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NADH Dehydrogenases: From Basic Science to Biomedicine

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This review article is concerned with two on-going research projects in our laboratory, both of which are related to the study of the NADH dehydrogenase enzyme complexes in the respiratory chain. The goal of the first project is to decipher the structure and mechanism of action of the proton-translocating NADH-quinone oxidoreductase (NDH-1) from two bacteria, *Paracoccus denitrificans* and *Thermus thermophilus* HB-8. These microorganisms are of particular interest because of the close resemblance of the former (*P. denitrificans*) to a mammalian mitochondria, and because of the thermostability of the enzymes of the latter (*T. thermophilus*). The NDH-1 enzyme complex of these and other bacteria is composed of 13 to 14 unlike subunits and has a relatively simple structure relative to the mitochondrial proton-translocating NADH-quinone oxidoreductase (complex I), which is composed of at least 42 different subunits. Therefore, the bacterial NDH-1 is believed to be a useful model for studying the mitochondrial complex I, which is understood to have the most intricate structure of all the membrane-associated enzyme complexes. Recently, the study of the NADH dehydrogenase complex has taken on new urgency as a result of reports that complex I defects are involved in many human mitochondrial diseases. Thus the goal of the second project is to develop possible gene therapies for mitochondrial diseases caused by complex I defects. This project involves attempting to repair complex I defects in the mammalian system using *Saccharomyces cerevisiae NDI1* genes, which code for the internal, rotenone-insensitive NADH–quinone oxidoreductase. In this review, we will discuss our progress and the data generated by these two projects to date. In addition, background information and the significance of various approaches employed to pursue these research objectives will be described.

KEY WORDS: NADH dehydrogenase; quinone; complex I; NDH-1; proton pump; *NDI1*; mitochondrial diseases; gene therapy.

INTRODUCTION

It is generally accepted that bacterial NADH dehydrogenases (NADH-quinone oxidoreductases) can be divided into at least three groups (Yagi, 1993; Yagi *et al.*, 1998). These are the proton-translocating NADH–quinone (Q) oxidoreductase (NDH-1) (Yagi *et al.*, 1998; Dupuis *et al.*, 1998a; Smith *et al.*, 2000), the NADH–Q oxidoreductase which lacks an energy-coupling site (NDH-2) and, thus, is not involved in proton translocation (Yagi, 1991; Smith *et al.*, 2000), and, finally, the sodium-translocating NADH–Q oxidoreductase (Na^+-NDH) (Nakayama *et al.*, 1998; Pfenninger-Li *et al.*, 1996; Zhou *et al.*, 1999; Nakamura *et al.*, 2000). On the other hand, mitochondrial NADH dehydrogenases can be divided into only two groups, the proton-translocating NADH–Q oxidoreductase (complex I) and the NADH–Q oxidoreductase lacking an energy-coupling site (Marres *et al.*, 1991; Yagi, 1991; Moller *et al.*, 1993). The mitochondrial enzymes lacking energy-coupling sites can be further divided into two groups in terms of their topology. The group designated NDI faces the matrix side of the mitochondria, while the group designated NDE faces the cytoplasmic compartment (de Vries and Grivell, 1988; Marres *et al.*, 1991; de Vries *et al.*, 1992; Luttik *et al.*, 1998). The characteristics of these various NADH dehydrogenases are summarized in Table I. On the basis of these characteristics, the

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Table I. Properties of NADH Dehydrogenases

	NDH-1	Na^+-NDH	NDH-2	Complex I	NDI	NDE
Ion-pump	H^+	Na^+	—[a]	H^+	—	—
Cofactors	FMN	FAD 1-2 FMN[c] (covalently bound)	FAD	FMN	FAD	n.d.[b]
	2[2Fe–2S] 6–7[4Fe–4S]	1[2Fe–2S]	—	2[2Fe–2S] 6[4Fe–4S]	—	n.d.
Subunit composition	13–14	6	1	42–43 (seven are encoded by mitochondrial DNA)	1	1

[a]— , none.
[b]Not determined.
[c]Na^+-NDH from *Vibrio alginolyticus* (Nakamura *et al.*, 2000) and *Vibrio harveyi* (Zhou *et al.*, 1999) were reported to house two and one covalently bound FMN, respectively.

bacterial NDH-1 enzymes appear to be counterparts of the mitochondrial complex I, while the bacterial NDH-2 enzymes are homologs of the mitochondrial NDI.

Since 1986, when our first paper concerned with the NDH-1 of *Paracoccus denitrificans* was published (Yagi, 1986), approximately one and a half decades have passed. During this time our laboratory has pursued not only further studies on the *Paracoccus* NDH-1 but has also engaged in the pursuit of other objectives concerning the NADH dehydrogenases (Kitajima-Ihara and Yagi, 1998; Seo *et al.*, 1998, 1999, 2000). Much has been accomplished over this span of time. Thus, it is time to summarize our progress in understanding the NADH dehydrogenase. This article represents such a summary and is concerned with past and on-going progress in the area of NADH dehydrogenase research in this laboratory (see Table II).

STRUCTURE AND FUNCTION STUDIES CONDUCTED ON THE BACTERIAL NDH-1 ENZYME

The mitochondrial complex I enzyme is a multisubunit protein–lipid complex that bears the oxidative phosphorylation coupling site 1. Complex I preparations from bovine heart (Hatefi *et al.*, 1985; Hatefi, 1985; Skehel *et al.*, 1998) and *Neurospora crassa* (Weiss *et al.*, 1991; Videira, 1998) are believed to be composed of at least 42 and 35 unlike polypeptides, respectively. This complexity has hampered progress in understanding many aspects of the structure and function of the mitochondrial complex I. Although model systems with a reduced level of complexity have been sought, most bacterial systems do not resemble the mammalian system sufficiently to serve as a model. *Paracoccus*, on the other hand, appears

Table II. A Chronological Table of Research on NADH Dehydrogenases in This Laboratory

Year	Research
1986	Isolation of NADH dehydrogenase complex from *Paracoccus* membranes (Yagi, 1986)
1988	Identification of DCCD-binding subunit of bovine heart complex I (Yagi and Hatefi, 1988)
1990	Identification of the NADH-binding subunit of *Paracoccus* NDH-1 (Yagi and Dinh, 1990)
1991	Cloning and DNA sequencing of the *NQO1* encoding the NADH-binding subunit of *Paracoccus* NDH-1 (Xu *et al.*, 1991b)
1993	Complete DNA sequencing of the gene cluster encoding *Paracoccus* NDH-1 (Xu *et al.*, 1993)
1994	Expression of the 25 kDa iron–sulfur subunit (Nqo2) of *Paracoccus* NDH-1 (Yano *et al.*, 1994a)
1996	Property and stoichiometry of the peripheral subunits of *Paracoccus* NDH-1 (Takano *et al.*, 1996)
1997	Cloning and DNA sequencing of the gene cluster encoding *Thermus* NDH-1 (Yano *et al.*, 1997)
1998	Rescue by the expressed yeast Ndi1 of the respiration of complex I-deficient mammalian cells (Seo *et al.*, 1998)
1999	Identification of pyridaben-binding subunit of NDH-1/complex I (Schuler *et al.*, 1999)
2000	Characterization of the membrane domain Nqo7 subunit of *Paracoccus* NDH-1 (Di Bernardo *et al.*, 2000)
	Functional expression of the yeast *NDI1* gene into the growth-arrested human cells (Seo *et al.*, 2000)

to have a respiratory chain that closely resembles that of mitochondria (Stouthamer, 1992). For example, the respiratory chain of aerobically grown *Paracoccus* contains coupling site 1 (Stouthamer, 1980) and exhibits EPR signals similar to those of the mitochondrial complex I (Albracht *et al.*, 1980; Meinhardt *et al.*, 1987). The cytochrome *c* oxidase of this organism is also similar to the mitochondrial cytochrome *c* oxidase in terms of electron carriers (cytochromes aa_3, 3Cu) (Ludwig and Schatz, 1980). However, it is composed of only four polypeptides (Iwata *et al.*, 1995) as compared to the 13 unlike polypeptides identified in the mitochondrial oxidase (Tsukihara *et al.*, 1996). Furthermore, the quinol-cytochrome *c* oxidoreductase complex of *Paracoccus* is composed of only three unlike polypeptides (Yang and Trumpower, 1986), whereas the mitochondrial enzyme contains 11 unlike polypeptides (Xia *et al.*, 1997; Iwata *et al.*, 1998). These data indicated that the *Paracoccus* respiratory chain might provide an excellent model system for the more complex mitochondrial chain. Therefore, it was of interest to attempt the isolation of a complex I-like enzyme system from *P. denitrificans* membranes in the hope that it too might be structurally simpler than its mammalian counterpart and might better lend itself to the study of its structure and mechanism of electron transfer and proton translocation. When this goal was pursued, the resulting, isolated, NADH dehydrogenase was discovered to be composed of 10 unlike polypeptides which contained noncovalently bound FMN, nonheme iron, and acid-labile sulfide (Yagi, 1986). Furthermore, the isolated *Paracoccus* NADH dehydrogenase complex cross-reacted with antisera directed against bovine heart complex I and against the protein fraction derived from the bovine heart complex I. Unfortunately, the preparation proved to be insensitive to rotenone, which is a specific inhibitor of the mammalian complex I/NDH-1 (Yagi *et al.*, 1998), suggesting that the isolated preparation may lack some subunits. This work was the first to demonstrate that the bacterial NDH-1 contains noncovalently bound FMN as cofactors. These results encouraged us to proceed with this project.

The first step in elucidating the structure and mechanism of action of this *Paracoccus* NDH-1 enzyme was to identify and characterize the function of the various polypeptides in this enzyme complex. First, we attempted to identify the NADH-binding subunit of the *Paracoccus* NDH-1 by using ^{32}P-labeled NAD(H) (Yagi and Dinh, 1990). This work was based on a report by Chen and Guillory (1981) in which these investigators used a tritiated photoaffinity NAD analog to identify the 51-kDa polypeptide of bovine heart complex I as the NADH-binding subunit of the mammalian complex I. In our hands the ^{32}P-labeled NAD(H) was irreversibly bound to the *Paracoccus* NADH dehydrogenase complex by UV irradiation. Analysis of the data illustrated that the radioactivity was incorporated into a single polypeptide of molecular weight 50 kDa. The labeling was shown to be specific, in that the 50-kDa protein band was protected from [^{32}P]NAD labeling in the presence of NADH, but not in the presence of NADP(H). In addition, antiserum to the NADH-binding subunit of bovine heart complex I cross-reacted with this polypeptide. It was, thus, concluded that the 50-kDa subunit of the *Paracoccus* NDH-1 is the NADH-binding subunit (Yagi and Dinh, 1990).

One of the advantages of using a bacterial system as a model is the high probability that the structural genes of a particular enzyme complex will constitute an operon. This has been illustrated for other respiratory chain enzyme complexes (Kurowski and Ludwig, 1987; Ishizuka *et al.*, 1990) as well as for the ATP synthases (Walker *et al.*, 1984). In the mitochondrial system, because genes coding for individual subunits of a multienzyme complex are often not located together, it is difficult to demonstrate conclusively that a polypeptide is a component of a particular enzyme complex rather than a copurified contaminant unless functional and/or structural justification can be provided. However, this difficulty is eliminated when the genes of an enzyme complex constitute an operon, since a polypeptide encoded by a structural gene in an operon is generally found to be an essential component of the enzyme complex encoded by this operon (Tzagoloff *et al.*, 1990; Nobrega *et al.*, 1990). Two prerequisites for understanding the structure and mechanism of such an intricate enzyme complex as the NDH-1/complex I are the identification of all the essential components of the enzyme complex as well as a knowledge of the primary structure of each subunit. Both of these prerequisites would be realized if an operon could be located. Therefore, an attempt was made to clone and sequence the gene encoding the NADH-binding subunit of the *Paracoccus* NDH-1. It was hoped that this data might provide information regarding both the structure of this enzyme complex and also the possible presence of an operon carrying the genes of this complex. For this purpose, the *Paracoccus* NADH-binding subunit was purified and subjected to N-terminal amino acid sequencing analysis. The resulting sequence data provided the 37 N-terminal amino acid residues of this subunit (Xu *et al.*, 1991b). On the basis of this amino acid sequence, probes of oligonucleotides were constructed and used to screen the *Paracoccus* genomic library. Unfortunately, after 6-months, no positive results had yet been obtained. Thus, upon the advice of our colleagues this approach was abandoned in favor of PCR, which was still a new technology at that time. When PCR was used to construct probes, the cloning of the structural gene (designated *NQO1*) for

the *Paracoccus* NADH-binding subunit was completed without further difficulty (Xu *et al.*, 1991b). Concurrently, the cDNA sequences of the bovine heart NADH-binding subunit were reported by two groups, Walker's and Attardi's (Pilkington *et al.*, 1991; Patel *et al.*, 1991). A comparison of the sequences of the NADH-binding subunit from the mammalian and bacterial sources revealed approximately 64% identity. In addition, when partial DNA sequencing of the regions surrounding the *NQO1* gene was carried out, sequences homologous to the 24, 49, and 75-kDa subunits of the bovine complex I were detected, suggesting that the structural genes of the *Paracoccus* NDH-1 do constitute a gene cluster. The entire DNA sequence of the gene cluster encoding the *Paracoccus* NDH-1 was subsequently determined (Xu *et al.*, 1991a,b, 1992a,b, 1993 Yagi *et al.*, 1992, 1993). The gene cluster of the *Paracoccus* NDH-1 was found to contain 14 structural genes and 6 URFs, designated *NQO1-14* and URF1-6, respectively (Yagi *et al.*, 1993). It should be noted that the structural genes encoding bacterial counterparts for the seven complex I hydrophobic subunits encoded by mtDNA (ND1-6 and 4L) are also present in this *Paracoccus* gene cluster (Chomyn *et al.*, 1985, 1986).

In addition to subunit composition and sequence information, the determination of subunit stoichiometry and subunit topology of the *Paracoccus* NDH-1 is also a prerequisite to understanding the structure and mechanism of action of this enzyme complex. The use of membrane preparations together with subunit-specific antibodies as probes is a reliable method for determining the subunit stoichiometry of membrane-bound enzyme complexes (Matsuno-Yagi and Hatefi, 1984; Belogrudov and Hatefi, 1994). This technique was thus employed to examine the structure of the *Paracoccus* NDH-1. Using antibodies directed against the individual *Paracoccus* Nqo1–Nqo6 and Nqo9 subunits, the subunit stoichiometry of these *Paracoccus* NDH-1 subunits was determined to be 1 mol of each subunit per mol of the enzyme complex (Takano *et al.*, 1996; Yano and Yagi, 1999). In terms of elucidating the topology of the NDH-1 complex, it is known that treatment of membrane preparations under alkaline conditions or with chaotropes extracts the peripheral part of the membrane enzyme complexes leaving the membrane-bound section intercalated in the lipid bilayer (Fujiki *et al.*, 1982; Azevedo and Videira, 1994). When this approach was employed on *Paracoccus* membranes, the Nqo1–Nqo6 and Nqo9 subunits were extracted from the *Paracoccus* membranes by these treatments while the Nqo7, Nqo8, and Nqo10–14 subunits were not. These results suggest that the Nqo1–Nqo6 and Nqo9 subunits are localized in the peripheral part of the *Paracoccus* NDH-1 *in situ* (Takano *et al.*, 1996), while the Nqo7, Nqo8, and Nqo10–14 subunits are membrane bound (Yano and Yagi, 1999). The extraction and expression experiments, however, also provided some indication that the two subunits, Nqo6 and Nqo9, may interact with the membrane segment. Thus, for example, these subunits were expressed in the membrane fraction in *E. coli.* Therefore, these two subunits may act as connectors between the peripheral and the membrane segments of the NDH-1 (Takano *et al.*, 1996; Yano and Yagi, 1999).

The data discussed above provided a great deal of information in terms of the NDH-1 subunit identity, sequence, stoichiometry, and topology. However, the location of the cofactors and iron–sulfur clusters was still unknown. In an attempt to identify which subunits bound the iron–sulfur clusters, the deduced primary structures of the *Paracoccus* NDH-1 subunits were compared against the consensus sequence known for different types of iron–sulfur clusters (Matsubara and Saeki, 1992; Johnson, 1994) Based on these data it was possible to speculate as to which of the NDH-1 subunits bind iron–sulfur clusters. The Nqo1, Nqo3, and Nqo9 subunits all contain at least one tetranuclear iron–sulfur cluster binding site consensus sequence. The Nqo9 subunit bears two putative tetranuclear cluster-binding sites. Furthermore, the Nqo2 and Nqo3 subunits bear possible binuclear iron–sulfur cluster-binding sites, although the consensus sequence for binuclear iron–sulfur binding sites is not as well established as that for tetranuclear iron–sulfur cluster binding sites (Yagi *et al.*, 1993). Additional data, obtained by comparing the homologous subunits of the *Paracoccus* NDH-1 enzyme with the known iron–sulfur binding subunits of bovine heart complex I (Hatefi *et al.*, 1985; Hatefi, 1985; Ohnishi *et al.*, 1985; Ragan, 1987) supported the hypothesis that the Nqo1, 2, 3, 9, and 6 subunits are probably associated with iron–sulfur clusters. These comparison data, although suggestive, were far from conclusive. Therefore, it was important to clarify whether these subunits actually ligate iron–sulfur clusters and, if so, to assign the specific EPR-detectable iron–sulfur clusters to the individual subunits. For this purpose, two approaches appeared to be applicable. One was resolution of both the *Paracoccus* NDH-1 subunits and the mammalian complex I subunits followed by a direct comparison of the two sets of subunits. As described by Ragan *et al.* (1982) and Ohnishi *et al.* (1985), separation of each individual subunit from these enzyme complexes without damaging the iron–sulfur clusters is difficult because of the harsh conditions required to separate each subunit. In addition, the limited amount of each subunit obtained using this technique hampers progress in their characterization. The other approach was to overexpress the genes encoding the individual *Paracoccus* subunits. If this overexpression

were successful, problems resulting from limitation of available material would vanish. Furthermore, if the selected host cells were equipped with the machinery required for incorporation of cofactors and native structural folding of the subunits, the need to resolve each subunit, intact, from the entire complex would become unnecessary, because the subunit could be isolated and characterized directly from the host. In addition, once native subunits were expressed, determination of the residues in that subunit, which ligate the cofactors, would become a possibility through site-directed mutagenesis. Given these options and the obvious advantages of the second approach, we attempted to express the genes encoding the putative cofactor bearing subunits of the *Paracoccus* NDH-1 in *Escherichia coli.* This host was selected because it is also known to contain an NDH-1 enzyme (Friedrich, 1998). The results, which are presented as a summary of our present knowledge regarding the subunit assignments of EPR-detectable iron–sulfur clusters, are outlined in Table III (a more detailed description of the subunit assignments of the EPR-detectable iron–sulfur clusters is available in a review by Yano and Ohnishi in this issue) (Crouse *et al.*, 1994; Yano *et al.*, 1994a,b, 1995, 1996, 1997, 1999). Although most of the EPR detectable clusters could be assigned, the location of center N2 remained uncertain. Some questions also remained in terms of the clusters in the Nqo3 subunit. Thus, for example, the *Thermus* Nqo3 subunit and its homologs in certain other bacteria contain the additional putative iron–sulfur cluster binding sequence motif, $C^{256}xxC^{259}xxxC^{263}x_{27}C^{291}$ (*Thermus* numbering) (Yano *et al.*, 1997). It was assumed, based on thermodynamic studies of EPR signals of *E. coli* NDH-1, that this motif, which was not present in the *Paracoccus* Nqo3 subunit, in the NDH-1 would ligate a [2Fe–2S] cluster (Friedrich, 1998; Oh and Bowien, 1998). In order to verify this hypothesis, we attempted to express the DNA fragment encoding this motif as well as the full-length *Thermus NQO3* gene in *E. coli.* The results indicated that instead of a [2Fe–2S] cluster, the expressed subunit segment ligates a single [4Fe–4S] cluster whose EPR signals exhibit axial symmetry. The same EPR signals derived from this [4Fe–4S] cluster were also observed in the full-length *Thermus* Nqo3 subunit expressed in the *E. coli.* Furthermore, a comparison between this motif and the three-dimensional structural analyses of the formate dehydrogenase H in *E. coli* ($C^{8}xxC^{11}xxxC^{15}x_{23}C^{37}$) and the periplasmic nitrate reductase in the sulfate-reducing bacterium, *Desulfovibrio desulfuricans* ATCC27774 ($C^{13}xxC^{16}xxxC^{20}x_{24}C^{45}$) displays that these cysteine clusters ligate [4Fe–4S] clusters (Boyingtonb *et al.*, 1997; Oh and Bowien, 1998; Dias *et al.*, 1999). In addition, the crystal structural analyses of the formaldehyde ferredoxin oxidoreductase (FOR) and the aldehyde ferredoxin oxidoreductase from *Pyrococcus furiosus* indicate that a similar cysteine motif ($C^{284}xxC^{287}xxxC^{291}x_{199}C^{491}$, FOR numbering) in these ferredoxin oxidoreductases ligates a [4Fe–4S] cluster (Chan *et al.*, 1995; Hu *et al.*, 1999). Therefore, these data suggest that the $CxxCxxxCx_nC$ motif in the *Thermus* Nqo3 subunits may ligate [4Fe–4S] clusters rather than [2Fe–2S] clusters, as previously thought (E. Nakamaru-Ogiso, T. Yano, T. Ohnishi, T. Yagi (2001), unpublished results).

Table III. Identity and Location of Iron–Sulfur Cluster-Containing Subunits in NDH-1/Complex I

Subunit[a]	Type of cluster	Symmetry of cluster	EPR-detectable cluster
Nqo1/NuoF/FP51k	[4Fe–4S]	Rhombic	N3
Nqo2/NuoE/FP24k	[2Fe–2S]	Rhombic	N1a
Nqo3/NuoG/IP75k	[2Fe–2S]	Axial	N1b
	[4Fe–4S]	Rhombic	N4
	[4Fe–4S]	Rhombic	N5 (?)
	[4Fe–4S][b]	Axial	?
Nqo6/NuoB/PSST	[4Fe–4S] (?)	?	N2 (?)
Nqo9/NuoI/TYKY	[4Fe–4S]	?	N2 (?)
	[4Fe–4S]	?	N2 (?)

[a] Nqo, *P. denitrificans* and *T. thermophilus*; Nuo, *E. coli* and *Rhodobacter capsulatus*, FP51k, bovine complex I.

[b] As described in the text, our preliminary data suggests that the cysteine motif ($CxxCxxxCx_nC$) ligates [4Fe–4S] cluster in the *Thermus* Nqo3 subunit.

As described above, the membrane segment of the *Paracoccus* NDH-1 appears to be composed of the seven subunits, Nqo7, 8, and Nqo10–14 (Hatefi *et al.*, 1985; Yano and Yagi, 1999). In contrast to the peripheral segment, this membrane-bound segment is believed to be involved in the proton translocation and Q-binding activities of the *Paracoccus* NDH-1 (Yagi *et al.*, 1998). Therefore, understanding the properties and characteristics of the Nqo7, 8, and Nqo10–14 subunits is essential to understanding the mechanism of proton translocation of the *Paracoccus* NDH-1. Although, to date, a limited amount of information has been made available regarding the functional roles of the hydrophobic subunits in NDH-1 (Earley *et al.*, 1987;Yagi and Hatefi, 1988; Hofhaus and Attardi, 1993, 1995; Majander *et al.*, 1996; Dupuis *et al.*, 1998; Bai and Attardi, 1998; Falk-Krzesinski and Wolfe, 1998; Kurki *et al.*, 2000), little is known about the structural properties of these subunits. In addition, the successful expression of the hydrophobic subunits has not yet been reported. This expression is a prerequisite to further studies of the biochemical and physicochemical properties of these subunits. In order to address this issue, we elected to attempt the expression of the individual

Paracoccus NDH-1 hydrophobic subunits in *E. coli.* As describe above, this approach has been proved to be a powerful means of determining the subunit assignments of the cofactors in the NDH-1/complex I. The *Paracoccus* Nqo7 subunit, a counterpart of the mitochondrial ND3 subunit, was selected as our first candidate with hopes that a similar strategy could be employed for structural studies of the other hydrophobic subunits (Di Bernardo *et al.*, 2000). A GST-fused expression system was employed in our attempts, which yielded the successful expression of the Nqo7 subunit in the membrane fraction of *E. coli.* Subsequent to expression, this subunit was purified and partially characterized. This characterization involved determining the topology of the Nqo7 subunit in the *Paracoccus* membranes by immunochemical and chemical modification methods. As seen in Fig. 1 (Di Bernardo *et al.*, 2000), the N-terminal region of this Nqo7 subunit faces the cytoplasmic phase of the bacterium, while its C-terminal region is directed toward the periplasmic phase. A loop containing the unique cysteine at position 47 is also directed toward the periplasmic phase. The data further indicate that the Nqo7 subunit includes three transmembrane segments as predicted from the hydropathy plots. Similar topographical results were obtained for both the purified, intact Nqo7 subunit and for the GST-fused Nqo7 subunit, both expressed in *E. coli* membranes (Di Bernardo *et al.*, 2000). Based on this information, the D74 and E76 residues appear to be located in the middle (12th and 14th amino acid) of the 23-amino acid TM2 transmembrane segment. Interestingly, these two carboxyl residues are well conserved in counterparts of the *Paracoccus* Nqo7 subunit, suggesting that these two carboxyl residues may be involved in

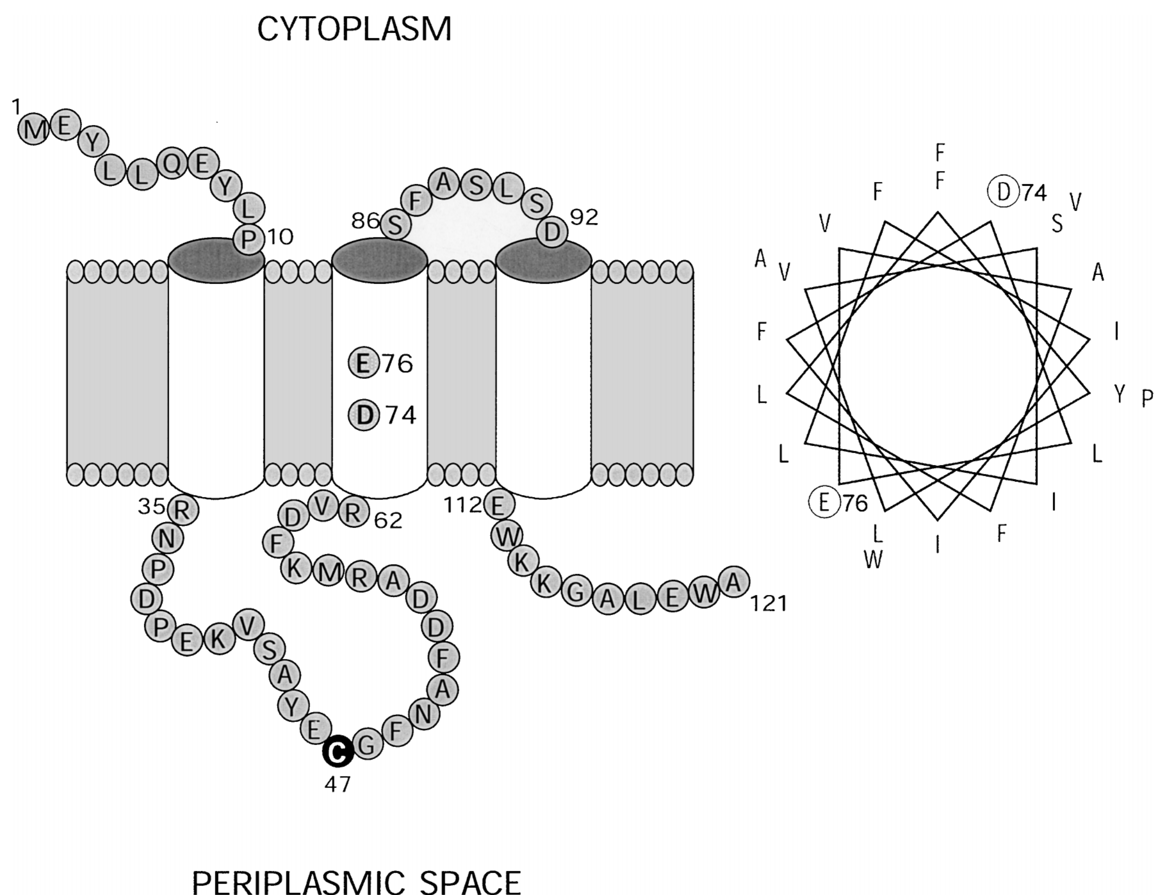

Fig. 1. Proposed topology of the *Paracoccus* Nqo7 subunit (left) and a helical wheel of the middle Nqo7 transmembrane segment containing the D74 and E76 residues (right). The three transmembrane segments of the *Paracoccus* Nqo7 subunit from the N- to the C-terminus are tentatively referred to as TM1, TM2, and TM3, respectively. The N-terminus of the subunit is exposed on the cytoplasmic side of the membrane. The C-terminus and the loop (R35 to R62) between TM1 and TM2 are exposed to the periplasmic space. As seen from the helical wheel of TM2, it is anticipated that the D74 and E76 residues may be located on opposite sides of the helical column.

the mechanism of proton translocation for this coupling site 1. However, this latter hypothesis remains to be proved experimentally.

GENE THERAPY OPTIONS FOR ADDRESSING COMPLEX I DEFECTS

As described above, it has been shown in recent years that structural and functional defects in complex I are involved in many human mitochondrial diseases (Schapira, 1998; Robinson, 1998). In particular, mutations and deletions of both the seven mitochondrially encoded subunits and the nuclear encoded subunits have been correlated with mitochondrial diseases. Thus, for example, dysfunction of complex I presents three problems (Kitajima-Ihara and Yagi, 1998): (1) impaired ability of the respiratory chain to oxidize NADH to NAD, resulting in lactic acidosis, (2) impaired ability of this enzyme complex to pump protons, which results in a decrease in the rate of ATP synthesis, and (3) production of superoxide radicals, causing DNA mutations, lipid peroxidation, and protein denaturation. Of these problems, the inability of mitochondria to oxidize NADH and the damage caused by superoxide radical production appear to present the most severe health problems. However, the impaired proton pumping, as long as it is limited to one of the three proton translocation sites, does not appear to present a serious health issue. Presently, mutations and deletions of the mtDNA-encoded subunits are not correctable. In addition, mutations resulting in multiple copies of any of the subunits encoded by the nuclear DNA are difficult to repair (de Grey, 2000). Unfortunately, the various chemical therapies employed to treat these diseases have been reported to be ineffective at the present time (Shoffner and Wallace, 1994; Chrzanowska-Lightowlers *et al.*, 1995).

In contrast to mammalian mitochondria, which are believed to contain only a single NADH-Q oxidoreductase, i.e., complex I, the mitochondria of *Saccharomyces cerevisiae* lack complex I, but instead have another type of NADH-Q oxidoreductase that is distinct from complex I in that it does not contain a proton translocation site and is rotenone-insensitive (de Vries and Grivell, 1988; Marres *et al.*, 1991; de Vries *et al.*, 1992). In fact, in *S. cerevisiae* mitochondria, two types of rotenone-insensitive NADH–Q oxidoreductases are present. One of these NADH–Q oxidoreductases (referred to as NDE) faces the intermembrane space, while the other (designated NDI) faces the matrix (Luttik *et al.*, 1998). The Ndi1 enzyme (the product of the *NDI1* gene) of the *S. cerevisiae* mitochondria consists of a single polypeptide chain with noncovalently bound FAD as a cofactor and no iron–sulfur clusters (de Vries and Grivell, 1988). This Ndi1 enzyme is the main entry point into the respiratory chain in this organism, just as complex I is in mammalian mitochondria. In contrast, however, the Ndi1 enzyme is reported to operate according to a two-electron reaction mechanism, whereas mammalian complex I is believed to be a one-electron reaction enzyme (de Vries and Grivell, 1988). The benefit of the two-electron mechanism present in the yeast Ndi1 enzyme is that it is not expected to result in damage from free radicals, since a two-electron reaction mechanism is much less likely to generate free radicals than a one-electron reaction mechanism. The *NDI1* gene encoding this *S. cerevisiae* enzyme has been cloned and sequenced by de Vries *et al.* (1992). The DNA sequence indicates the presence in the gene of an ORF of 1539 bp which is predicted to encode a precursor protein of 513 amino acid residues. Of these amino acid residues, the 26 N-terminal residues serve as the signal sequence for import into mitochondria.

The similarity in function between the mammalian complex I and the *S. cerevisiae* Ndi1 enzyme coupled with the benefits offered by the Ndi1 enzyme, i.e., the simplicity of its structure and its two-electron reaction mechanism, suggested that if the Ndi1 enzyme could be utilized to replace the complex I activity in complex I-deficient mammalian systems, problems (1) and (3) described above would be solved. This concept received support from a recent publication in which it was shown that this Ndi1 enzyme, expressed in *E. coli,* was able to perform as a member of the respiratory chain in the prokaryotic host cells (Kitajima-Ihara and Yagi, 1998). In addition, observations indicating that complex I-type enzymes and Ndi1-type enzymes can coexist in bacteria, plant, and fungal mitochondria (Yagi, 1991; Moller *et al.*, 1993; Luttik *et al.*, 1998; Smith *et al.*, 2000), suggests that neither the association of the Ndi1 type enzyme with the inner mitochondrial membranes nor its function will be negated or hindered by the presence of endogenous complex I. Based on this knowledge, it seems possible that one approach to coping with complex I defects may be to introduce an Ndi1-type enzyme into mammalian mitochondria. In order to provide an assessment of the capability of the yeast *NDI1* gene to repair complex I defects in mammalian cells, an attempt was made to express functionally active Ndi1 in complex I-deficient mammalian cells. As a first step toward this goal, the yeast *NDI1* gene was transcribed and translated in Chinese hamster cells (Seo *et al.*, 1998). The results indicated that the *NDI1* leader sequence successfully directed the enzyme to the mitochondria, since the expressed Ndi1 was determined to be incorporated predominantly in the mitochondria. Furthermore, the expressed Ndi1 enzyme was observed to restore the NADH oxidase activity of the complex I-deficient Chinese hamster mutant cell

line, CCL16-B2, which was isolated from lung fibroblasts by Scheffler and co-workers (Scheffler, 1974, 1986; Ditta *et al.*, 1976; DeFrancesco *et al.*, 1976). The restored NADH oxidase activity was shown to be due to the presence of the Ndi1 enzyme in that the activity was insensitive to rotenone, a specific complex I inhibitor, but was sensitive to flavone, an inhibitor of the yeast Ndi1.

The next step in exploring the potential usefulness of the *NDI1* gene for treatment of mitochondrial diseases caused by complex I deficiency involved verifying that the *NDI1* gene could be functionally expressed in the mitochondria of human cells bearing their own endogenous complex I. The results of this study indicated that the yeast *NDI1* gene can be successfully transcribed and translated in human embryonal kidney 293 cells (HEK 293) (Seo *et al.*, 1999). In addition, although the content of complex I in the HEK 293 mitochondria was unchanged by *NDI1* transfection, the expressed Ndi1 enzyme was shown to modify the characteristics of oxidative phosphorylation in these host cells. Thus, for example, the nontransfected HEK 293 cells could not survive in media containing the complex I inhibitors rotenone or 1-methyl-4-phenylpyridinium ion. However, the *NDI1*-transfected cells were resistant to these reagents.

Unfortunately, the transfection procedures employed in the above study, calcium phosphate precipitation, and lipofection methods using the pHook-2 vector, will have limitations when applied *in vivo.* First, transfection is either very difficult or practically impossible to achieve in many human cell lines. Second, mitochondrial defects often occur in nonproliferating or slowly proliferating tissues. Thus whereas most transfection procedures require rapidly dividing cells, in this case, successful therapy will require that the *NDI1* gene be expressed in differentiated and nondividing cells. Recently, however, adeno-associated virus (AAV) expression systems have been developed which allow expression of genes in nonproliferating cells (Muzyczka, 1994; Naldini *et al.*, 1996). AAV vectors have been used to deliver a number of different genes into a variety of target tissues both *in vitro* and *in vivo,* clearly demonstrating the potential of this virus in gene therapy for human diseases. Another advantage of the AAV vector is that in contrast to the more commonly used retroviral and adenoviral vectors, AAV is a nonpathogenic human parvovirus, which does not elicit antibodies against itself, and has been demonstrated to be useful for long-term expression of transgenes (Flotte *et al.*, 1993). In order to determine if this expression system might prove useful for delivery of the *NDI1* gene into nonproliferating cells, we used *NDI1*–recombinant AAV vectors to transfect growth-arrested human cells (Seo *et al.*, 2000). Not only was the transfection successful, but the transfected cells were shown to express functionally active Ndi1 enzyme. These results clearly support the concept of gene therapy for human diseases resulting from complex I deficiency. Our final goal, which is to use the yeast *NDI1* gene to repair complex I defects, remains to be accomplished. However, the results obtained so far suggest that although several hurdles remain to be surmounted, this strategy to cure complex I deficient diseases hold great promise.

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