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Complex I: A Chimaera of a Redox and Conformation-Driven Proton Pump?

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From phylogenetic sequence analysis, it can be concluded that the proton-pumping NADH:ubiquinone oxidoreductase (complex I) has evolved from preexisting modules for electron transfer and proton translocation. It is built up by a peripheral NADH dehydrogenase module, an amphipatic hydrogenase module, and a membrane-bound transporter module. These modules, or at least part of them, are also present in various other bacterial enzymes. It is assumed that they fulfill a similar function in complex I and related enzymes. Based on the function of the individual modules, it is possible to speculate about the mechanism of complex I. The hydrogenase module might work as a redox-driven proton pump, while the transporter module might act as a conformation-driven proton pump. This implies that complex I contains two energy-coupling sites. The NADH dehydrogenase module seems to be involved in electron transfer and not in proton translocation.

KEY WORDS: NADH:ubiquinone oxidoreductase; NADH dehydrogenase; complex I; modular evolution; Fe–S clusters; electron transfer; proton translocation; *Escherichia coli*.

INTRODUCTION

The proton-pumping NADH:ubiquinone oxidoreductase, also called complex I, is the first of the respiratory complexes providing the proton-motive force required for energy-consuming processes like the synthesis of ATP, active transport, and motion (Weiss *et al.*, 1991; Walker, 1992; Brandt, 1997). During the last years, the genomes of 30 bacteria, 10 eukarya, and 8 archaea have been deciphered. Sequencing of more than 300 other genomes is underway. These data revealed that homologs of complex I exist in bacteria, archaea, and in mitochondria and chloroplasts (Friedrich *et al.*, 1995; Friedrich and Weiss, 1997; Friedrich and Scheide, 2000). The proton-pumping NADH:ubiquinone oxidoreductase is a member of a large family of energy-converting oxidoreductases that accepts electrons from a hydride donor and passes them to a membrane-bound two-electron acceptor (Friedrich and Scheide, 2000). An attempt is made in this review to elucidate the mechanism of complex I based on conclusions drawn from its modular structure and evolution. Two modules of complex I are proposed to be involved in proton translocation, each of them representing one coupling site.

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MODULAR STRUCTURE OF COMPLEX I

The *Escherichia coli* complex I is a good example to visualize the modular structure of complex I. In general, 14 genes code for subunits of the bacterial complex I (Friedrich *et al.*, 1995). In *E. coli* the complex I genes are organized in the so-called *nuo* operon (from NADH:ubiquinone oxidoreductase; Weidner *et al.*, 1993) with the particularity that the genes *nuoC* and *D* are fused to one gene *nuoCD* (Braun *et al.*, 1998; Friedrich, 1998). The resulting 13 complex I subunits are named NuoA to NuoN. The *E. coli* complex was isolated in the presence of alkyl glycoside detergents at pH 6.0 (Leif *et al.*, 1995; Spehr *et al.*, 1999). The preparation has a molecular mass of approx. 550 kDa and is composed of the 13 subunits coded by the *nuo* genes (Leif *et al.*, 1995; Spehr *et al.*, 1999). It contains one flavin mononucleotide (FMN) and up to nine iron-sulfur (Fe–S) clusters. By means of biochemical procedures, the preparation can easily be cleaved, resulting in a fragmentation pattern that

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Table I. Properties of the *E. coli* Complex I and Its Fragments

Preparation	Electron transfer activity	Subunits	Cofactors
Complex I	NADH/ferricyanide	NuoA	
	NADH:ubiquinone	NuoB	1 × [4Fe–4S], N2
		NuoCD	
		NuoE	1 × [2Fe–2S], N1a
		NuoF	FMN; 1 × [4Fe–4S], N3
		NuoG	2 × [2Fe–2S], N1b, N1c[a]
			2 × [4Fe–4S], N4, N5
		NuoH	
		NuoI	2 × [4Fe–4S], N6a/N6b
		NuoJ	
		NuoK	
		NuoL	
		NuoM	
		NuoN	
NADH dehydrogenase fragment	NADH/ferricyanide	NuoE	1 × [2Fe–2S], N1a
		NuoF	FMN, 1 × [4Fe–4S], N3
		NuoG	2 × [2Fe–2S], N1b, N1c[a]
			2 × [4Fe–4S], N4, N5
Connecting fragment	None	NuoB	1 × [4Fe–4S], N2
		NuoCD	
		NuoI	2 × [4Fe–4S], N6a/N6b
Membrane fragment	None	NuoA	
		NuoH	
		NuoJ	
		NuoK	
		NuoL	
		NuoM	
		NuoN	

[a] This cluster has so far only been detected in the *E. coli* complex I.

helped to elucidate the modular structure of complex I (Leif *et al.*, 1995; Table I).

The soluble NADH dehydrogenase fragment is composed of the subunits NuoE, F, and G (Table I). One FMN and the EPR-visible Fe–S clusters N1b, N1c, N3, and N4 have been detected in this preparation (Leif *et al.*, 1995; Braun *et al.*, 1998). It has been shown that the homolog of NuoE of *Paracoccus denitrificans*, Nqo2, carries the Fe–S cluster N1a (Yano *et al.*, 1994). Since the typical binding motif is conserved, it is reasonable to assume that this Fe–S cluster is present in NuoE of the *E. coli* NADH dehydrogenase fragment as well, although it has not yet been detected. NuoF contains the NADH-binding site, the FMN, and the Fe–S cluster N3 as it has been shown for complex I from beef heart, *Neurospora crassa* and *P. denitrificans* (Chen and Guillory, 1981; Fearnley and Walker, 1992; Weidner *et al.*, 1993; Fecke *et al.*, 1994; Yano *et al.*, 1996; Ohnishi, 1998). NuoG is assumed to bind the Fe–S cluster N4 (Yano *et al.*, 1996; Ohnishi, 1998). The binuclear cluster N1c, which has so far been uniquely found in the preparation of the *E. coli* complex I, is most likely located on NuoG as well (Leif *et al.*, 1995). This subunit contains a typical cysteine motif for binding of such a cluster, which is not found in the homologous subunits of other organisms, with the exception of *Salmonella typhimurium* and *Thermus thermophilus* (Friedrich, 1998). From the latter, complex I has not been isolated so far. In addition, NuoG contains a binding site for a second tetranuclear cluster, most likely N5. It was controversal whether this cluster is a true cofactor of complex I because of its low concentration in complex I preparations (Ohnishi, 1979, 1998; Beinert and Albracht, 1982). N5 has recently been detected in the overproduced homolog of NuoG in *P. denitrificans*, Nqo3 (Yano *et al.*, 1995; Ohnishi, 1998; T. Ohnishi and T. Yano, pers. commun.). It is most likely present in stoichiometric amounts in complex I, but the major portion of the N5 spins may be in the $S = 3/2$ ground state leading to an underestimation of the spins in the $g = 2$ region (Yano *et al.*, 1995; Ohnishi, 1998; T. Ohnishi and T. Yano, pers. commun.).

The second, amphipathic fragment is presumed to connect the NADH dehydrogenase fragment with the membrane fragment and is, therefore, called connecting fragment (Leif *et al.*, 1995). The isolated connecting

fragment shows no reactivity toward NADH or quinones (Leif *et al.*, 1995). It is composed of the subunits NuoB, CD, and I and contains the EPR-detectable Fe–S cluster N2 (Leif *et al.*, 1995). This cluster is most likely located on subunit NuoB as indicated by EPR-spectroscopic analysis of site-directed mutants of *E. coli* and *Yarrowia lipolytica* (Friedrich, 1998; Ahlers *et al.*, 2000), although site-directed mutagenesis of the *Rhodobacter capsulatus* homolog of NuoI indicated that N2 could also be localized on this subunit (Chevallet *et al.*, 1997). The subunit NuoI contains two conserved binding motifs for the ligation of tetranuclear Fe–S clusters (Fearnley and Walker, 1992; Weidner *et al.*, 1993). However, it has recently been shown by means of combined UV/vis and EPR spectroscopy that complex I contains two additional Fe–S clusters located on NuoI that have not yet been deteced by EPR spectroscopy (Friedrich *et al.*, 2000; Rasmussen *et al.*, 2001). A redox difference absorption of a chromophore was detected in isolated complex I that did not arise from one of the known cofactors. This absorption is also present in the connecting fragment of the *E. coli* complex I and other enzymes containing a homolog of NuoI (Friedrich *et al.*, 2000; Rasmussen *et al.*, 2001). These Fe–S clusters have been named N6a and N6b because they both exhibit the same pH-independent midpoint potential (Rasmussen *et al.*, 2001). The close contact between NuoB and NuoI (see below) might explain why mutations in NuoI have an effect on cluster N2 on NuoB (Chevallet *et al.*, 1997).

Finally, the membrane fragment is composed of the seven hydrophobic subunits NuoA, H, J, K, L, M, and N, which are mitochondrially encoded in eukaryotes. NuoL, M, and N most likely share a common ancestor and arose by gene triplication (Kikuno and Miyata, 1985; Fearnley and Walker, 1992). They are related to subunits of a novel type of K^+ or Na^+/H^+ antiporter that has been detected in *Sinorhizobium meliloti* and *Bacillus* sp. C-125 (Putnoky *et al.*, 1998; Hamamoto *et al.*, 1994; Friedrich *et al.*, 1995; Friedrich and Weiss, 1997; Kosono *et al.*, 1999). Thus far, no cofactor has been detected in this fragment. However, there are some lines of evidence for the existence of a high-potential cofactor in complex I that might be located in the membrane fragment (Friedrich, 1998; Friedrich *et al.*, 2000; Schulte *et al.*, 1998, 1999). The chemical structure of this cofactor is not yet known, but from its UV/vis and FT–IR spectra it has been speculated to be a modified amino acid with a quinoid structure (Schulte *et al.*, 1999; Friedrich *et al.*, 2000; see also U. Schulte, this issue).

The three fragments described above correspond roughly to the modules from which the complex evolved and which are present in other bacterial enzymes as well. The NADH dehydrogenase fragment is capable of catalyzing the NADH/ferricyanide activity and represents the electron input module of complex I (Braun *et al.*, 1998). It is an evolutionarily conserved protein module for the reversible transformation of one two-electron to two one-electron transfer steps (Friedrich and Weiss, 1997). The NADH dehydrogenase module is also part of several bacterial NAD-dependent hydrogenases and formate dehydrogenases (Bowien and Schlegel, 1981; Pilkington *et al.*, 1991; Friedrich and Weiss, 1997; Oh and Bowien, 1998).

The connecting fragment represents a part of the hydrogenase module (Friedrich and Scheide, 2000). The four subunits comprising this fragment are homologous to subunits of a family of membrane-bound multisubunit hydrogenases (Böhm *et al.*, 1990; Sauter *et al.*, 1992; Friedrich *et al.*, 1993; Albracht, 1993; Weidner *et al.*, 1993). In addition to the subunits of the connecting fragment, the hydrogenase module (defined by sequence homology) also contains the hydrophobic subunits NuoH and NuoL, which are present in the preparation of the membrane fragment (Leif *et al.*, 1995). Beside these two subunits, the membrane fragment contains the transporter module (defined by sequence homology). The name of this module derives from the homology of NuoM and N to subunits of K^+ or Na^+/H^+ antiporter mentioned above (Friedrich and Scheide, 2000).

OCCURRENCE OF THE COMPLEX I HOMOLOGS

The complex I of most bacteria and mitochondria couples the transfer of electrons from NADH to a quinone with translocation of protons across the membrane (Weiss *et al.*, 1991; Walker, 1992; Brandt, 1997; Friedrich and Scheide, 2000). It has been reported that in some bacteria complex I might pump Na^+ ions (Krebs *et al.*, 1999; Steuber *et al.*, 2000). Generally, the bacterial complex consists of 14 different subunits and is considered as a structural minimal form of NADH:ubiquinone oxidoreductase (Weidner *et al.*, 1993; Xu *et al.*, 1993; Yano *et al.*, 1997; Dupuis *et al.*, 1998) . Seven subunits are peripheral proteins bearing all known redox groups of complex I. The remaining seven subunits are most hydrophobic proteins predicted to fold into 54 α-helixes across the membrane. Little is known about their function, but they are most likely involved in proton translocation (see below).

The mitochondrial complex I of eukaryotes was adapted from a bacterial progenitor in the course of endosymbiosis. In addition to the homologs of the 14 bacterial subunits, it contains up to 27 extra proteins. The homologs of the seven hydrophobic subunits are mitochondrially encoded in all eukaryotes (Walker, 1992; Schulte and Weiss, 1995). Most of the extra proteins of beef heart and *N. crassa* show a small but distinct

sequence identity (Schulte and Weiss, 1995; Videira, 1998), their function for the mitochondrial complex I, however, remains unclear. Two of them appear to be involved in a biosynthetic pathway with a specialized means for respiration (Schneider *et al.*, 1997). One is an acyl carrier protein with a phosphopantetheine group (Runswick *et al.*, 1991; Sackmann *et al.*, 1991) and the other belongs to a heterogeneous family of reductases/isomerases with a conserved nucleotide-binding site (Walker, 1992; Schulte *et al.*, 1999; see also U. Schulte, this issue).

Cyanobacteria contain homologs of 11 out of the 14 bacterial complex I genes. Three genes coding for NuoE, F, and G, which constitute the electron input part of complex I, namely, the NADH dehydrogenase module, are missing (Friedrich *et al.*, 1995; Friedrich and Scheide, 2000). It has been shown that cyanobacteria indeed contain a homolog of complex I (Berger *et al.*, 1991) with an electron input module different from the one of the bacterial complex (Boison *et al.*, 1999). The cyanobacterial complex is most likely involved in photosynthetic electron transfer involving photosystem I (Mi *et al.*, 1992) and it has been discussed that either ferredoxin, NADPH or NADH might function as electron donor (Berger *et al.*, 1993; Friedrich *et al.*, 1995; Guedeney *et al.*, 1996). The cyanobacterial complex I should, therefore, work either as ferredoxin:plastoquinone or NAD(P)H:plastoquinone oxidoreductase.

The plastidal complex of higher plants was adapted from a cyanobacterial progenitor by endosymbiosis, as revealed by the presence of the homologs of the 11 cyanobacterial complex I genes in the plastidal genome (Shimada and Sugiura, 1991; Ogawa, 1991; Friedrich *et al.*, 1995). Like in cyanobacteria, the plastidal complex is involved in cyclic electron transfer in the light and most likely in chlororespiration in the dark (Burrows *et al.*, 1998; Kofer *et al.*, 1998). A complex I preparation from pea thylacoids contains 16 subunits (Sazanov *et al.*, 1998). It has been proposed that this preparation includes the subunits constituting the NADH dehydrogenase part (Sazanov *et al.*, 1998). However, the genes coding for these subunits have not yet been detected (Grohmann *et al.*, 1996).

The complex I homolog of archaea works as $F_{420}H_2$ dehydrogenase (Bäumer *et al.*, 2000; Friedrich and Scheide, 2000). Like cyanobacteria, some archaea contain 11 of the 14 bacterial complex I subunits, while the subunits that comprise the NADH dehydrogenase module are missing. Most likely this module has been replaced by an $F_{420}H_2$ dehydrogenase module exhibiting an analogous function. $F_{420}H_2$ is a reversible hydride donor like NADH. The genes for an $F_{420}H_2$:menaquinone oxidoreductase have been found in the hyperthermophilic sulfate-reducing archaeon *Archaeoglobus fulgidus* (Klenk *et al.*, 1997). In the methanogenic archaeon *Methanosarcina mazei* methanophenazine, a hydrophobic 2-hydroxyphenazine with an ether-bridged polyisoprenoid side chain has been identified as electron acceptor indicating the presence of a $F_{420}H_2$:methanophenazine oxidoreductase (Abken *et al.*, 1998; Bäumer *et al.*, 2000; Brüggemann *et al.*, 2000). In both organisms, the complex I homolog is the main generator of proton-motive force (Kunow *et al.*, 1994; Deppenmeier *et al.*, 1999). The electron donor of the complex I homolog of the crenarchaeon *Aeropyrum pernix* is not yet known (Kawarabayasi *et al.*, 1999; Friedrich and Scheide, 2000).

All members of the complex I family contain homologs of 11 subunits that should constitute the structural framework for proton translocation and quinone binding, despite the fact that the electron donor, the electron input module, and the electron acceptor may vary (Friedrich and Scheide, 2000).

MODULAR EVOLUTION OF COMPLEX I

Analysis of phylogenetic calculations revealed the modular evolution of complex I (Friedrich and Weiss, 1997; Friedrich and Scheide, 2000; Fig. 1). The electron transfer proteins NuoB and NuoD, which are also present in nowadays soluble [NiFe] hydrogenases can be traced back to the oldest ancestor. NuoB and the small subunit of the hydrogenases share the binding motif for the ligation of one tetranuclear Fe–S cluster. Most of the amino acids that are presumed to constitute a proton pathway in the large subunit of the hydrogenases are conserved in NuoD, while the residues that ligate the active [NiFe] center of hydrogenases are not conserved (Friedrich *et al.*, 1993; Albracht, 1993; Volbeda *et al.*, 1995; Friedrich and Weiss, 1997). The combination of the common ancestor of complex I and the soluble hydrogenases with the ferredoxin-type subunit NuoI, the ion-translocating NuoL, as well as NuoH, which is involved in quinone binding, gave rise to a common ancestor of complex I and a family of membrane-bound multisubunit hydrogenases. This common ancestor is present in the nowadays complex I as hydrogenase module (Fig. 1).

The family of the membrane-bound multisubunit hydrogenases includes the Ech hydrogenase of *Methanosarcina barkeri*, the hydrogenase 3 and 4 of the formate hydrogenlyase system of *E. coli*, the CO-induced hydrogenase of *Rhodospirillum rubrum*, and several other enzymes from bacteria and archaea (Böhm *et al.*, 1990; Fox *et al.*, 1996a; Andrews *et al.*, 1997; Künkel *et al.*,

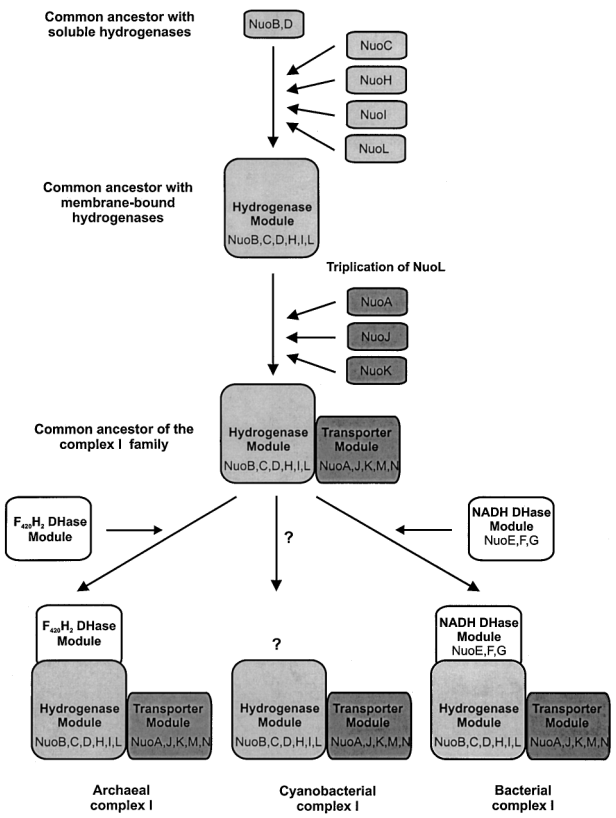

Fig. 1. Hypothetical scheme of the modular evolution of complex I. An ancestral hydrogenase made up by the progenitors of NuoB and D (upper block) evolved by addition of a ferredoxin (NuoI), a transport protein (the progenitor of NuoL, M, and N), a quinone-binding protein (NuoH), and a protein of yet unknown function (NuoC). This led to the formation of the common ancestor of complex I and the membrane-bound hydrogenases. This enzyme was equipped with further proteins by triplication of the transporter protein NuoL and addition of NuoA, J, and K leading to the common ancestor of complex I of bacteria, cyanobacteria, and archaea. The bacterial and the archaeal complex I emerged by acquisition of the NADH dehydrogenase module (NuoE, F, and G) and the $F_{420}H_2$ dehydrogenase module, respectively. The nature of the module adapted to build the cyanobacterial complex is not yet known. It cannot be excluded that the latter possibly evolved also by adaption of the NADH dehydrogenase module or from alterations of the already existing hydrogenase module. The hydrogenase module is shown in light grey, the transporter module in dark grey, and the different electron input modules in white.

1998; Meuer *et al.*, 1999; Teerstegen and Hedderich, 1999; Friedrich and Scheide, 2000). These hydrogenases seem to be involved in electron transfer from low-potential electron donors to protons leading to the production of hydrogen (Teerstegen and Hedderich, 1999). They all contain homologs of the hydrophilic subunits NuoB, C, D, and I and of the hydrophobic subunits NuoH and L. In some cases, a homolog of NuoC is missing and NuoI has been replaced by polyferredoxins, which might fulfill a related function. Some of these hydrogenases contain three to ten additional hydrophobic subunits, which show no sequence homology to complex I subunits (Teerstegen and Hedderich,1999; Friedrich and Scheide, 2000).

Upon evolutionary division, the membrane part of complex I was equipped with further membrane proteins by acquisition of NuoA, J, and K and by triplication of the gene of the ion-translocating protein NuoL (Fig. 1). Nothing is known about the function of NuoA, J, and K, but homologs of the seven hydrophobic subunits including NuoA, J, and K are present in all members of the complex I family. The subunits NuoM and N are related to bacterial antiporters (see above), therefore, the membrane-bound extension of the hydrogenase module was called the transporter module (Friedrich and Scheide, 2000). The hydrogenase module together with the transporter module build the common ancestor of the complex I homologs of bacteria, archaea, and eukarya (Fig. 1).

The "classical" complex I of bacteria and eukarya adapted the NADH dehydrogenase module, which led to the formation of nowadays proton-pumping NADH: quinone oxidoreductase (Fig. 1). The homolog in archaea adapted the $F_{420}H_2$ dehydrogenase module, which led to the formation of the $F_{420}H_2$:quinone oxidoreductase. It remains an open question whether the cyanobacterial complex I homolog was equipped with its own electron input module for ferredoxin or NAD(P)H or whether it evolved by alterations of the already existing hydrogenase module. From the modular structure and evolution of complex I it is clear that nowadays homologs of complex I are made up of three major domains: a variable electron input module, a conserved hydrogenase, and transporter module (Fig. 1).

A MODULAR MECHANISM FOR COMPLEX I?

It is reasonable to assume that the function of the modules is similar in complex I and the related bacterial enzymes. Thus, one can speculate about the function of the individual modules within complex I. The NADH dehydrogenase module is not present in the complex I homologs of cyanobacteria and archaea, while it is found in other soluble enzymes. These enzymes like the NAD^+-reducing hydrogenase or formate dehydrogenase from *Ralstonia eutropha* are not directly involved in energy coupling (Massanz *et al.*, 1998; Oh and Bowien, 1998). Furthermore, the Gibbs free energy available from the electron transfer reactions between the cofactors of this module is not sufficient to drive proton translocation (Sled' *et al.*, 1993; Braun *et al.*, 1998; Friedrich, 1998; Ohnishi, 1998). The isolated NADH dehydrogenase fragment from *E. coli* has been reduced with NADH and the reoxidation kinetics of the FMN and the Fe–S clusters N1b, N1c, N3, and

N4 have been followed by UV/vis and EPR spectroscopy (Scheide *et al.*, 2001). It was demonstrated that all cofactors reoxidize with the same rate, which would be unlikely if one of the electron transfer reactions would be coupled to proton translocation (Scheide *et al.*, 2001). Taking together, it is very unlikely that the electron transfer within the NADH dehydrogenase module is coupled with proton translocation, although it contains most of the known cofactors of complex I. Its role may be to collect electrons and pass them to the hydrogenase module in order to keep the Fe–S clusters of this module in a reduced state (Fig. 2).

The hydrogenase module is homologous to the nowadays membrane-bound multisubunit hydrogenases. There are some indications that members of the family of membrane-bound multisubunit hydrogenases might constitute a site of energy conservation. The strongest evidence for this suggestion stems from the finding that *R. rubrum* is able to grow on CO, which is converted to CO_2 and H_2 in the dark. Therefore, this reaction has to be coupled with energy conservation. As the CO-induced hydrogenase is the only membrane-bound enzyme in this reaction, it is most likely the involved proton pump (Fox *et al.*, 1996b). It has been proposed that the hydrogenase 4 of the *E. coli* formate hydrogenlyase system is a proton pump as well (Andrews *et al.*, 1997). Although this reaction does not provide sufficient energy under standard conditions, it is assumed that the reaction becomes more exergonic under low hydrogen partial pressures (Andrews *et al.*, 1997). *Methanosarceina barkeri* is capable of synthesizing CO_2 and H_2 from CO under certain growth conditions. This reaction is coupled with a proton translocation (Bott *et al.*, 1986; Bott and Thauer, 1989). It is assumed that the Ech hydrogenase is the energy-converting enzyme in this metabolic pathway (Künkel *et al.*, 1998). Because of that the membrane-bound hydrogenases are most likely proton pumps with a stoichiometry of 1 H^+/e^-.

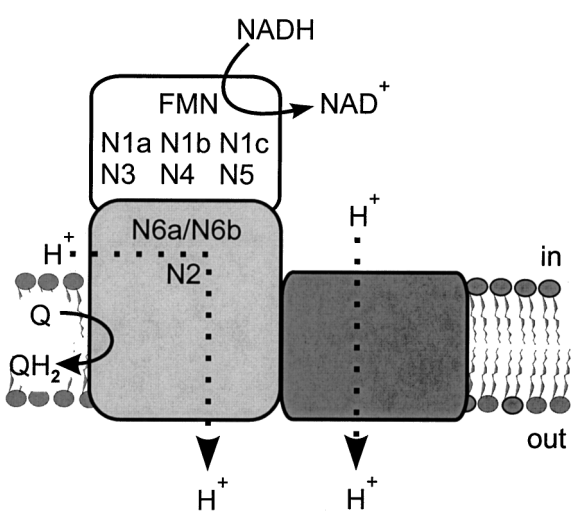

Fig. 2. Hypothetical mechanism of complex I. The NADH dehydrogenase module shown in white accepts electrons from NADH and transfers them to the hydrogenase module (light grey) via the FMN and the Fe–S clusters N1a, N1b, N1c, N3, N4, and N5. The electrons are accepted by the Fe–S clusters N6a and N6b. Further electron transfer to N2 and finally ubiquinone is linked with a redox-driven proton translocation. It is assumed that the overall electron transfer mediates conformational changes to the transporter module (dark grey) by a yet unknown coupling mechanism. The transporter module would provide a second, conformation-driven coupling site.

This would imply that the hydrogenase module provides one coupling site in complex I as well, although there are a few differences between the hydrogenases and complex I. The hydrogenases contain a [NiFe] active site, which is missing in complex I (van der Spek *et al.*, 1996) and react with hydrogen, which is not a substrate for complex I. On the other hand, complex I uses quinones as electron acceptors, which are not a substrate for the hydrogenases (Meuer *et al.*, 1999). Inhibitor-insensitive complex I mutants of *R. capsulatus* and labeling experiments with inhibitors of the complex I quinone-binding site have suggested that the homologs of NuoB, D, and H are involved in quinone binding (Earley *et al.*, 1987; Darrouzet *et al.*, 1998; Schuler *et al.*, 1999; Prieur *et al.*, 2001). All these subunits are part of the hydrogenase module (Fig. 2). Therefore, it was assumed that during evolution of complex I, the common ancestor with the membrane-bound hydrogenases has lost its [NiFe] active site and gained the ability to react with quinones (Friedrich and Scheide, 2000). For substrate reduction, both enzymes, complex I as well as the membrane-bound hydrogenases, use protons from the cytoplasmic site of the membrane (Weiss *et al.*, 1991; Walker, 1992; Brandt, 1997; Meuer *et al.*, 1999). Furthermore, most of the amino acids proposed to form a proton channel within the large subunit of hydrogenases are conserved in the homologous complex I subunit (see above). This might indicate that the overall topology of the proton pump in the hydrogenase module of complex I and the membrane-bound hydrogenases is rather similar.

The hydrogenase module is assumed to accept electrons from the NADH dehydrogenase module by means of the Fe–S clusters N6a and N6b on NuoI. Within this module, the electrons are transferred via cluster N2 on NuoB to the quinone reduction site (Fig. 2). The proton translocation coupled with this electron transfer might be accomplished by NuoL, which belongs to the ion-translocating proteins. From sequence comparisons, it has been proposed that the homologs of NuoI represent a special class of an 8Fe–ferredoxin, which works as the electrical driving unit for a proton pump (Albracht and Hedderich, 2000).

The sequence motif for binding of the two 4Fe–4S clusters contains conserved acidic amino acids that might get protonated upon reduction of the Fe–S clusters. It is assumed that the electron transfer involving these Fe–S clusters is charge compensated by the protonation of these amino acids (Albracht and Hedderich, 2000). However, it has been shown experimentally that the midpoint potential of the Fe–S clusters on this subunit, N6a and N6b (Table I), is not pH dependent (Friedrich *et al.*, 2000; Rasmussen *et al.*, 2001). On the other hand, the midpoint potential of N2 located on NuoB is pH dependent. This has led to the assumption that N2 is involved in proton translocation (Ingledew and Ohnishi, 1980; Ohnishi, 1998) and its role as a redox Bohr group has been discussed (Brandt, 1997). The pH dependence of N2 exhibits a negative slope implying that reduction of N2 is coupled to a proton uptake and reoxidation of N2 leads to a deprotonation of the redox Bohr group (Ingledew and Ohnishi, 1980; Brandt, 1997).

Recently, it has been shown by means of combined electrochemistry and FT–IR spectroscopy that the electron transfer involving N2 is coupled with a protonation/deprotonation of an acidic amino acid (Hellwig *et al.*, 2000). Electrochemically induced FT–IR difference spectra of the *E. coli* complex I were obtained at a potential where the FMN and all Fe–S clusters were oxidized, but N2 was still reduced and at a potential where the FMN and all Fe–S clusters including N2 were oxidized. The difference FT–IR spectra revealed changes in the protein structure and in the protonation states of amino acids that are due to the redox reaction of N2 (Hellwig *et al.*, 2000).

The main feature of the difference spectra is a positive absorption around 1710 cm^{-1}, which has been attributed to protonated aspartic or glutamic side chains and two negative absorptions at 1556 and 1410 cm^{-1} attributed to the respective signals for the deprotonated forms. These data indicate that an Asp or Glu side chain is protonated coupled to the oxidation of N2 and, therefore, does not represent the protonation of a redox Bohr group associated with N2. It has been proposed that the proton being released from the redox Bohr group upon oxidation of N2 is picked up by the acidic amino acid as observed by FT–IR spectroscopy (Hellwig *et al.*, 2000). It is possible that such a proton transport chain driven by the redox reaction of N2 represents one energy-coupling site in complex I. It can be speculated that the protonation of an acidic amino acid associated with the oxidation of N2 is the molecular switch involved in proton translocation in the hydrogenase module of complex I as well as in the membrane-bound multisubunit hydrogenases. The hydrogenase module might, therefore, represent a conserved device for redox-driven proton translocation.

The FT–IR difference spectra contained even more information. The electrochemically induced redox difference spectra of the fully oxidized minus the fully reduced complex I show strong positive and negative signals in the amide I region from 1700 to 1600 cm^{-1} (Hellwig *et al.*, 2000). They reflect large reorganizations of the polypeptide backbone accompanied with the reaction of complex I. In this respect, it is striking that the transporter module contains the subunits NuoN and M, which are homologous to K^+ and Na^+/H^+ antiporters (Friedrich and Weiss, 1997). Transporters translocate substrates across the membrane by changing their conformation (Sahin-Toth *et al.*, 2000). The modular evolution of complex I and the large conformation changes associated with the reaction of complex I give rise to the idea that the transporter module of complex I might represent a second coupling site in complex I that works as a conformation-driven proton pump (Fig. 2).

It has been known for some time, that binding of substrates or inhibitors to complex I or the reduction of the complex leads to changes in the observed fragmentation or crosslink pattern (Rossi *et al.*, 1965; Gondal and Anderson, 1985; Kotlyar and Gutman, 1992; Sled' and Vinogradov, 1993; Belogrudov and Hatefi, 1994; Yamaguchi *et al.*, 1998). It is not yet clear whether these conformation changes are related to proton pumping in complex I, although it has been proposed that energy coupling in complex I may involve protein conformation changes as a key step (Yamaguchi *et al.*, 1998; Sazanov *et al.*, 2000).

Recent electron microscopic investigations in our group using the isolated complex I from *E. coli* have shown that the complex is indeed made up of three major domains, as depicted in Fig. 2 (B. Böttcher *et al.*, in preparation). The molecular mass of these three domains fit nicely with the masses of the modules as derived from the corresponding DNA sequences. Electron micrographs of oxidized and reduced complex I single particles revealed a different conformation of the complex depending on its redox state (B. Böttcher *et al.*, in preparation). These data fit with the idea that the transporter module represents a conserved device for conformation-driven proton translocaction.

Based on this, one can speculate that complex I contains two coupling sites and is a chimera of a redox-driven and a conformation-driven proton pump. The redox-driven part would be provided by the hydrogenase module with the redox reaction of cluster N2 as the molecular switch for proton pumping. The conformation-driven part would be provided by the transporter module with the subunits NuoM and N as the second coupling site within complex I. The coupling between the electron transfer reaction and the conformational changes remains to be established.

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