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MINI-REVIEW

Bacterial NADH–Quinone Oxidoreductases

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Abstract

The NADH–quinone oxidoreductases of the bacterial respiratory chain could be divided in two groups depending on whether they bear an energy-coupling site. Those enzymes that bear the coupling site are designated as NADH dehydrogenase 1 (NDH-1) and those that do not as NADH dehydrogenase 2 (NDH-2). All members of the NDH-1 group analyzed to date are multiple polypeptide enzymes and contain noncovalently bound FMN and iron-sulfur clusters as prosthetic groups. The NADH–ubiquinone-1 reductase activities of NDH-1 are inhibited by rotenone, capsaicin, and dicyclohexylcarbodiimide. The NDH-2 enzymes are generally single polypeptides and contain noncovalently bound FAD and no iron-sulfur clusters. The enzymatic activities of the NDH-2 are not affected by the above inhibitors for NDH-1. Recently, it has been found that both of these types of the NADH–quinone oxidoreductase are present in a single strain of bacteria. The significance of the occurrence of these two types of enzymes in a single organism has been discussed in this review.

Key Words: NADH–quinone oxidoreductase; bacteria; *Paracoccus*; energy-coupling site 1.

Introduction

The NADH–quinone (Q) oxidoreductases of the respiratory chain catalyze electron transfer from NADH to quinone. In addition to this function, the enzyme complexes in certain bacteria and mitochondria bear a pump which transports protons across membranes (Hatefi, 1985; Ragan, 1987; Yagi, 1988, 1989). The NADH–Q oxidoreductase is extremely important; not only in this enzyme complex the point of entry for the major fraction of electrons

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that traverse the respiratory chains, but also, as reported recently (Ramsay *et al.*, 1989a; Shoffner *et al.*, 1989; Ramsay *et al.*, 1989b; Scholte, 1988; Holt *et al.*, 1988; Wallace *et al.*, 1988), many human mitochondrial diseases involved structural and functional defects at the level of this enzyme complex, which is composed of more than 25 unlike polypeptides (Hatefi, 1985; Leonard *et al.*, 1987; Yagi, 1988, 1989). As far as our knowledge extends, it may safely be said that the mitochondrial NADH–Q oxidoreductase has one of the most intricate structures of any membrane-bound proteins.

Paracoccus denitrificans is a gram-negative soil bacterium (John and Whatley, 1975, 1977). *Paracoccus* membranes appear to have a respiratory chain that closely resembles that of mitochondria (John and Whatley, 1977; see also other articles in this volume). The cytochrome oxidase of this organism is similar to the mitochondrial cytochrome oxidase in terms of electron carriers (cytochrome aa_3, 2Cu) (Ludwig and Schatz, 1980), but it is composed of only three unlike polypeptides as compared to at least 13 unlike polypeptides in mitochondrial oxidase (Kadenbach *et al.*, 1987; Haltia *et al.*, 1988, 1989). Likewise, the ubiquinol (UQH_2)–cytochrome *c* oxidoreductase of *Paracoccus* membrane is composed of only three unlike polypeptides (Yang and Trumpower, 1986), whereas the mitochondrial enzyme contains at least eight unlike polypeptides (Weiss, 1987). On the basis of these findings suggesting that *Paracoccus* membranes are similar to, although structurally simpler than, mammalian mitochondria in many biochemical aspects (John and Whatley, 1975), John and Whatley (1975) have hypothesized an evolutionary relationship between *Paracoccus* membranes and mitochondria.

In 1968, a Japanese group (Imai *et al.*, 1968a,b) found that the NADH–Q oxidoreductase segment of the *Paracoccus* respiratory chain closely resembles the same segment of the mitochondrial respiratory chain. They showed that the NADH–Q oxidoreductase segment of *Paracoccus* membranes bears an energy-coupling site, is rotenone sensitive, and contains EPR-visible iron-sulfur (FeS) clusters. We have purified the NADH–Q oxidoreductase from *Paracoccus* membranes (Yagi, 1986). This NADH dehydrogenase complex from *Paracoccus* membranes is composed of approximately 10 unlike polypeptides, suggesting that the *Paracoccus* NADH–Q oxidoreductase, like the cytochrome oxidase and the UQH_2–cytochrome *c* oxidoreductase, is also simpler than its mitochondrial counterpart. Therefore, the *Paracoccus* enzyme complex appears to provide a useful model system to study the structure and mechanism of action of mitochondrial NADH–Q oxidoreductase. In addition, *Thermus thermophilus* HB-8 membranes have been reported to contain an NADH–Q oxidoreductase bearing the energy-coupling site (Meinhardt *et al.*, 1989). The enzyme complex has been isolated from the bacteria and shown to be composed of around 10 unlike polypeptides (Yagi *et al.*, 1988). In addition to a simpler structure, the enzyme complex from

T. thermophilus has the great advantage of extreme stability to heat. Furthermore, it has been reported that *Escherichia coli* and *Rhodobacter capsulata* also contain energy-transducing NADH–Q oxidoreductases (Matsushita *et al.*, 1987; Baccarini-Melandri *et al.*, 1973).

To date several excellent reviews regarding the mammalian mitochondrial NADH–Q oxidoreductase have been published (Hatefi, 1985; Hatefi *et al.*, 1985; Ragan, 1987). However, with the exception of the Na-transporting NADH–Q oxidoreductase of *Vibrio alginolyticus* (Unemoto and Hayashi, 1989), there is no review on the bacterial NADH–Q oxidoreductase. Therefore, we attempt, in this review, to describe the present state of knowledge of the NADH–Q oxidoreductase not only of *Paracoccus* but also of several other bacteria.

Bacterial NADH–Quinone Oxidoreductase

It has been reported that preparations suggestive of two types of NADH dehydrogenases have been fractionated from *E. coli* (Bragg and Hou, 1967; Hendler and Burgess, 1974). In addition, crossed immunoelectrophoresis experiments have suggested the existence of two types of NADH dehydrogenase not only in *E. coli* but also in *Micrococcus lysodeikticus* (Owen *et al.*, 1980a,b; Owen and Salton, 1975). Recently, both types of NADH–Q oxidoreductase have been isolated from *T. thermophilus* HB-8 and *E. coli* (Yagi *et al.*, 1988; Hayashi *et al.*, 1989). Further characterization of these two types of NADH dehydrogenase has illustrated that one of them contains an energy-coupling site whereas the other does not. In light of these results, it appears that the NADH–quinone oxidoreductases of the bacterial respiratory chains can be divided into at least two groups (Yagi, 1988, 1989; Yagi *et al.*, 1988). One group includes those enzymes that bear an energy-coupling site. These enzymes are designated NADH dehydrogenase 1 (NDH-1). The enzymes of the other group lack an energy-coupling site and are referred to as NADH dehydrogenase 2 (NDH-2).

NDH-1

Composition. At the present time, the existence of NDH-1 has been reported in several strains. These include *P. denitrificans* (Imai *et al.*, 1968; Stouthamer, 1980), *T. thermophilus* HB-8 (Meinhardt *et al.*, 1990), *E. coli* (Matsushita *et al.*, 1987), *R. capsulata* (Baccarini-Melandri *et al.*, 1973), and *V. alginolyticus* (Unemoto and Hayashi, 1989).

The isolated *Paracoccus* NADH–Q oxidoreductase is composed of approximately ten unlike polypeptides and contains noncovalently bound

Table I. Properties of the FeS Clusters of NADH-Q Oxidoreductase (NDH-1) Segments of *P. denitrificans*, *T. thermophilus* HB-8, *E. coli*, and Bovine Heart Mitochondria

Organisms	Cluster	Field positions $g_{x,y,z}$	E_m (mV)	Cluster structure
P. denitrificans[a]	N1a	1.918, 1.937, 2.029	−150	Binuclear
	N1b	1.929, 1.941, 2.019	−260	Binuclear
	N2	1.919, 1.923, 2.050	−130	Tetranuclear
	N3	1.861, 1.935, 2.014	−240	Tetranuclear
	N4	1.878, 1.935, 2.093	−270	Tetranuclear
T. thermophilus HB-8[b]	N1a	1.93, 1.94, 2.02	−274	Binuclear
	N2	1.89, 1.95, 2.04	−304	Tetranuclear
	N3	1.80, 1.83, 2.06	−289	Tetranuclear
E. coli[c]	N1	1.92, 1.935, 2.03	−220	Binuclear
	N2	1.90, 1.91, 2.05	−240	Tetranuclear
Bovine heart[d]	N1a	1.91, 1.95, 2.03	−370	Binuclear
	N1b	1.92, 1.94, 2.02	−245	Binuclear
	N2	1.92, 1.92, 2.05	−20	Tetranuclear
	N3	1.86, 1.93, 2.04	−245	Tetranuclear
	N4	1.87, 1.93, 2.10	−245	Tetranuclear

[a]From Meinhardt *et al.* (1987).
[b]From Meinhardt *et al.* (1990).
[c]From Meinhardt *et al.* (1989).
[d]From Ingledew and Ohnishi (1980).

FMN and FeS clusters as prosthetic groups (Yagi, 1986). The ratio of FMN to nonheme iron (Fe) to acid-labile sulfide (S^{2-}), experimentally determined for the isolated enzyme complex, was 1 : 13–14 : 11–12. As seen in Table I, EPR studies of the NADH–Q oxidoreductase segment of *Paracoccus* membranes exhibit the presence of four confirmed FeS clusters (one binuclear and three tetranuclear) (Albracht *et al.*, 1980; Meinhardt *et al.*, 1987) akin to NADH–Q oxidoreductase of mammalian mitochondria (complex I) (Hatefi, 1985). This means an FMN : Fe : S^{2-} ratio of 1 : 14 : 14, which is roughly the same as the ratio obtained with the isolated *Paracoccus* enzyme. Meinhardt *et al.* (1987) have reported the presence of another binuclear cluster designated as N1a in the NADH–Q oxidoreductase segment of *Paracoccus* membranes. However, N1a appears to be quite labile since it decomposes within a week even when stored at liquid-nitrogen temperatures. In addition, the NADH–ubiquinone-1 (UQ_1) reductase activity of *Paracoccus* membranes is sensitive to rotenone even after the signals of cluster N1a are lost. Therefore, it remains to be seen whether cluster N1a is an essential component of *Paracoccus* NADH–Q oxidoreductase. Among the confirmed iron-sulfur clusters described above, only the E_m value of the cluster N2 exhibits pH dependence (−60 mV/pH), suggesting that cluster N2 is probably concerned with H^+ translocation at coupling site 1. Similar pH dependence was observed in the E_m value of cluster N2 of bovine NDH-1.

The NDH-1 isolated from *T. thermophilus* HB-8 membranes is composed of approximately 10 unlike polypeptides (Yagi *et al.*, 1988). This preparation contained noncovalently bound FMN, nonheme iron, and acid-labile sulfides. The ratio of FMN to nonheme iron to acid-labile sulfide was 1 : 11–12 : 7–9. EPR studies showed that three iron-sulfur clusters (one binuclear and two tetranuclear) were presented in the NDH-1 isolated from *T. thermophilus* HB-8 (Meinhardt *et al.*, 1990). These results are consistent with those using membrane-bound *T. thermophilus* NDH-1. To date, the effect of pH on the E_m of these three FeS clusters has not been elucidated. Furthermore, four species of stable semiquinone EPR signals were detected in *T. thermophilus* HB-8 membranes (Meinhardt *et al.* 1990). One of the four signals, which exhibits $E_{m,9} = -100$ mV, may belong to the NDH-1 because this semiquinone species was almost completely destabilized in the presence of high concentrations of 2-heptyl-4-hydroxyquinoline-*N*-oxide (250 μM) which inhibits the NDH-1. These results have suggested single electron transfer to and from Q in the *Thermus* NDH-1. In contrast, two major ubisemiquinone species have been observed in bovine Complex I with the E_m values of -115 and -169 mV (King and Suzuki, 1984). The former and the latter are assigned, respectively, to the $Q/Q^{\cdot-}$ and $Q^{\cdot-}/QH_2$ couples.

The *E. coli* NDH-1 probably contains noncovalently bound FMN (Hayashi *et al.*, 1989). EPR studies using membranes of this organism suggest that there might be at least two FeS clusters in the NDH-1 segment (one is binuclear and the other is tetranuclear) (Meinhardt *et al.*, 1989). Both clusters are extremely labile, even when the membranes are stored at 4°C.

As described above, all the NDH-1 enzymes isolated at present appear to contain noncovalently bound FMN and iron-sulfur clusters as prosthetic groups. In addition, the isolated bovine and *Neurospora* complex I also have similar prosthetic groups. It is likely that these prosthetic groups are a common feature among energy-transducing NADH-Q oxidoreductases. On the other hand, as seen in Table I, a high-potential tetranuclear cluster equivalent to mitochondrial FeS cluster N2 was found in *Paracoccus*, but not in *T. thermophilus* HB-8 nor in *E. coli* NDH-1.

Characterization of Subunits. In the case of bovine complex I, some of the subunits have been characterized (Galante and Hatefi, 1979; Pilkington and Walker, 1989; Fearnley *et al.*, 1989; Runswick *et al.*, 1989). For example, Chen and Guillory (1981) have shown, using a tritiated photoaffinity NADH analogue, that the M_r 51,000 polypeptide of bovine complex I is the NADH-binding subunit. Recently, it was shown that *N,N′*-dicyclohexylcarbodiimide (DCCD) inhibits enzyme activities of the NDH-1 of various organisms (Yagi, 1987), and that the DCCD-binding subunit of bovine NDH-1 is the mitochondrial ND-1 gene product (Yagi and Hatefi, 1988). Studies of Earley *et al.* (1987) have suggested that the rotenone-binding subunit of bovine

Table II. Comparison of Amino Acid Composition of NAD(H)-Binding Subunit of *P. denitrificans*, *T. thermophilus* HB-8, and Bovine NADH–Q Oxidoreductase Complex (NDH-1)

	Percentage residues		
Amino acid	*P. denitrificans*[a]	HB-8[a]	Bovine heart[b]
Alanine	11.08	11.57	10.13
Arginine	7.62	5.92	6.67
Aspartic acid	8.97	6.91	7.98
Glutamic acid	12.92	13.78	10.98
Glycine	11.71	9.46	12.08
Histidine	1.40	1.20	2.00
Isoleucine	6.50	5.13	6.58
Leucine	7.96	10.66	6.89
Lysine	2.83	6.83	5.70
Methionine	3.30	1.08	2.01
Phenylalanine	3.53	2.88	3.95
Proline	5.21	4.75	5.53
Serine	4.01	3.68	4.33
Threonine	4.94	5.56	5.17
Tyrosine	2.38	2.50	2.16
Valine	5.64	8.09	6.98
Polarity[c]	42.7	43.9	42.8
M_r	50,000	47,000	51,000

[a]The NADH binding subunit of *Paracoccus* NADH–Q oxidoreductase was isolated by preparative SDS-PAGE (Yagi and Hatefi, 1988). For amino acid analysis, the isolated subunit was re-electrophoresed and re-isolated. The purified NADH binding subunit (20 μg) was hydrolyzed in an evacuated and sealed tube with 6 N HCl at 110°C for 24 hours, and amino acid analysis was carried out on a Beckman 7300 amino acid analyzer.
[b]From the result reported by Galante and Hatefi (1979).
[c]Determined according to Capaldi and Vanderkooi (1972).

NDH-1 is also the ND-1 gene product. In contrast, except for the identification of the NADH-binding subunit in *Paracoccus* and *Thermus* (see below), nothing is known about the characteristics of the subunits of bacterial NDH-1 (Yagi, 1988, 1989; Yagi and Dinh, 1990; Xu and Yagi, unpublished results).

In order to identify the NADH-binding subunit of the *Paracoccus* NDH-1, purified enzyme was irradiated by UV light in the presence of [adenylate-^{32}P] NAD. Radioactivity was incorporated exclusively into a single polypeptide with M_r 50,000 (Yagi and Dinh, 1990). Similar results were obtained when [adenylate-^{32}P] NADH was used. The labeling of the M_r 50,000 polypeptide was diminished when UV irradiation of the enzyme with [^{32}P] NAD was performed in the presence of NADH, but not in the presence of NADPH, which is not a substrate for the *Paracoccus* NDH-1. The labeled polypeptide isolated by preparative SDS gel electrophoresis was shown to

cross-react with antiserum to the NADH-binding subunit (M_r = 51,000) of bovine NDH-1. In addition, antibody to the M_r 50,000 polypeptide of *Paracoccus* NDH-1 cross-reacted with the NADH-binding subunit of bovine complex I (Xu and Yagi, unpublished results). As shown in Table II, the amino acid composition of the *Paracoccus* NADH-binding subunit was also very similar to that of the bovine NADH-binding subunit. These chemical and immunological results indicate that the M_r 50,000 polypeptide is the NADH-binding subunit of *Paracoccus* NDH-1.

The same strategy was used for determination of the NADH-binding subunit of *T. thermophilus* HB-8 NDH-1. An M_r 47,000 polypeptide was labeled by [^{32}P]NAD(H) (Xu and Yagi, unpublished results). The labeling of the M_r 47,000 polypeptide with [^{32}P] NAD was decreased in the presence of NADH or deamino-NADH, but not in the presence of NADP(H). Antibodies to the bovine or the *Paracoccus* NADH-binding subunit failed to cross-react with the NADH-binding subunit of *T. thermophilus* HB-8.

Catalytic Properties. In *Paracoccus*, *T. thermophilus*, and *E. coli* membranes, the NDH-1 is able to utilize not only NADH but also deamino-NADH as a substrate. The K_m^{app} values for NADH in NADH–UQ_1 and NADH–$K_3Fe(CN)_6$ reductase activities were determined to be approximately 10 μM in all three of these organisms (see Table III). In contrast, NADPH does not act as a substrate for bacterial NDH-1 because the enzyme activities of the NDH-1 in the presence of NADPH are less than 2% of those using NADH. Ubiquinone-1 is commonly used for enzyme assays of the NADH–Q oxidoreductase regardless of the type of enzyme. As shown in Table III, there are no significant differences in the K_m^{app} values for UQ_1 between *Paracoccus* and *E. coli*, these K_m^{app} values both lying in the range of 10–15 μM. Although menaquinone-6 (MQ_6) but not ubiquinone is a constituent of *T. thermophilus* HB-8 membranes, the *Thermus* NDH-1 is able to reduce UQ_1 as well as MQ_1

Table III. K_m^{app} Values for NADH and UQ_1 of NADH–UQ_1 Reductase Activities of *P. denitrificans*, *T. thermophilus* HB-8, and *E. coli* NADH–Q Oxidoreductase

	K_m^{app} (μM)	
Organisms	for NADH	for UQ_1
P. denitrificans		
Membrane-bound NDH-1	14	14
isolated NDH-1	13	14
T. thermophilus HB-8		
Isolated NDH-1	10	—
Isolated NDH-2	12	—
E. coli		
Membrane-bound NDH-1	14.7	9.0
Membrane-bound NDH-2	50	13.3

(Yagi *et al.*, 1988; Meinhardt *et al.*, 1990). This is not surprising since even rhodoquinone has been reported to act as a substrate for bovine complex I whose physiological substrate is UQ_{10} (Kita *et al.*, 1988). In fact, duroquinone is also used for the enzyme assays of mammalian mitochondrial complex I because the substitution of these quinones for UQ_1 has little effect on the sensitivity to rotenone. In view of these results, substrate specificity of quinone-binding site of the NDH-1 appears to be fairly low. However, with respect to the bacterial NDH-1, the sensitivity to rotenone of the enzyme activities with duroquinone as the substrate is somewhat lower as compared to that with UQ_1 as the substrate, suggesting that duroquinone is able to accept electrons not only at the quinone side but also at the NADH side of the rotenone inhibition site in these enzyme complexes. The NADH–$K_3Fe(CN)_6$, NADH–menadione, and NADH–2,6-dichlorophenol-indophenol (DCIP) reductase activities were all unaffected by inhibitors of energy-coupling site 1 (e.g., retenone, piericidin A, or capsaicin). In addition, kinetic analysis of NADH–$K_3Fe(CN)_6$ reductase activity of bovine complex I suggests that NADH and $K_3Fe(CN)_6$ compete with each other at the same site on the enzymes (see below). Therefore, these activities do not appear to be accompanied by H^+ translocation. Nevertheless, a low level of H^+ translocation coupled to NADH–$K_3Fe(CN)_6$ reductase activity has been observed in *E. coli* membranes in the presence of KCN (Matsushita *et al.*, 1987). This apparent contradiction remains unsolved.

At the present time, aside from the results listed above, little is known about the reaction mechanism of the NDH-1. Inhibitors have been shown to be useful tools to probe the pathways of electron transfer and proton translocation of the ubiquinol–cytochrome *c* oxidoreductase, suggesting that a similar approach may work for NDH-1. Rotenone, piericidin A, capsaicin, and DCCD are well known as potent inhibitors of NDH-l in various organisms. However, there are some exceptions. Rotenone inhibition of deamino-NADH–UQ_1 reductase activity of *E. coli* NDH-1 is at most 50% even at high concentrations of the inhibitor. An additional complication with these inhibitors is their lack of specificity. Thus, piericidin A inhibits enzyme activities of the NDH-2 in *Bacillus subtilis* membranes and *Saccharomyces cerevisiae* submitochondrial particles. DCCD inhibits NADH–$K_3Fe(CN)_6$ reductase activity of *Paracoccus* membranes. Capsaicin inhibition appears to correlate better with the presence of coupling site 1 than inhibition by either rotenone, piericidin A, or DCCD. Kinetic analyses of inhibitory effect of capsaicin suggest that capsaicin probably inhibits by binding to the quinone-binding site of NDH-l (Shimomura *et al.*, 1989; Yagi, 1990). The inhibition of *Paracoccus* NDH-l activity by both capsaicin and rotenone can be reversed by washing the membranes with a medium containing bovine serum albumin (Meijer *et al.*, 1978; Yagi, 1990). Therefore, capsaicin appears to be a useful

reagent to investigate the role of quinones in the energy-coupling site associated with NDH-1.

As described above, it has been reported that DCCD binds to the mitochondrial ND-1 gene product and inhibits NADH–UQ_1 reductase activity of NDH-1 of various organisms. In ATP synthases, complex III and cytochrome oxidase, the subunits to which DCCD binds are, respectively, a proteolipid, cytochrome *b* (and/or possibly a subunit of M_r 8,000), and subunit III (Azzi *et al.*, 1984; Yagi, 1987). The proteolipid of the ATP synthase has been shown to be essential for proton translocation in this energy-transducing system. Furthermore, subunit III of cytochrome oxidase seems to be important for efficiency energy coupling. It is conceivable that in the NDH-1, the ND-1 gene product (DCCD-binding protein) is involved in proton translocation. In addition, it has been reported that rotenone also binds to the mitochondrial ND-1 gene product but the rotenone-binding site is different from the DCCD-binding site. Therefore, it might be of interest to study whether capsaicin and piericidin A bind to the same polypeptide of the NDH-1. Besides these inhibitors, dequalinium chloride, its derivatives, and tinopal AN have been reported to inhibit NADH–UQ_1 reductase of *Paracoccus* membranes (Anderson *et al.* 1989; Phillips and Kell, 1982). Recent reports suggest that the binding sites of these inhibitors might not be associated with the rotenone-binding site. *p*-Chloromercuribenzoate, 7-chloro-4-nitrobenz-2-oxa-1,3-diazole, and tetranitromethane, which are modifiers of amino acid residues, also inhibit NADH–UQ_1 reductase activities of NDH-1 (Yagi, unpublished results), suggesting yet other ways of probing the mechanism of NDH-1 activity.

Although the $H^+/2e$ stoichiometry of proton translocation coupled to electron transfer by bacterial NDH-1 has not been determined by a direct method, the difference of $H^+/2e$ between the system bearing sites 1, 2, and 3 and that bearing only sites 2 and 3, obtained using *Paracoccus* spheroplasts, suggests that the $H^+/2e$ value at site 1 is in the range of 2.5–5.5 (Stouthamer, 1980). Without precise information about $H^+/2e$ we are unable to speculate on the mechanism of the energy coupling in the bacterial NDH-1. In the case of bovine complex I, several types of proton-translocation mechanism are proposed (see Hatefi, 1985; Ragan, 1987; Krishnamoorthy and Hinkle, 1988). Whether the same mechanism operates in the bacterial NDH-1 remains to be seen.

Paracoccus cells grown under sulfate-limited or rotenone-present conditions have been shown to lack rotenone senstivity, site 1 coupling, and EPR signals for cluster N2 (Meijer *et al.* 1977). As described above, the E_m values of the cluster N2 of *Paracoccus* and bovine NDH-1 appear to be pH-dependent. These results suggest that the subunit carrying cluster N2 may be responsible for proton translocation and rotenone sensitivity. Nevertheless,

as seen in Table I, the high-potential tetranuclear cluster equivalent to mitochondrial and *Paracoccus* FeS cluster N2 was not found in *T. thermophilus* HB-8 or in *E. coli* NDH-1. Therefore, it remains to be seen what role this high-potential tetranuclear cluster plays in mitochondria and *Paracoccus* and why it is absent in *T. thermophilus* HB-8 or *E. coli* NDH-1.

NDH-2

Composition. Table IV compares the characteristics of NDH-2 enzymes isolated from various bacteria. It is clear that the NDH-2 is widely present in bacteria. Most of these enzymes contain noncovalently bound FAD as a prosthetic group and no FeS clusters. However, a single polypeptide enzyme believed to be NDH-2 was isolated from *Thermus aquaticus*, and was found to contain FMN instead of FAD (Walsh *et al.*, 1983). The possibility exists, however, that the isolated enzyme is a fragment of the NDH-1 because inhibitory effects of rotenone and capsaicin on NADH oxidase and NADH–UQ_1 reductase activities of membranes of this organism suggested that this bacterium has the NDH-1 (Yagi, unpublished results). Furthermore, it has been reported that the NDH-2 is present not only in bacteria but also mitochondria of *S. cerevisiae* (de Vries and Grivell, 1988) and in plant mitochondria (Palmer and Moller, 1982; More and Rich, 1980). In contrast, there have been no reports suggesting the existence of the NDH-2 in *Paracoccus* membranes.

Genes of the NDH-2 of *E. coli* (Young *et al.*, 1981) and alkalophilic *Bacillus* YN-1 (Xu *et al.*, 1991) have been cloned and their DNA sequences determined. As shown in Fig. 1, there are three homologous regions

Table IV. Characteristics of NDH-2 Isolated from Various Organisms

Organisms	M_r of subunit (kDa)	Flavin	Ratio of flavin to subunit (mol/mol)	Reference
Bacillus subtilis	63	FAD	—	Bergsma *et al.*, 1982
Bacillus caldotenax	44	FAD	—	Kawada *et al.*, 1981
alkalophilic *Bacillus* YN-1	65	FAD	1	Hisae *et al.*, 1983
Escherichia coli	47	FAD	1	Jaworowski *et al.*, 1981
Bacillus stearothermophilus	43	—	—	Mains *et al.*, 1980
Thermus thermophilus HB-8	53	FAD	1	Yagi *et al.*, 1988
Thermus aquaticus	50	FMN	0.05	Walsh *et al.*, 1983
Sulfolobus acidocaldarius	50	—	—	Wakao *et al.*, 1987
Photobacterium phosphoreum	—	FAD	—	Imagawa and Nakamura, 1978
Halobacterium cutirubrum	—	FAD	—	Hochstein and Dalton, 1973
Saccharomyces cerevisiae mitochondria	53	FAD	0.9	de Vries and Grivell, 1988

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           6
a) E. coli KIVIVGGGAGGLEMATQLGHKLGRKKKAKITLVD
   YN-1    DVLVVGGGPAGASSAIYAARKGIR----TGIVAD
           210

           170
b) E. coli NIAIVGGGATGVELSAELHNAVKQLHSYGYKGLTNE
   YN-1    DVAVIGGGNSGVEAAIDLAGIV--------NHVTVLE
           349

           298
c) E. coli VVEPTLQTRDPDIYAIGDC
   YN-1    VVDSHGATNVPGVFAAGDC
           462
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Fig. 1. Homologies between *E. coli* NDH-2 and alkalophilic *Bacillus* YN-1 NDH-2.

between these two bacterial NDH-2 primary structures as predicted by the DNA sequences. Two of them (Fig. 1a,b) exhibit the particular sequences known as a Rossman fold which are believed to form a $\beta\alpha\beta$ unit with ADP-binding properties (Wierenga *et al.*, 1986). Because the NDH-2 of both bacteria contains FAD as a prosthetic group, it is possible that one of the $\beta\alpha\beta$ regions may represent a binding site for FAD and the other for NADH. Furthermore, we can assume that the quinone-binding sites of NDH-2 of various organisms share homologous structures with each other, because UQ_1 acts as a substrate for all known NDH-2's. It is therefore possible that the third homologous region (Fig. 1c) may constitute the quinone-binding site.

Removal of 60 residues from the N-terminus of alkalophilic *Bacillus* NDH-2 had little effect on the NADH dehydrogenase activity of this enzyme and yet resulted in complete loss of its ability to bind to liposomes. In view of these results, the 60 residues of the N-terminus of alkalophilic *Bacillus* NDH-2 appear to function as its anchor into membranes (Xu *et al.*, 1985, 1989). However, a corresponding region has not been found in *E. coli* NDH-2. Recently, the primary structure of the N-terminal region between 1–25 of *Thermus* NDH-2 has been determined (Xu and Yagi, unpublished results). Although no homology to the alkalophilic *Bacillus* N-terminal membrane anchor was founded, a sequence suggestive of a nucleotide-binding site (GXGXXG) is present, indicating that *Thermus* NDH-2 might be homologous to its *E. coli* counterpart in terms of amino acid sequence rather than to alkalophilic *Bacillus.*

Catalytic Properties and Mechanism of Action. NADH is able to act as a substrate for the NDH-2 but NADPH is not. Although the NDH-2 of *T. thermophilus* HB-8 membranes and *S. cerevisiae* mitochondria are able to react with deamino-NADH as a substrate as well as NADH, their *E. coli* and *B. subtilis* counterparts cannot catalyze deamino-NADH oxidation.

Therefore, in *E. coli* membranes, use of deamino-NADH allowed activity measurements specifically limited to NDH-1, providing a selective assay for study of the characteristics of NDH-1 in the presence of NDH-2. The NDH-2 is not inhibited by rotenone, capsaicin, or DCCD. To date, only a single NDH-2 inhibitor, flavone, has been reported. This compound acts as an inhibitor for the NDH-2 of *Arum maculalum* and *S. cerevisiae* mitochondria (Cook and Cammack, 1984; de Vries and Grivell, 1988).

In the case of *E. coli*, the K_m^{app} value for NADH of NADH–UQ_1 reductase activity by the NDH-2 appears to be approximately eight times higher than the same values for the NDH-1 enzyme (Matsushita *et al.*, 1987). In contrast, there are not significant differences between the NDH-1 and the NDH-2 in terms of the K_m^{app} values for UQ_1 (Yagi and Calhoun, unpublished results). The K_m^{app} value for NADH of NADH–UQ_1 reductase activity by *Thermus* NDH-2 is similar to those of NDH-1 in *T. thermophilus* HB-8 (Yagi *et al.*, 1988).

Using the NDH-2 isolated from marine bacterium, *Photobacterium phosphoreum*, Imagawa and Nakamura (1978) have reported that the reaction of NADH–DCIP reductase of this enzyme follows a ping-pong bi bi mechanism. Similar results have been obtained for reaction of NADH–$K_3Fe(CN)_6$ reductase of bovine NDH-1 (Dooijewaard and Slater, 1976a, 1976b). These results imply that NADH and DCIP [or $K_3Fe(CN)_6$] react at the same or overlapping sites on the enzymes. In contrast, the reaction of NADH–DCIP reductase by the NDH-2 of *Halobacterium halobium* conforms to the order bi bi mechanism (Hochstein, 1975). To our knowledge, there has been no report regarding the reaction mechanism of NADH–UQ_1 reductase by the NDH-1 or the NDH-2.

Conclusions and Perspectives

As described above, *Paracoccus* membranes grown under sulfate-limited conditions have been reported to lack cluster N2 signal in the NDH-1 segment and energy-coupling site 1. Two possible explanation were given by Meijer *et al.* (1978). (1) The loss or modification of N2 leaves to a "short circuit" of the proton-translocating oxidoreduction segment normally associated with the NDH-1. (2) The growth conditions mentioned lead to the synthesis of a nonproton-translocating NADH–Q oxidoreductase. In addition, *E. coli* membranes grown under the same conditions as above have been reported to lack EPR signals due to FeS clusters in NADH–Q oxidoreductase segment but show electron-transport activity from malate to oxygen as high as normal membranes. Furthermore, there seems to exist a mechanism in certain bacteria to regulate the relative populations of the two

distinct types of NADH–Q oxidoreductase in response to various growth conditions. It is well known that in the case of cytochrome oxidase, oxygen controls the content of the two types of this enzyme complex (heme aa_3 and heme *o*). Although researchers are inclined to study the NADH–Q oxidoreductase bearing energy-coupling site, the author would like to emphasize the importance of not only the NDH-1 but also the NDH-2 that exists widely in bacterial membranes and plant and fungus mitochondria.

Since the earlier work concerning the NADH–Q oxidoreductase in *E. coli* by Bragg and Hou (1967) was published, more than three decades have passed. Stouthamer and his colleagues and Young and his colleagues have contributed to the elucidation of structure and function of bacterial NADH–Q oxidoreductases, and yet progress in the study of bacterial NADH–Q oxidoreductase seems to be slower than progress in the mammalian system. One of the reasons appears to be the presence of these two distinct types of the enzymes in bacteria which have not been characterized until lately. These problems have been partially overcome at present. It seems to the author that the structural simplicity and possibility of gene manipulation present in the bacterial systems provide exciting options in the continued study of this enzyme. The application of these results to the mammalian complex I is important not only in the field of bioenergetics but also in clinical medicine.

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