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The Origin of Cluster N2 of the Energy-Transducing NADH–Quinone Oxidoreductase: Comparisons of Phylogenetically Related Enzymes[1]

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NADH–quinone (Q) oxidoreductase is a large and complex redox proton pump, which utilizes the free energy derived from oxidation of NADH with lipophilic electron/proton carrier Q to translocate protons across the membrane to generate an electrochemical proton gradient ($\Delta\tilde{\mu}_{H^+}$). Although its molecular mechanism is largely unknown, recent biochemical, biophysical, and molecular biological studies have revealed that particular subunits and cofactors play an essential role in the energy-coupling reaction. Based on these latest experimental data, we exhaustively analyzed the sequence information available from evolutionarily related enzymes such as [NiFe] hydrogenases. We found significant and conserved sequence differences in the PSST/Nqo6/NuoB, 49kDa/Nqo4/NuoD, and ND1/Nqo8/NuoH subunit homologs between complex I/NDH-1 and [NiFe] hydrogenases. The alterations, especially in the postulated ligand motif for cluster N2 in the PSST/Nqo6/NuoB subunits, appear to be evolutionarily important in determining the physiological function of complex I/NDH-1. These observations led us to propose a hypothetical evolutionary scheme: during the course of evolution, drastic changes have occurred in the putative cluster N2 binding site in the PSST/Nqo6/NuoB subunit and the progenitors of complex I/NDH-1 have concurrently become to utilize a lipophilic electron/proton carrier such as Q as its physiological substrate. This scheme provides new insights into the structure and function relationship of complex I/NDH-1 and may help us understand its energy-coupling mechanism.

KEY WORDS: NADH–quinone oxidoreductase; complex I; NDH-1; iron–sulfur cluster, energy transduction; evolution.

INTRODUCTION

NADH–quinone (Q) oxidoreductase (EC 1.6.99.3) is located in the inner membrane of mitochondria or in the cytoplasmic membrane of bacteria and plays an important role in the energy transduction. The mitochondrial enzyme is dubbed as complex I whereas the bacterial counterpart is called NDH-1. The enzyme complex is a main entry site of reducing equivalents to the respiratory chain. The complex I/NDH-1 oxidizes NADH, which is derived from the metabolism of sugars and fatty acids, and transfers electrons to a lipophilic electron/proton carrier Q (ubiquinone, menaquinone, plastoquinone, or rhodoquinone, etc.).

$$\mathrm{NADH} + \mathrm{Q} + \mathrm{H^+} + n\mathrm{H^+_{in}} \leftrightarrow \mathrm{NAD^+} + \mathrm{QH_2} + n\mathrm{H^+_{out}}\ (n\text{: number of pumped protons})$$

Coupled to the oxidation-reduction reaction, the enzyme complex pumps protons from one side of the membrane to the other, generating an electrochemical proton gradient ($\Delta\tilde{\mu}_{H^+}$).

[1] Key to abbreviations: complex I, mitochondrial proton-pumping NADH:ubiquinone oxidoreductase; NDH-1, bacterial energy-transducing NADH:quinone oxidoreductase; Fe–S, iron–sulfur cluster; FMN, flavin mononucleotide; EPR, electron paramagnetic resonance; SMP, submitochondrial particles; Q, quinone; SQ, emiquinone; $\Delta\tilde{\mu}_{H^+}$, electrochemical proton gradient; E_m, midpoint redox potential; $F_{420}H_2$, (*N*-L-lactyl-γ-glutamyl)-L-glutamic phosphodiester of 7,8-didemethyl-8-hydroxy-5-deazariboflavin-5′-phosphate, reduced form; TDP, trifluoromethyldiazirinyl-pyridaben.

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Complex I/NDH-1 is the least understood enzyme complex in the oxidative phosphorylation system and its molecular mechanism of action remains unknown. However, recently its physiological importance has increasingly been elevated. It is generally accepted that some neurodegenerative disorders such as LHON disease, Leigh syndrome, and Parkinson's disease are associated with complex I deficiency (Wallace, 1992; Liang and Wong, 1998). Complex I activity may be impaired by genetic mutations in mitochondrial (mt) and nuclear (n) DNA as well as environmental substances, such as drugs and pesticides (Wallace, 1992; Smeitink and van den Heuvel, 1999; Betarbet *et al*., 2000). This type of decrease of complex I activity may reduce mitochondrial oxidative phosphorylation and cellular activity.

Our ultimate goal is to understand the molecular mechanism by which complex I/NDH-1 utilizes the redox energy to translocate protons (Belogrudov and Hatefi, 1994; Dutton *et al*., 1998; Yagi *et al*., 1998; Brandt, 1999). Recently, significant progress has been made and it has become apparent that particular subunits and cofactors play essential roles in the energy-coupling reaction of the enzyme complex. It is generally accepted that the electron transfer from cluster N2 to the Q pool is linked to the energy-coupling reaction. Recent biochemical analyses have clearly pointed out specific subunits (PSST/Nqo6/NuoB, 49kDa/Nqo4/NuoD, and ND1/Nqo8/NuoH), as key players in such coupling reactions (Leif *et al*., 1995; Darrouzet and Dupuis, 1997; Okun *et al*., 1999; Schuler *et al*., 1999).

It is also important to realize the effect of what this evolution has had on the development of these subunits and cofactors. The rapidly growing genomic sequence database provides useful information that helped us track the evolutionary path of complex I/NDH-1 (Friedrich and Scheide, 2000). This allows us to trace the history of several essential subunits and cofactors. Our analysis reveals that significant sequence alterations occurred in the past, especially in the postulated cluster N2 binding site in the PSST/Nqo6/NuoB subunit, and that such evolutionary events seem to be critical turning points in determining the enzymic properties of complex I/NDH-1.

In this mini-review, we briefly summarize the current understanding of complex I/NDH-1 and discuss the probable origin of cluster N2 as revealed by comparative sequence analyses with phylogenetically related enzymes.

GENERAL PROPERTIES OF MITOCHONDRIAL COMPLEX I AND BACTERIAL NDH-1

Complex I from bovine heart mitochondria has been studied extensively in the past (Hatefi, 1985). Bovine heart complex I is composed of at least 42 subunits and its total molecular mass is estimated to be about 900 kDa (Walker, 1992). Seven subunits, called ND1-6 and 4L, are encoded in mtDNA and the others (35 subunits) are encoded in nDNA. All latter subunits, as well as cofactors, are synthesized and transported to the mitochondrial inner membrane for assembly and maturation of complex I. Hence, the biosynthesis of complex I includes many steps and involves a number of genes, most of which remain to be identified.

The bacterial NDH-1 has a much simpler subunit composition than that of its mitochondrial counterparts, however, it shares many structural and enzymic properties similar to the other respiratory enzyme complexes, such as quinol-cytochrome *c* oxidoreductase (complex III) and terminal oxidase (complex IV) (Yagi *et al*., 1998). The NDH-1 has been isolated from several bacteria and extensively studied in *Paracoccus denitrificans* (Yagi *et al*., 1993), *Escherichia coli* (Friedrich, 1998), *Rhodobacter capsulatus* (Dupuis *et al*., 1998), and *Thermus thermophilus* HB-8 (Yano *et al*., 1997). In general, the NDH-1 is composed of 14 subunits, all of which are conserved in mitochondrial complex I. Therefore, the 14-subunit structure represents a minimal functional unit necessary to catalyze the energy-transducing NADH-quinone oxidoreductase reaction. Although a high-resolution three-dimensional structure is not available, it is known that complex I/NDH-1 seems to exhibit a unique L-shape appearance composed of a hydrophobic membrane and a hydrophilic promontory section (Grigorieff, 1998; Guènebaut *et al*., 1998). The membrane part is composed of seven hydrophobic ND subunits, whereas the promontory part contains most of the hydrophilic subunits.

Recently, genomic sequences of a number of microorganisms revealed that NDH-1 gene homologs are widely distributed across the prokaryote kingdom from bacteria to archaea. These complex I/NDH-1 and its homologous enzymes possess structural and functional variations (Friedrich and Scheide, 2000), indicating that they may have diverged from a common ancestor during the course of evolution.

ELECTRON TRANSFER COMPONENTS

In general, complex I/NDH-1 contains one molecule of noncovalently bound FMN and as many as eight Fe–S clusters ($2 \times$ [2Fe–2S] type and $6 \times$ [4Fe–4S] type) as electron transfer components (Ohnishi, 1998). An additional Fe–S cluster (designated N1c) seems to be present in some bacterial NDH-1, such as *E. coli* (Leif *et al*.,

1995) and *T. thermophilus* (Yano *et al*., 1997). Six Fe–S clusters are detectable by EPR spectroscopy and designated N1a and N1b for [2Fe–2S] clusters and N2, N3, N4, and N5 for [4Fe–4S] clusters. Identification of the location of these cofactors is of great importance in determining the electron transfer pathway. Based on sequence analyses and biochemical studies, such as subunit expression experiments, locations of some of the Fe–S clusters have been determined thus far. The NADH-oxidizing unit, called flavoprotein (FP) subcomplex, is composed of the 51kDa/Nqo1/NuoF and the 24kDa/Nqo2/NuoE subunits. This unit contains FMN and clusters N1a and N3 (Ohnishi *et al*., 1985; Yano *et al*., 1996). FMN is an indispensable component for the oxidation of NADH that initiates the electron transfer reaction (Yano *et al*., 1996). The 75kDa/Nqo3/NuoG subunit contains three Fe–S clusters N1b, N4, and elusive N5 in its N-terminal domain, wiring electrons from the NADH-oxidizing unit to cluster N2 (Yano *et al*., 1995). An additional Fe–S cluster (N1c) seems to be located in this subunit (Leif *et al*., 1995; Yano *et al*., 1997). Currently, the location of cluster N2 is a critically important issue because the cluster is directly involved in Q reduction and proton translocation (Ohnishi, 1998). We will discuss the unique properties of cluster N2 in detail later.

Three Fe–S clusters are not fully detectable by EPR spectroscopy for unknown reasons. One of these is cluster N5, the presence of which has been reported for mitochondrial complex I from bovine heart (Ohnishi, 1975) and from yeast *Yallowia lipolytica* (Djafarzadeh *et al*., 2000). The EPR signals of cluster N5 have also been detected in the NDH-1 from *R. sphaeroides* (Sled' *et al*., 1993). Recently, our laboratory obtained experimental evidence that cluster N5 is located in the 75kDa/Nqo3/NuoG subunit. The 75kDa/Nqo3/NuoG subunit contains 11 fully conserved cysteine residues and 1 fully conserved histidine residue in the N-terminal region (Yano *et al*., 1995). This Fe–S cluster-binding domain is homologous to the N-terminal region of Fe-only hydrogenases. The high-resolution three-dimensional structure depicts that the corresponding region coordinates one [2Fe–2S] and two [4Fe–4S] clusters (Peters *et al*., 1998). One of the [4Fe–4S] clusters is ligated by 1 histidine and 3 cysteine residues. Based on this and other information, we propose that the histidine-coordinating [4Fe–4S] cluster corresponds to cluster N5 in complex I/NDH-1 and the novel properties of cluster N5 can be attributed to the mixed ligand coordination (T. Yano, E. Nakamura-Ogino, T. Yogi, and T. Ohnishi, 2000, unpublished results).

Two repeats of typical binding motifs for [4Fe–4S] clusters (CxxCxxC . . . CP . . . CxxCxxC . . . CP) indicated that the TYKY/Nqo9/NuoI subunit contains 2 × [4Fe–4S] clusters. The expression and *in vitro* Fe–S cluster reconstitution experiments of the *P. denitrificans* Nqo9 subunit have shown some molecular properties of the bound 2 × [4Fe–4S] clusters (Yano *et al*., 1999). The complicated EPR spectra clearly revealed the presence of 2 × [4Fe–4S] clusters in the Nqo9 subunit. This subunit has been considered to bear cluster N2 (Chevallet *et al*., 1996). However, the other candidate, the PSST/Nqo6/NuoB subunit, appears to be more qualified as the cluster N2 binding site for several reasons. If this is so, it remains to be clarified why 2 × [4Fe–4S] clusters in the TYKY/Nqo9/NuoI subunit are not readily detectable by EPR spectroscopy *in vivo* (Friedrich *et al*., 2000).

MULTIPLE UBISEMIQUINONE (SQ) SPECIES DETECTED IN COMPLEX I SEGMENT OF THE MITOCHONDRIAL RESPIRATORY CHAIN

It is known that the activated and tightly coupled bovine heart SMP exhibits ubisemiquinone (SQ) free-radical EPR signals at $g = 2.004$ during the steady state oxidation of NADH or succinate (Vinogradov *et al*., 1995; van Belzen *et al*., 1997; Ohnishi *et al*., 1998). At least three distinct SQ species are associated with complex I. All exhibit strikingly different characteristics from each other in terms of physicochemical properties, sensitivities to uncoupler and to complex I/NDH-1 inhibitors, and pH dependence (Yano *et al*., 2000; Magnitsky *et al*., 2001). Thus, clearly multiple Q-binding sites are present in complex I and which function differently from each other. Some of the molecular properties of these complex I-associated SQ species are briefly summarized below.

Fast-Relaxing SQ_{Nf} Species

The SQ_{Nf} species is observed only in tightly coupled SMP and is highly sensitive to uncoupler, indicating that this species is significantly affected by $\Delta\tilde{\mu}_{H^+}$. The SQ_{Nf} species is pH sensitive and can only be detected within a narrow pH range (7.0–8.5) (Yano *et al*., 2000). The resolved SQ_{Nf} EPR spectrum exhibited a narrow line width ($\Delta H_{pp} = \sim 8.4$ gauss) that remained unchanged between pH 7.0 and 8.5 (Yano *et al*., 2000; Magnitsky *et al*., 2001). Based on the observation that its line width was not affected by H_2O/D_2O exchange, it is suggested that the SQ_{Nf} is in an anionic form ($Q^{\bullet -}$) (T. Yano and T. Ohnishi, 2000, unpublished results). It has been suggested that the SQ_{Nf} species is located close to cluster N2. The distance between the SQ_{Nf} species and cluster N2 was estimated to be 8–11 Å based on the following observations: (1) The splitting of the

$g_{//}$ signal of cluster N2 caused by a magnetic interaction between these two spin systems (N2 and SQ_{Nf}) (Vinogradov *et al*., 1995); (2) the spin-relaxation enhancement effects of cluster N2 on the SQ_{Nf} species (Magnitsky *et al*., 2001). This close topographical relationship between the SQ_{Nf} and cluster N2 further suggests that the direct electron transfer taking place from cluster N2 to the primary electron acceptor Q (in the Q_{Nf} site) produces the SQ_{Nf} species in a $\Delta\tilde{\mu}_{H^+}$-dependent manner (Yano *et al*., 2000).

Slow-Relaxing SQ_{slow} Species (SQ_{Ns} and SQ_{Nx})

The slow-relaxing SQ_{slow} species (SQ_{Ns} and SQ_{Nx}) is insensitive to uncoupler (Ohnishi *et al.*, 1998; Magnitsky *et al*., 2001). The SQ_{slow} signal amplitude progressively increased as pH was raised from 6.5 to 9.0, suggesting that either the SQ_{Ns} or the SQ_{Nx} species is in an anionic form (Yano *et al*., 2000). Relatively weak spin-relaxation enhancement of the SQ_{Ns} suggests that its location is far from cluster N2 (estimated >30 Å) (Vinogradov *et al*., 1995). Although these properties of the SQ_{slow} species are reminiscent of those of the SQ_i species in the bc_1 complex, its topological location and thermodynamic properties should be determined in order to better define its roles.

CLUSTER N2: A SPECIALLY DESIGNED ELECTRON TRANSFER COMPONENT

Cluster N2 has been singled out from other Fe–S clusters because of its distinct properties (Ohnishi, 1998). In addition to its high and pH-dependent E_m values and sensitivity to the $\Delta\tilde{\mu}_{H^+}$, the mutual magnetic interaction with the uncoupler-sensitive SQ_{Nf} species, as described above, is a clear indication of its direct role in Q reduction as well as coupled proton translocation. Two subunits, PSST/Nqo6/NuoB and TYKY/Nqo9/NuoI, have been subjected to close scrutiny for the location of cluster N2 (Ohnishi, 1998). These two candidate subunits are amphiphatic. When the whole subunits were individually expressed in *E. coli*, they were both produced in the membranes. The hydrophilic Fe–S cluster domain of the TYKY/Nqo9/NuoI subunit appears to be anchored into the membrane by its hydrophobic N-terminal stretch, probably similar to the Rieske Fe–S protein in complex III (Harnisch *et al.*, 1985). In the PSST/Nqo6/NuoB subunit, hydrophobic patches are scattered throughout the subunit. The subunit may have more extensive contacts with the membrane subunits of the enzyme complex (Yano and Yagi, 1999). This is a criterion expected for the cluster N2 binding subunit which would establish electron transfer to lipophilic Q molecules (Yano *et al*., 2000).

Although we cannot completely rule out the possibility that the TYKY/Nqo9/NuoI subunit bears cluster N2, several lines of experimental data and homology analysis favor the PSST/Nqo6/NuoB subunit. Among 14 subunits, the PSST/Nqo6/NuoB subunit has the highest homology among complex I from various sources. Photoaffinity labeling experiments revealed that the PSST/Nqo6/NuoB subunit has a tight binding site for complex I/NDH-1 inhibitors, which is also conserved (Schuler *et al*., 1999). Labeling of the PSST/Nqo6 subunit by TDP is tightly correlated with the inhibition of NADH oxidation activity, and is completely prevented by other potent complex I inhibitors such as rotenone, piericidin A, and bullatacin. Most of the structurally diverse inhibitors of complex I/NDH-1 exclusively interfere with the electron transfer step between cluster N2 and the Q pool (Friedrich *et al*., 1994; Degli Esposti, 1998; Miyoshi, 1998; Okun *et al*., 1999). It is concluded that the PSST/Nqo6/NuoB subunit is directly involved in the electron transfer from cluster N2 to the Q pool, further suggesting that the subunit contains cluster N2 (Schuler *et al*., 1999).

A ROOT OF COMPLEX I/NDH-1

Recent accumulation of large amounts of sequence information has provided us with an opportunity to track how complex I/NDH-1 has evolved. It is suggested that the present form of complex I/NDH-1 has evolved from an ancestor common to [NiFe] hydrogenases by acquiring several preexisting modules, which provide additional functions (Friedrich and Weiss, 1997; Friedrich and Scheide, 2000). A primitive form of hydrogenase can be seen as a water-soluble [NiFe] hydrogenase composed of a large and a small subunit. The large subunit contains a [NiFe] center, where asymmetric splitting of the hydrogen molecule ($H_2 \leftrightarrow H^+ + H^- \leftrightarrow 2H^+ + 2e^-$) or its reverse reaction takes place. The small subunit contains multiple Fe–S clusters that transfer electrons between the [NiFe] center and electron carriers such as ferredoxin. The hydrogenase large subunit is similar to the 49kDa/Nqo4/NuoD subunit of complex I/NDH-1 whereas the N-terminal region of the small subunit ligating a $[4Fe–4S]_{proximal}$ cluster is homologous to the PSST/Nqo6/NuoB subunit (Albracht, 1993).

At an early stage of its evolution, a water-soluble [NiFe] hydrogenase might have associated with membranes by acquiring membrane proteins which were ancestors of the ND1 and the ND2/4/5 subunits, followed by further changes in Fe–S cluster binding proteins (*e.g.*, TYKY/Nqo9/NuoB subunit). Some descendants

Table I. Comparisons between Complex l/NDH-1 and [NiFe] Hydrogenase Enzyme Complexes

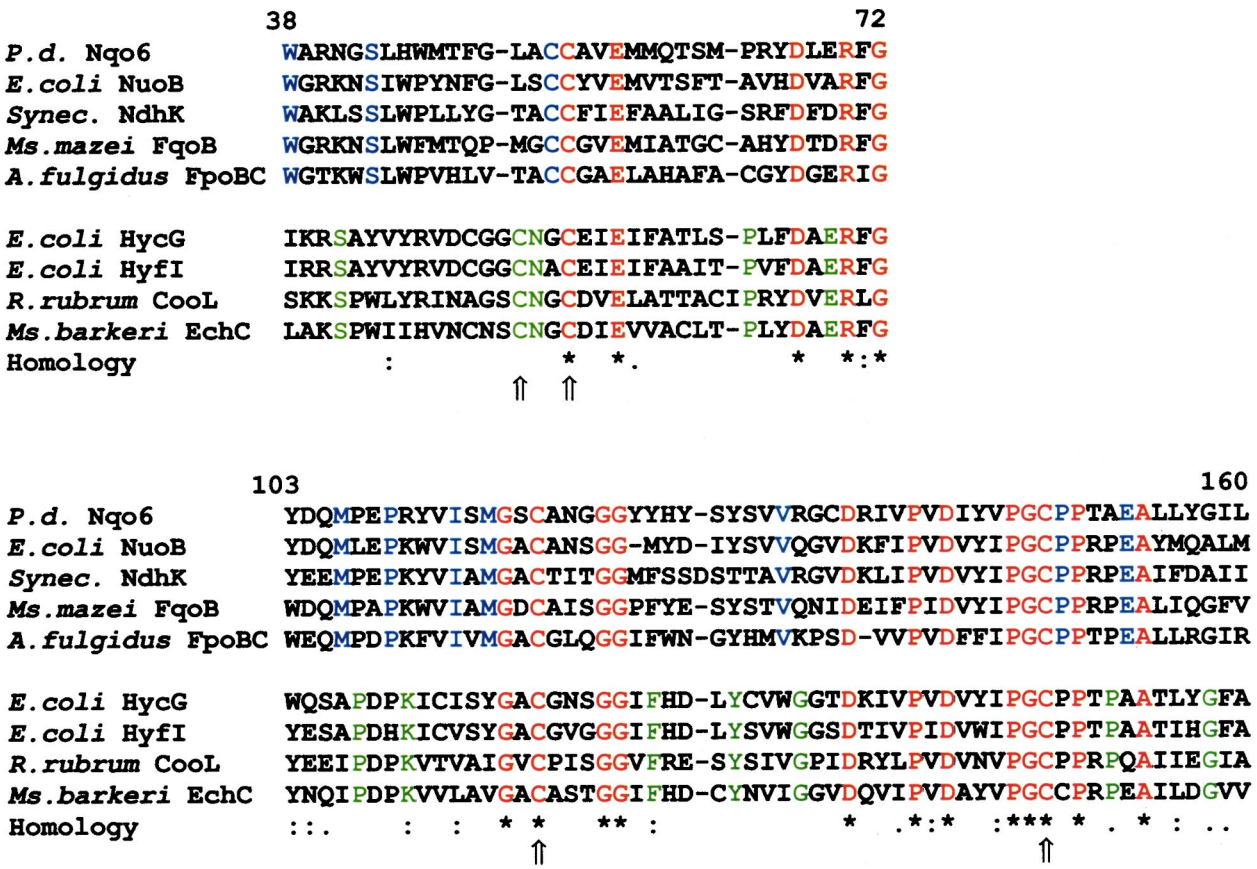
Organisms and enzymes	Physiological reactions	49-kDa subunit homologs	PSST homologs	TYKY homologs	NDI homologs	ND2, 4, or 5 homologs
P. denitrificans	$NADH + Q^a$	Nqo4/NuoD	Nqo6/NuoB	Nqo9/Nuol	Nqo8/NuoH	Nqo12,13,14
E. coli (Nqo/Nuo)	$\rightarrow NAD^+ + QH_2$	No cofactor	[4Fe–4S]	2 × [4Fe–4S]		NuoL,M,N,
Synechocystis.	$2 \times Fd_{red}{}^b + PQ^a$	Ndh	NdhK (PsbG)	Ndhl	Ndha	NdhB,D,F
sp. (Ndh)	$\rightarrow 2 \times Fd_{ox} + PQH_2$	No cofactor	[4Fe–4S]	2 × [4Fe–4S]		
A. fulgidus	$F_{420}H_2 + MQ^c$	FpoD	FpoB	Fpol	FpoH	FpoL,M,N
(Fpo)	$\rightarrow F_{420} + MQH_2$	No cofactor	[4Fe–4S]	2 × [4Fe–4S]		
Ms. mazei	$F_{420}H_2 + MPhe^d$	FqoD	FqoBC[e]	Fqol	FqoH	FqoL,M,N
(Fqo)	$\rightarrow F_{420} + MPheH_2$	No cofactor	[4Fe–4S]	2 × [4Fe–4S]		
Ms. barkeri	$2 \times Fd_{red} + 2H^+$	EchE	EchC	EchF	EchB	EchA
(Ech)	$\rightarrow 2 \times Fd_{ox} + H_2$	[NiFe]	[4Fe–4S]	2 × [4Fe–4S]		
R. rubrum	$CO + H_2O$	CooH	CooL	CooX	CooK	CooM
(Coo)	$\rightarrow CO_2 + H_2$	[NiFe]	[4Fe–4S]	2 × [4Fe–4S]		

[a] *Pracoccus. denitrificans* NDH-1 utilizes ubiquinone (UQ) whereas *E. coli* NDH-1 is thought to use menaquinone (MQ). Cyanobacteria contain plastoquinone (PQ).
[b] Its physiological electron donor has not yet been identified. NAD(P)H and ferredoxin (Fd) are considered.
[c] This menaquinone (MQ) contains a saturated alkyl tail at its 6 position.
[d] Methanophenazine.
[e] FqoBC is a fusion protein between the PSST/Nqo6/NuoB and 30kDa/Nqo5/NuoC subunits.

of such a primitive complex can be found and may include formate hydrogen-lyases (Hyc and Hyf) from *E. coli* (Bohm *et al*., 1990; Sauter *et al*., 1992; Andrews *et al*., 1997), [NiFe] hydrogenase (Ech) from *Methanosarcina barkeri* (Kunkel *et al*., 1998; Meuer *et al*., 1999), and CO-induced hydrogenase (Coo) of *Rhodospilillum rubrum* (Fox *et al*., 1996a,b). These membrane-bound enzyme complexes catalyze the formation of hydrogen from protons in their peripheral part (Table I). Complex I/NDH-1 might have branched out at some stage of evolution and gained a NADH-oxidizing module in order to catalyze the NADH-Q oxidoreductase reaction. Some other members of these complexes may have acquired different modules to use different substrates, such as ferredoxins [or NAD(P)H] and $F_{420}H_2$. They may have evolved to yield the putative ferredoxin[NAD(P)H]:plastoquinone oxidoreductases in cyanobacteria and $F_{420}H_2$:menaquinone oxidoreductase in archaea, *Archaeroglobus fulgidus* (Fqo) (Kunow *et al*., 1994; Bruggemann *et al*., 2000), respectively.

WHEN DID Q REDUCTASE REACTION EMERGE?

A question remains to be answered in the module evolution hypothesis. When and how did complex I/NDH-1 obtain the ability to utilize a lipophilic electron carrier Q? It has been proposed that when a [NiFe] hydrogenase became membrane-bound with ND1 and ND2/4/5 homologs, the progenitor of complex I/NDH-1 might have used lipophilic electron carriers (Friedrich and Weiss, 1997; Friedrich and Scheide, 2000). When we compared the primary sequences of complex I/NDH-1 subunits to those of related members of the [NiFe] hydrogenase family, we noticed significant differences in the primary sequences of some subunits (Fig. 1A and B). Figure 1A shows the sequence alignments of hydrogenase small subunit homologs and PSST/Nqo6/NuoB subunit homologs. Regions containing ligand cysteine residues of a $[4Fe–4S]_{proximal}$ cluster are highly conserved among the [NiFe] hydrogenases (CxxC . . . GxCxxxG . . . PGC) (Fig. 1A). As depicted in their X-ray structures, the $[4Fe–4S]_{proximal}$ cluster is located adjacent to the [NiFe] center in the large subunit and plays an important role in transferring electrons. On the other hand, it is apparent that the PSST/Nqo6/NuoB subunit also conserves four cysteine residues (CC . . . GxCxxxG . . . PGC). However, the arrangement of the first two cysteine residues differs from the arrangement found in the hydrogenase homologs. It should be emphasized that regardless of the types of lipophilic electron carriers they may use (ubiquinone, menaquinone, plastoquinone, rhodoquinone, or methanophenazine), the PSST/Nqo6/NuoB homologs of all enzyme complexes listed in Fig.1A (see Table I) and others (not shown) belong to the complex I/NDH-1 group. Consistent with this observation, the [NiFe] hydrogenases listed in Fig. 1 and others contain the intact [NiFe] center binding motif (RxCxxC . . . DPCxxC). They apparently catalyze the hydrogenase reaction using



A

B

Fig. 1. Sequence comparisons between complex I/NDH-1 and [NiFe] hydrogenases. In (A) the N-terminal and C-terminal regions of the small subunit homolog containing four ligand residues for a [4Fe–4S]$_{proximal}$ cluster and the corresponding regions of the PSST/Nqo6/NuoB subunits are shown. Cysteine residues coordinating a [4Fe–4S]$_{proximal}$ cluster in the small subunits are shown by (⇑). In (B), a comparison of the N-terminal stretch of the ND1/Nqo8/NuoH subunits of complex I/NDH-1 with the corresponding region of their [NiFe] hydrogenase counterparts is shown. This is the most conserved loop region between the predicted first and second α-helixes. The corresponding position of the human pathological ND1/3460 mutation is indicated by (↓). The sequences were aligned using CLASTALW. Fully conserved, highly conserved, and moderately conserved amino acid residues in both groups are indicated by (*), (:), and (.), respectively. Fully conserved amino residues in both groups are also shown by red letters. Amino acid residues conserved in only either group are shown in blue (for complex I/NDH-1 group) and in green (for [NiFe] hydrogenase group). Numbering is according to *P. denitrificans*. The amino acid sequences were obtained from GeneBank as follows: *P. denitrificans* Nqo6 (P29918) and Nqo8 (AAA25595); *E. coli* NuoB (AAC75347) and NuoH (CAA48367); *Synechocystis* sp. PCC6803 NdhK(PsbG) (BAA18284) and NdhA (P26522); *Ms. mazei* FpoB (AAF65732) and FpoH (AAF65735); *A. fulgisdus* FqoBC (AAB89421) and FpoH (NP_070658); *E. coli* HycG (AAC75761) and HycD (AAC75764); *E. coli* HyfI (P77668) and HyfC (AAB88565); *Ms. barkeri* EchC (CAA76119) and EchB (CAA76188); *R. rubrum* Cool (AAC45118) and CooK (AAC45117).

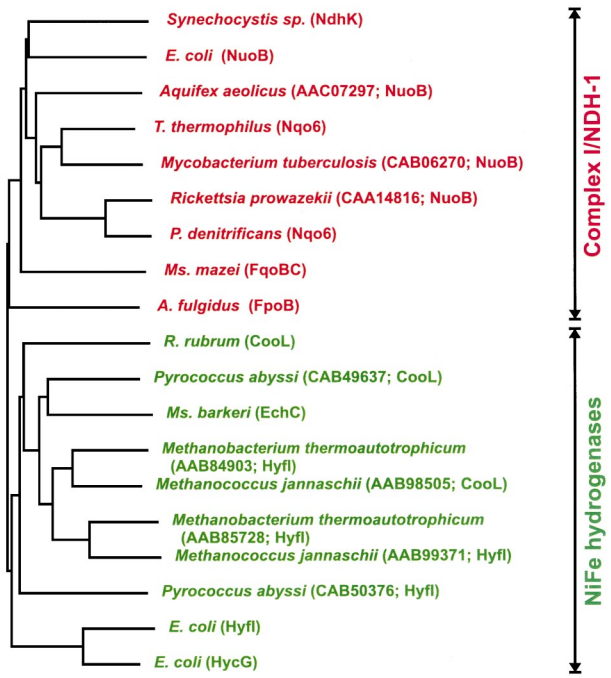


Fig. 2. Unrooted phylogenetic tree for the PSST/Nqo6/NuoB subunits of complex I/NDH-1 family and the homologous subunits of [NiFe] hydrogenase family. The tree was constructed using the neighbor-joining method. In addition to the amino acid sequences listed in Fig. 1, additional homologous subunit sequences from various sources were included in the analysis. Their accession numbers and homologous proteins are shown in parentheses, respectively. More extensive phylogenetic analyses including more various organisms are available on a web site (http:www.uni-dusseldolf.de/WWW/Mathnat/biochem).

ferredoxins (Meuer *et al*., 1999) or peripherally associated electron input devices (Fox *et al*., 1996a,b; Andrews *et al*., 1997). In contrast, the 49kDa/Nqo4/NuoD subunits of complex I/NDH-1 group do not contain the [NiFe] center binding motif (data not shown). In addition, there is currently no concrete experimental evidence that supports the possibility that these [NiFe] hydrogenase enzyme complexes utilize Q or its equivalents as their physiological substrates. Conversely, no significant sequence alteration can be found in the TYKY/Nqo9/NuoI subunit homologs between the complex I/NDH-1 and the [NiFe] hydrogenase groups (data not shown). These observations suggest that significant sequence alterations seen in the two lineages might have occurred in the specific subunits associated with changes in their physiological reactions during the course of evolution (Table I and Fig. 2). We can readily imagine that such a significant change in the ligand motif in the PSST/Nqo6/NuoB subunit must have dramatically transformed its possible [4Fe–4S] cluster. Since two adjacent cysteine residues (-CC-) cannot concomitantly ligate a [4Fe–4S] cluster, the new [4Fe–4S] cluster in PSST/Nqo6/NuoB subunits had to recruit a noncysteinyl ligand residue from elsewhere (Ahlers *et al*., 2000). The identity of this ligand residue remains to be determined. Hence, the [4Fe–4S] cluster in the PSST/Nqo6/NuoB subunit exhibits unique properties and functions.

These sequence comparisons in conjunction with their possible physiological reactions imply one hypothetical evolutionary scheme. Dramatic changes in the ligand coordination of the [4Fe–4S] cluster have occurred in the PSST/Nqo6/NuoB subunit (*birth of cluster N2*), and the complex I/NDH-1 progenitors have concurrently been able to interact with lipophilic electron carriers like Q (*birth of Q reductase*). The establishment (redirection) of the electron transfer pathway to Q, for instance, may have led to the degeneration of the [NiFe] center from the 49kDa/Nqo4/NuoD (Fig. 3). This process possibly has occurred as ambient redox states and a membrane Q pool became more oxidative due to the appearance of oxygen in the atmosphere.

Q-REDUCTION SITE(S)

Our hypothetical evolutionary scheme proposes that the Q-binding site(s) might have arisen over several subunits (Fig. 3). The most likely subunits are the PSST/Nqo6/NuoB and the 49kDa/Nqo4/NuoD subunits. Concomitant with the significant changes in the cluster N2-binding site in the PSST/Nqo6/NuoB subunit, the Q-interaction site (the Q_{Nf} site) has appeared in some

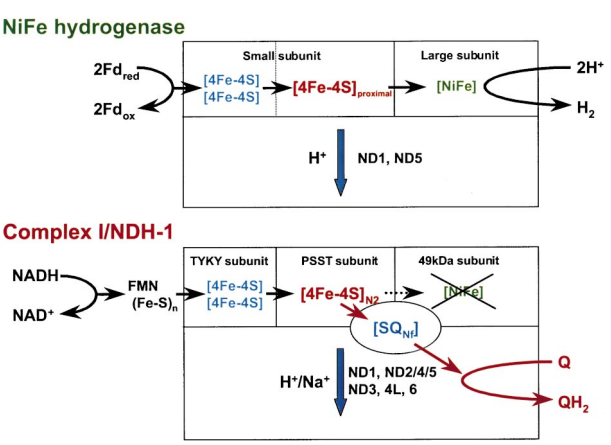


Fig. 3. A schematic presentation of hypothetical electron and H^+/Na^+ transfer reactions in [NiFe] hydrogenase and complex I/NDH-1. Electron transfer pathways are shown by arrows. The coupled H^+/Na^+ translocation is presented by blue arrows. Open boxes represent core regions for the redox coupled H^+/Na^+ translocation reactions. The observed Q intermediate, SQ_{Nf} species, is shown in an open oval. Details of the Q reduction reaction are not known.

regions of these subunits surrounding cluster N2 in order to accept electrons from it (Yano, *et al*., 2000). The specific labeling of the PSST/Nqo6/NuoB subunit by TDP strongly supports its direct role in Q reduction (Schuler *et al*., 1999). The finding of an inhibitor-sensitive amino acid residue Val407 (*Rhodobacter capsulatus* numbering) in the 49kDa/Nqo4/NuoD subunit suggests that the subunit is part of the Q-reduction site (Darrouzet and Dupuis, 1997; Dupuis *et al*., 1998). The Val residue is located in the C-terminal end of the subunit, at the position that corresponds to one of four Cys ligand residues of a [NiFe] center of the large subunit of [NiFe] hydrogenases.

The ND1/Nqo8/NuoH has been considered to play a key role in the Q-reduction reaction. This subunit is the binding sites for complex I inhibitors, DCCD (Yagi and Hatefi, 1988), a photoaffinity rotenone analog (Earley and Ragan, 1984), dihydrorotenone (Earley *et al*., 1987), and TDP (Schuler *et al*., 1999). The photoaffinity TDP labeling experiments have shown that, although its labeling is not correlated with inhibition of the enzyme, the ND1/Nqo8/NuoH subunit is a highly specific interaction site for TDP among hundreds of mitochondrial proteins (Schuler *et al*., 1999). Therefore, it seems very likely that the ND1/Nqo8/NuoH subunit also plays a unique role in binding of inhibitors and Q.

Our sequence analysis revealed that some conserved sequence alterations did indeed occur in the ND1/Nqo8/NuoH subunits of complex I/NDH-1 group associated with the changes in their physiological reactions. These changes include an insertion of seven to nine amino acid residues in the most highly conserved loop region between the predicted first and second transmembraneous α-helixes (Fig. 1B). Some differences in the fully conserved amino acid residues are also found in this region (Fig. 1B) and other regions (not shown) between the complex I/NDH-1 and the [NiFe] hydrogenase groups. Interestingly, the highly conserved loop region of human ND1 subunit contains an Ala residue (indicated by an arrow in Fig. 1B) whose substitution to Thr (ND1/3360) is known to be a primary cause of LHON disease (Majander *et al*., 1991, 1996; Wallace *et al*., 1995). Mutagenesis experiments of the *P. denitrificans* Nqo8 subunit have shown that the corresponding region plays an important role in Q reduction (Zickermann *et al*., 1998). These experimental data appear to be in parallel to our evolutionary scheme. Therefore, our analyses predict that the loop region of the ND1/Nqo8/NuoH subunit, shown in Fig. 1B, is involved in the Q-binding site.

Our evolutionary scheme illustrates that complex I/NDH-1 has gained the ability to utilize lipophilic electron carriers, such as Q, by altering amino acid sequences of particular subunits at some stages in the past. This view is rather similar to the proposed evolutionary path of quinol oxidase, which is thought to have evolved from cytochrome *c* oxidase by acquiring quinol oxidation sites in subunits I and II (Castresana and Saraste, 1995).

REDOX-LINKED $H^+(Na^+)$ TRANSLOCATION

The proton translocation system most likely operates in the membrane subunits ND1 and/or ND2/4/5 of complex I/NDH-1, which are also conserved among the [NiFe] hydrogenase group (Table I). It is known that the ND2, ND4, and ND5 subunits are homologous to each other and share their roots with Na^+/H^+ or K^+/H^+ antiporters (Kikuno and Miyata, 1985). It is noteworthy that the energy-conserving hydrogenase reactions have been proposed for *Ms. berkeri* hydrogenase (Ech) and *R. rubrum* CO-induced hydrogenase (Coo) (Meuer *et al*., 1999). Since these enzyme complexes do not use Q, a redox-linked proton-translocation mechanism not involving Q appears to be functional. This suggests that such a redox-coupled energy-transducing system already existed in the past. It seems likely that during the path of evolution of complex I/NDH-1, the Q oxidation–reduction reaction has been integrated into the preexisting $H^+(Na^+)$ translocation system and that more elaborate energy-coupling systems might have developed in the membrane portion (Fig. 3). If so, this system may allow some members of the NDH-1 family to translocate Na^+ ion (Krebs *et al*., 1999; Steuber *et al*., 2000).

FINAL REMARK

Besides the enzyme complexes discussed in this review, some other membrane-bound oxidoreductases such as NADH:ferredoxin oxidoreducase (*nfr*) from *Rhodobacter capsulatus* (Kumagai *et al*., 1997) and methyl-H_4MPT:HS-CoM methyltransferase (*mtr*) from *Methanobacterium thermoautotrophicum* (Hedderich *et al*., 1999; Tersteegen and Hedderich, 1999) have also been proposed to catalyze the energy-conserving oxidoreductase reactions possibly without involving Q. The oxidoreductase reactions appear to take place in their peripherally localized subunits and are linked to the translocation of H^+ or Na^+ across the membrane. The Na^+-NQR from *Vibrio alginolyticus* also exhibits structural features similar to those of the enzyme complexes mentioned above (Hayashi *et al*., 1995). This type of redox-linked energy-coupling mechanism, which is largely unknown, may be common in nature. Therefore,

it is of great importance to understand functional roles of the membrane subunits. Recently, interesting information has been reported for some ND subunits of complex I/NDH-1 (Di Bernardo *et al*., 2000; Sazanov *et al*., 2000; Sazanov and Walker, 2000).

The comparisons of the phylogenetically related enzymes in connection with their physiological reactions have provided new insights into the structure and function relationship of complex I/NDH-1.

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