substrate is to be determined; accordingly the individual tetrapyrrole ring substituents have been omitted

ligating cysteines of NifD, N and Y. Thus, it is possible that the proposed NflD dimer contains a bridging intersubunit [4Fe4S] cluster. Considering a homodimeric $(NflD)_2$ structure, an intriguing question will be whether the asymmetric substrate binds to an active site located between the two monomers or whether there are two identical active sites located within each monomer. In any case, probably several different proton donating residues are required for the three reductive protonation steps during cofactor F_{430} biosynthesis.

4.4 Protein Protein Interactions and Proposed Reaction Catalyzed by NflH/NflD

Based on the assumptions described above, it is reasonable to suggest that the homodimeric reductase $(NflH)_2$ interacts with the homodimeric catalytic $(NflD)_2$ component to form a $(NflH)_2$/$(NflD)_2$ complex (Fig. 1d). Such a complex would represent a minimal version in order to achieve nitrogenase-like catalysis (Hu and Ribbe 2015). The necessity of ATP-binding and hydrolysis for complex formation and electron transfer from $(NflH)_2$ to $(NflD)_2$ is very likely but remains to be shown experimentally.

The reaction catalyzed within the catalytic $(NflD)_2$ is most likely comprised of two 1,2 reductive protonation steps, in order to stereospecifically reduce the C12-C13 and C18-C19 double bonds, and one 1,4 reductive protonation in order to convert the C20-C4 diene to a single C1-N1 double bond (Fig. 5). Structural and biochemical analysis of the NflH/NflD system in the future will reveal the amino acid residues involved in these reactions and will possibly provide insights into the evolutionary relationship between NflH/NflD, DPOR, COR and nitrogenase.

5 Concluding Remarks

The enzymatic mechanisms of DPOR and COR (and most likely of NflH/NflD) indicate a high degree of plasticity for the reductive protonation of differing tetrapyrrole molecules. However, the individual substrates differ with respect to the redox state of the conjugated ring system. A deeply buried substrate binding pocket devoid of water molecules or unspecific proton donors might be nature's design to avoid unspecific 'tetrapyrrole over-reduction'. The overall amount of precisely positioned proton donors might account for the individual two, four or six electron reductions catalyzed by DPOR/COR, COR or NflH/NflD, respectively. Furthermore, the redox potential of the involved [4Fe4S] clusters (reductase and catalytic component) might be also relevant for the respective tetrapyrrole reducing activity. With respect to this, the further elucidation of the catalytic differences of DPOR, COR and NflH/NflD also might have implications for the further understanding of nitrogenase catalysis.

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Ethical Approval The authors declare that this article does not contain any studies with human participants or animals.

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