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Minireview

Bacterial NADH-Quinone Oxidoreductases: Iron-Sulfur Clusters and Related Problems

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Many bacteria contain proton-translocating membrane-bound NADH-quinone oxidoreductases (NDH-1), which demonstrate significant genetic, spectral, and kinetic similarity with their mitochondrial counterparts. This review is devoted to the comparative aspects of the iron-sulfur cluster composition of NDH-1 from the most well-studied bacterial systems to date.: *Paracoccus denitrificans*, *Rhodobacter sphaeroides*, *Escherichia coli*, and *Thermus thermophilus*. These bacterial systems provide useful models for the study of coupling Site I and contain all the essential parts of the electron-transfer and proton-translocating machinery of their eukaryotic counterparts.

KEY WORDS: NADH-quinone oxidoreductase: NDH-1; iron-sulfur cluster; *Paracoccus denitrificans*; *Rhodobacter sphaeroides*; *Escherichia coli*; *Thermus thermophilus*.

INTRODUCTION

Mitochondrial NADH:ubiquinone oxidoreductase (Complex I, coupling Site I) plays a central role in the oxidation of NADH, the reducing product of cellular metabolism, by the respiratory chain. Complex I is the most complicated and least understood energy-transducing proton-motive device of the respiratory chain. Mammalian NADH:ubiquinone oxidoreductase consists of 41 subunits (Arizmendy *et al.*, 1992a,b; Walker, 1992) and contains the flavin mononucleotide (FMN) molecule, at least five iron-sulfur clusters, and one to two bound ubiquinone species as prosthetic groups (Ohnishi, 1979; Beinert and Albracht, 1982; Suzuki and King, 1983; Burbaev *et al.*, 1989). Because of the striking complexity of the eukaryotic mitochondrial Complex I, considerable efforts were made to obtain simpler prokaryotic Site I model systems.

Many bacterial membrane-associated electron-transfer enzyme complexes, including NADH : quinone oxidoreductase, demonstrate significant spectral, genetic, and kinetic similarity with their mitochondrial counterparts (Trumpower, 1990; Anraku and Gennis, 1987; Yagi, 1991) and provide useful and adequate models for energy-transduction studies. Besides, bacterial systems have a series of rather obvious advantages over mammalian sources for the study of membranous respiratory complexes. (i) The ease of genetic manipulations including: overexpression, amplification, or disruption of genes encoding complexes; generation of random and site-directed mutants; comparison of DNA-determined amino acid sequences to define structural cognation and important conserved motifs within the proteins from different taxonomic groups. (ii) Bacteria tend to contain redundant electron-transfer systems (Anraku, 1988) expressed in the form of multiple enzymes, such as alternative oxidases or multiple dehydrogenases. Thus, for instance, in most bacterial systems

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there are two distinct membrane-bound NADH dehydrogenases (NDH-1 and NDH-2) (Yagi, 1986). Only the former consists of multiple subunits, contains FMN and FeS clusters, and works as a coupling site. Due to this redundancy, bacteria are capable of growth under a variety of conditions utilizing different branches of their electron-transport systems, thus allowing the modification of one system into a nonfunctional enzyme and maintenance of a viable organism. This redundancy, in some cases, makes it difficult to carry out kinetic studies of the enzyme, but through the use of selective substrates [such as deamino-NADH with *E. coli* (Matsushita *et al.*, 1987) and *Rb sphaeroides* NDH-1] or specific inhibitors, these difficulties can be overcome. (iii) Usually bacterial systems provide a simpler polypeptide composition of electron-transfer complexes as compared with their mitochondrial cousins. This is the case for the cytochrome bc_1 complexes and cytochrome aa_3 oxidases (Ludwig and Schatz, 1980; Yang and Trumpower, 1986), but apparently not for the NADH dehydrogenase complexes, which are still extremely complicated systems in bacteria.

To date, the most well-studied bacterial systems with respect to NADH dehydrogenase are *P. denitrificans*, *Rb. sphaeroides*, *E. coli*, and *T. thermophilus*. From all of these species NDH-1 has been obtained in purified form (Yagi, 1986; Yagi *et al.*, 1988; Hayashi *et al.*, 1989; Yumoto *et al.*, 1992; H. Leif *et al.*, unpublished data; Fukumori *et al.*, unpublished data). Redox components of these enzymes have been characterized both *in situ* and in isolated NDH-1 preparations (Meinhardt *et al.*, 1987, 1989, 1990; Fukumori *et al.*, unpublished data). NDH-1 gene clusters of *P. denitrificans* (Xu *et al.*, 1991a,b, 1992a,b, 1993) and *E. coli* (Weidner and Weiss, 1992; Weidner *et al.*, 1993) have been sequenced and similar work on photosynthetic bacteria is in progress (Dupuis, 1992).

Since genetic and structural aspects of energy-coupled NADH dehydrogenase complexes from different bacteria are reviewed in detail in recent papers (Berks and Ferguson, 1991; Weiss *et al.*, 1991; Yagi, 1991, 1993; Walker, 1992; Pilkington *et al.*, 1993; reviews in this volume), this article will focus mostly on the composition of iron-sulfur clusters in NDH-1 from the aforementioned bacterial species.

Paracoccus Denitrificans

The cytoplasmic membrane of *P. denitrificans* shows a remarkable similarlity to the inner mitochondrial membrane from functional organization of the aerobic electron transport chain to the fine spectral and thermodynamic properties of intrinsic redox components of the respiratory complexes (John and Whatley, 1975; Stouthamer, 1980, Albracht *et al.*, 1980); therefore this bacterium rightfully got the name "a free-living mitochondrion." Like the mitochondrial complex, *P. denitrificans* NADH dehydrogenase contains five EPR[7] detectable iron-sulfur clusters (N1a through N4) with spectral and thermodynamic properties almost identical to the mitochondrial counterparts (see Table I). This fact indicates that the local protein surroundings of the FeS clusters are very close, if not the same, in *P. denitrificans* and mammalian Complex I. Taking into account the latter consideration and the fact that the *g*-values of binuclear FeS clusters are very sensitive to their microenvironment (Blumberg and Peisach, 1974; Dugad *et al.*, 1990), it is absolutely amazing that binuclear cluster N1a in *P. denitrificans*, which demonstrates the same EPR properties as its mitochondrial counterpart, has an almost 200 mV higher (−160 mV vs. ~ −400 mV) midpoint potential. By rough comparison of the putative [2Fe–2S] cluster binding sites' sequences in *P. denitrificans* and bovine heart, one can see that the nearest neighborhood of conserved cysteine residues in the NQO3 gene product and the homologous 75 kDa subunit is much closer than that in the NQO2 gene product and the 24 kDa polypeptide (Xu *et al.*, 1991a,b; Pilkington and Walker, 1989; Runswick *et al.*, 1989). Thus, it seems not unreasonable to suggest that the [2Fe–2S] cluster designated as N1a in *P. denitrificans* is localized in the 25 kDa subunit (NQO2 gene product), and that the difference in the clusters' environments could provide the higher midpoint potential as compared with N1a in mammalian system.

Another puzzle concerns the stoichiometry of N1a and N1b clusters. From redox potentiometric titration data it was found that both of them are in stoichiometric amounts when compared to cluster N2 (Meinhardt *et al.*, 1987) and, in accordance to their midpoint potentials, should be reducible by NADH. On the other hand, quantitation of N1 content in NADH reduced subbacterial particles have revealed a stoichiometry of 1 : 1 with the N2 cluster instead of

[7] Abbreviations: UQ_1, ubiquinone-1; MQ_1, menaquinone-1; DB, 2,3-dimethoxy-5-methyl-6-decylbenzoquinone; DCCD, *N,N'*-dicyclohexylcarbodiimide; d-NADH, reduced nicotinamide hypoxanthine dinucleotide (deamino-NADH); EPR, electron paramagnetic resonance.

Table I. Properties of the FeS Clusters of NADH-Q Oxidoreductase (NDH-1) Segments of Bacterial Respiratory Chain in Comparison with Bovine Heart Mitochondra

Organisms	Clusters	Field position			E_m (mV)	−60 mV/pH
		g_x	g_y	g_z		
P. denitrificans[a]	N1a [2Fe–2S]	2.03	1.94	1.92	−150	+
	N1b [2Fe–2S]	2.02	1.94	1.92	−260	−
	N2 [4Fe–4S]	2.05	1.92	1.92	−130	+
	N3 [4Fe–4S]	2.01	1.94	1.88	−240	−
	N4 [4Fe–4S]	2.09	1.94	1.88	−270	−
Rb. sphaeroides[b]	N1a [2Fe–2S]	2.02	1.93	1.93	−380	?
	N1b [2Fe–2S]	2.02	1.94	1.93	−240	−
	N2 [4Fe–4S]	2.05	1.93	1.92	−130	+
	N3 [4Fe–4S]	2.05	1.93	1.87	−230	−
	N4 [4Fe–4S]	2.08	1.94	1.89	−240	−
	?N5 [4Fe–4S]	2.07	1.92	1.89	~ − 250	?
T. thermophilus[c]	N1a [2Fe–2S]	2.02	1.94	1.92	< −350	+
	N1b [2Fe–2S]	2.02	1.94	1.93	−270	−
	N2 [4Fe–4S]	2.04	1.95	1.89	−300	−
	N3 [4Fe–4S]	2.04	1.95	1.89	−440	−
	N4 [4Fe–4S]	2.06	1.83	1.80	−290	−
E. coli[d]	N1a [2Fe–2S]	2.03	1.94	1.94	−380	n.d.
	N1b [2Fe–2S]	2.03	1.94	1.94	−260	n.d.
	?N1c [2Fe–2S]	1.99	1.95	1.92	−240	n.d.
	?N2 [4Fe–4S]	2.06	1.94	1.93	−160	n.d.
	N3 [4Fe–4S]	2.09	1.94	1.89	−240	n.d.
	N4 [4Fe–4S]	2.05	1.91	1.90	−270	n.d.
Bovine heart[e]	N1a [2Fe–2S]	2.03	1.95	1.91	−370	+
	N1b [2Fe–2S]	2.02	1.94	1.92	−250	−
	N2 [4Fe–4S]	2.05	1.93	1.92	−150	+
	N3 [4Fe–4S]	2.04	1.93	1.86	−250	−
	N4 [4Fe–4S]	2.10	1.94	1.89	−250	−
	N5 [4Fe–4S]	2.07	1.93	1.90	−260	−

[a] Meinhardt *et al.* (1987); [b] Y. Fukumori *et al.*, unpublished data; [c] Meinhardt *et al.* (1990); [d] H. Leif *et al.*, unpublished data; [e] Ingledew and Ohnishi (1980).

the expected 2 : 1 value (Albracht *et al.*, 1980). The reason for this discrepancy is still waiting for an explanation and may elucidate the role of the N1a cluster in electron transfer.

The E_m value of cluster N2 in *P. denitrificans* is pH-dependent, as it is in mitochondria, but exhibits a pK of the reduced form at pH 7.6 (Meinhardt *et al.*, 1987). Cluster N1a also elicits a pH-dependent midpoint potential, but over a very narrow pH interval (from 6.5 to 7.5). Although both clusters in mammalian Complex I were considered to be directly involved in the proton-translocation mechanism (Ohnishi, 1973; Gutman *et al.*, 1972), it is not clear whether these peculiarities of N1a and N2 cluster behavior in *P. denitrificans* reflect any change in the proton-translocating mechanism with changing pH. If they really do, one can expect variable H^+/e^- stoichiometry and that by varying pH it would be possible to separate the function of two proposed subsites in coupling Site I (Ragan, 1990; Kotlyar *et al.*, 1990; A. Vinogradov, this volume).

Rhodobacter sphaeroides

Rhodobacter sphaeroides and other purple photoheterotrophic bacteria have been extensively studied with regard to their primary photochemistry and bioenergetics. When these bacteria are cultivated aerobically in the dark, they are able to utilize their respiratory chain which is very similar to the mitochondria and is composed of coupling Site I (NADH dehydrogenase) (Baccarini-Melandri *et al.*, 1973), Site II (cytochrome bc_1 complex) (Takamiya *et al.*, 1982), and Site III (cytochrome aa_3 complex) (Gennis *et al.*, 1982). Cytochrome bc_1 complex and cytochrome aa_3 oxidase have been isolated and were

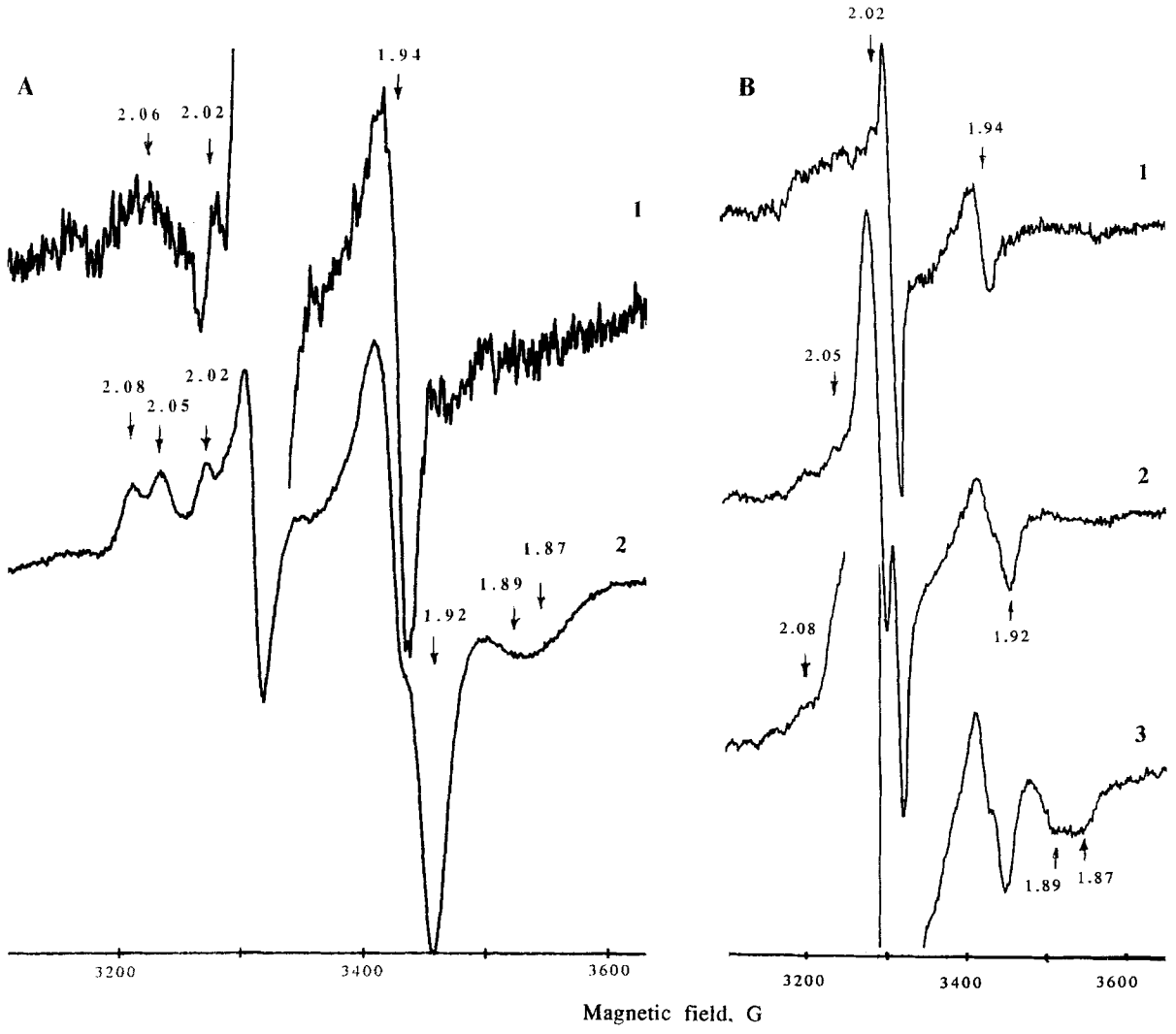

Fig. 1. A. Differential "−358 mV minus −157 mV" EPR spectra of potentiometrically poised chromatophores, prepared from aerobically dark-grown *Rb. sphaeroides* GA cells. Chromatophores (28.5 mg/ml) were suspended in 20 mM MOPS (pH 7.0), 0.1 M KCl, and 5 mM $MGSO_4$ and potentiometrically titrated in the presence of the following redox dyes: methyl viologen, benzyl viologen (40 μM of each), 2-hydroxy-1,4-naphthoquinone, indigotetrasulfonate, indigotrisulfonate, indigodisulfonate, neutral red, phenosafranine, safranine-T, (50 μM of each), 2-hydroxy-1,4-naphthoquinone-2-sulfonate (33 μM), and pyocyanine (10 μM). Spectra were measured at 28 K and 2 mW microwave power (1) and 10.5 K and 10 mW (2). Other EPR conditions: microwave frequency 9.2 GHz, modulation amplitude 12.5 G, modulation frequency 100 kHz, scanning rate 250 G/min, time constant 0.064 sec. B. EPR spectra of deamino-NADH-reduced *Rb. sphaeroides* GA chromatophores prepared from aerobically dark-grown cells. Chromatophores (23 mg/ml) were suspended in 50 mM MOPS (pH 7.0), 0.1 M KCl, and 5 mM $MgSO_4$, and treated with 2 nmol piericidin A per mg protein, and the sample was frozen 6 sec after d-NADH addition. Spectra were measured at 27 K and 2 mW microwave power (1), 14 K and 1 mW (2), and 7.6 K and 20 mW (3). Other EPR condition as in (A).

found to contain redox components very similar to those of the mitochondrial counterparts (Yun *et al.*, 1990; Cao *et al.*, 1992). It is also known that this bacterium contains some low-potential iron-sulfur clusters (Zannoni and Ingledew, 1983). We have conducted detailed EPR, thermodynamic, and kinetic analysis of the Site I system in *Rb. sphaeroides* and *Rb. capsulatus*. These bacteria, as well as *E. coli*, contain two NADH dehydrogenases, NDH-1 and NDH-2 (La Monica *et al.*, 1976). The former is very sensitive to the conventional Site I inhibitors (see Table II) and, in contrast to NDH-2, is able to use deamino-NADH as a substrate with almost the same V_{max} and K_m as for NADH. As exemplified in Fig. 1, EPR spectra of

Table II. Comparative Affinities of NDH-1 Segments of Bacterial Respiratory Chains to Site I inhibitors

	I_{50} (μM)		
	Rotenone	Piericidin A	Annonin VI[e]
Rb sphaeroides GA chromatophores[a]	0.24	0.07	0.88
E. coli NKSO2 membranes[b]	8.5	4.5	0.03
T. thermophilus HB-8 membranes[c]	0.35	0.30	0.13
T. thermophilus HB-8 isolated NDH-1[c,d]	23	20	43

[a] Spectrophotometric measurements of inhibitory effect on deamino NADH oxidase activity.
[b] NDH-2-Depleted mutant [Calhoun and Gennis, 1992]. Polarographic measurements of inhibitory effect on NADH oxidase reaction.
[c] Spectrophotometric measurement of inhibitory effect on NADH : DB reductase activity of NDH-1.
[d] Isolated NDH-1 was reconstituted with 1 mg of asolectin per mg protein before activity measurements.
[e] Loundershausen *et al.* (1991).

Rb sphaeroides chromatophores reduced both with deamino-NADH (B) and potentiometrically (A) demonstrate the existence of iron-sulfur clusters N1a, N1b, N2, N3, N4, and, probably, N5. Spectral and thermodynamic characteristics of these centers are very close to those of the mitochondrial counterparts (see Table I). Such a similarity together with additional attractive possibilites for a very fast flash-induced ubiquinone reduction and energization of chromatophore membrane and for direct quantitative calibration of H^+/e^- stoichiometry using electrochromic carotenoid bandshift (Cotton *et al.*, 1989) makes photosynthetic bacteria a perfect model for the study of the energy-transducing mechanism in coupling Site I.

Escherichia coli

The *E. coli* energy-coupled NADH dehydrogenase complex has the most dramatic history of investigation. For many years a controversy existed concerning the presence of coupling Site I in this organism. This controversy was resolved in 1987 through the work of Matsushita *et al.* (1987) who demonstrated the presence of two NADH dehydrogenase complexes. One complex (NDH-1), in contrast to the other (NDH-2), contains iron-sulfur clusters, is able to oxidize deamino-NADH, and is coupled to the generation of $\Delta\mu H^+$. The physiological role of this "double entrance" system remains a mystery. Recent *in vivo* study of the bioenergetic efficiency of the *E. coli* aerobic respiratory chain has revealed that both NDH-1 and NDH-2 almost equally share the electron flux from reduced nucleotides (Calhoun *et al.*, 1993); therefore, the presence of NDH-2 leads to a decrease of total energetic efficiency (P/O ratio) of respiration by performing the role of an endogenous uncoupler of Site I. Apparently, the electron flow partitioning should be under strong metabolic and/or genetic control, but almost nothing is known for now about the mechanisms of this regulation (Spiro *et al.*, 1989).

The iron-sulfur-containing NADH dehydrogenase complex of *E. coli* has been one of the most difficult of the bacterial systems to characterize spectroscopically. EPR analysis of FeS clusters in NDH-1 *in situ* using conventional Site I inhibitors (rotenone and piericidin A) has been hindered by the relatively low sensitivity of *E. coli* to these compounds (see Table II) and by the presence of the inhibitor-insensitive NDH-2. Potentiometric analysis is impeded due to the presence of numerous membrane-bound oxidoreductases which also contain FeS clusters and whose number and amount vary widely depending on growth conditions and the strain (Ingledew and Poole, 1984). Besides, the extreme lability of NDH-1 makes it difficult to work on the Site I segment of the respiratory chain in this bacterium (Matsushita *et al.*, 1987; Meinhardt *et al.*, 1989). Early work on the iron-sulfur clusers in *E. coli* K12 (Ingledew *et al.*, 1980) demonstrated the presence of two low-potential clusters, one binuclear and one tetranuclear, although the functional role of these clusters was not investigated. Later two additional iron-sulfur clusters with low midpoint potentials were identified in *E. coli* strain GR19N as probable intrinsic components of NDH-1 (Meinhardt *et al.*, 1989). A breakthrough was made in this field very recently when H. Weiss laboratory succeeded in cloning and sequencing the *E. coli* NDH-1 gene cluster (Weidner *et al.*, 1993; Friedrich *et al.*, this volume). They have found that the homologues for all subunits (51, 24, 75, 23 (TYKY-)[8] and 20 kDa (PSST-)[8]) containing cystein-rich motifs in bovine heart (Walker, 1992), and *P. denitrificans* (Yagi, 1991, 1993) are also present in the *E. coli* genome.

[8] A one-letter code of the sequence of amino acids 1–4 was used in order to distinguish subunits with the same molecular weight (according to Walker, 1992).

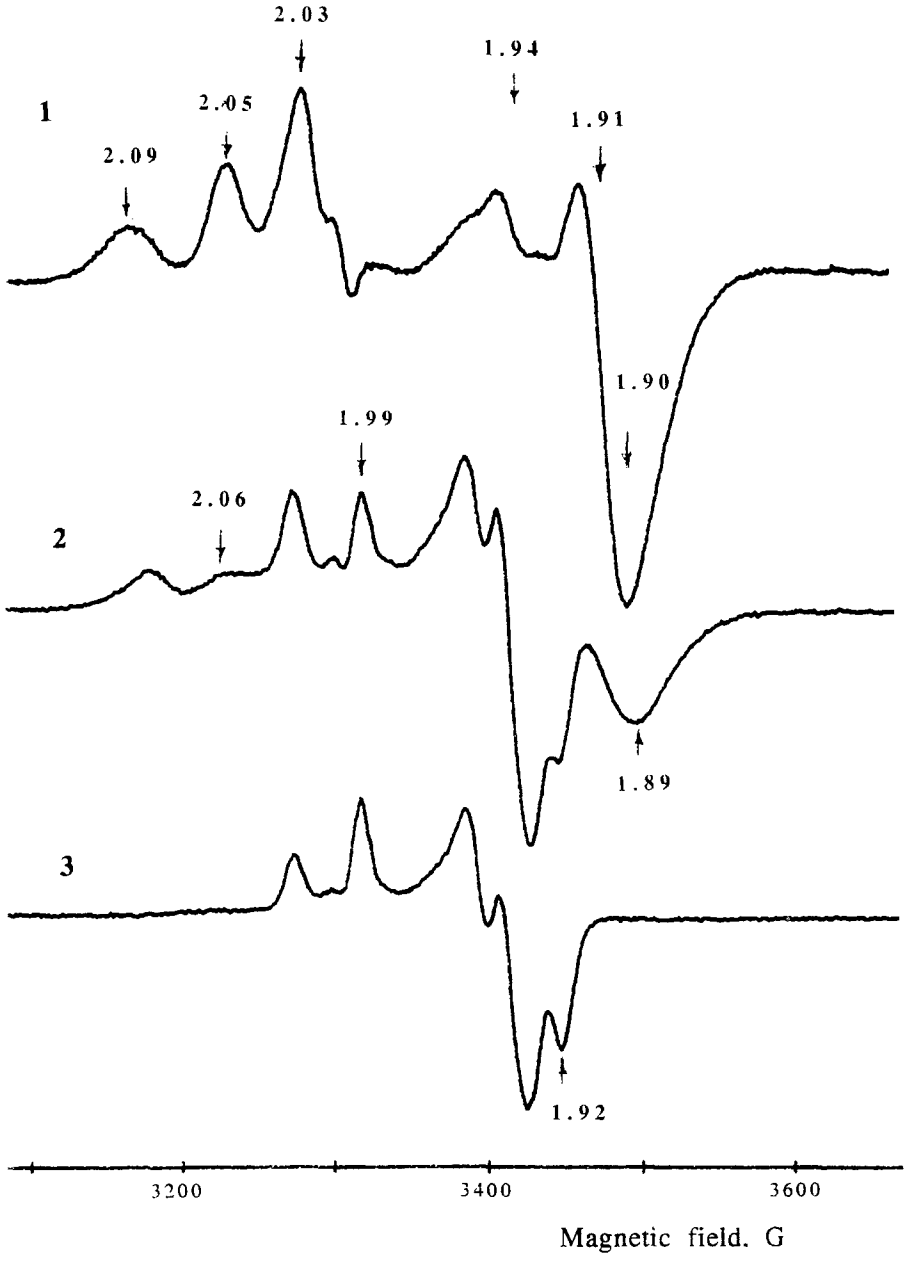

Fig. 2. EPR spectra of NADH-reduced isolated NDH-1 from *E. coli* (18 mg/ml in 50 mM MES, pH 6.0). EPR conditions as in Fig. 1A except microwave power and temperature, which were: 10 mW, 7.8 K (1), 5 mW, 15 K (2), and 1 mW, 25 K (3).

Our EPR analysis of membranes from mutant strains of *E. coli* with genetically deleted NDH-2 (NKSO2) and succinate dehydrogenase complex (MWC231) (Calhoun and Gennis, 1993) together with analysis of NDH-1 isolated in H. Weiss's laboratory (H. Leif *et al.*, unpublished data) has revealed the presence of most likely six FeS clusters (see Fig. 2 and Table I) (manuscript in preparation): three binuclear and three tetranuclear. The very-low-potential [2Fe–2S] center with an axial-type signal may correspond to the mammalian N1a cluster. The existence of a high-potential [4Fe–4S] cluster *in situ* is still subject to doubt since its concentration is much less relative to the other clusters. According to spectral similarity a [4Fe–4S] cluster with axial symmetry ($g_{x,y,z} = 2.05$, 1.91, 1.90) might be the counterpart of the mitochon-

Table III. Kinetic Characteristics of Membrane-Bound and Purified *T. thermophilus* NDH-1 Interaction with Quinone Acceptors (Hepes, pH 7.5, 100 μM NADH)

Acceptor	Parameter	Membrane-bound NDH-1	Purified NDH-1[a]
UQ_1	K_m (μM)	0.75	~5
	V_{max} (μmole/min mg)	0.80	—
DB	K_m (μM)	5.8	8.4
	V_{max} (μmole/min mg)	0.02	6.6
MQ_1	K_m (μM)	0.59	>100
	V_{max} (μmole/min mg)	0.90	—

[a] Reconstituted with 1 mg asolectin per mg protein.

drial cluster N-2, but it has a much lower midpoint potential. The pH dependence of the E_m values of all clusters is currently under investigation. It is worthwhile noting that fully conserved, very characteristic sequence motifs for the putative N-2 binding site in mammalian TYKY and PSST and *P. denitrificans* NQO9 and NQO6 gene products (Dupuis *et al.*, 1991; Arizmendi *et al.*, 1992a; Xu *et al.*, 1992a, 1993) were also found in *E. coli* NUO9 and NUO2 gene products (Weidner *et al.*, 1993), although the degree of sequence identity between *E. coli* and bovine is significantly less than between *P. denitrificans* and bovine.

An interesting peculiarity of the *E. coli* system is the presence of a third binuclear cluster (N1c) with a very unusual EPR spectrum ($g_{x,y,z} = 1.99, 1.95, 1.92$). Such a low g_z value for a binuclear FeS clusters usually indicates a change in the ferric atom covalency and has been observed for soluble ferredoxins only in the presence of harsh chaotropic agents like 1.5 M guanidine (Blumberg and Peisach, 1974). Since in membrane preparations its spectrum is strongly overlapped with $g = 2.00$ free radical resonance, it is not clear whether this cluster is a product of some other [2Fe–2S] cluster deterioration during NDH-1 isolation or is an intrinsic component of the enzyme.

Thermus thermophilus

After a series of unsuccessful attempts to purify intact (or in other words, sensitive to conventional inhibitors and capable of proton translocation) NADH : quinone reductase from mesophilic bacteria (Yagi, 1986; Hayashi *et al.*, 1989; Y. Fukumori, unpublished data), researchers have fixed their eyes upon the NADH dehydrogenase of thermophilic bacteria which were expected to be the most promising system for purification, reconstitution, and crystallization due to the extreme stability of their proteins against heat as well as against denaturating environments (Kagawa, 1978; Fee *et al.*, 1986).

The respiratory chain of *T. thermophilus* also possesses two membrane-bound NADH-dehydrogenase complexes (Yagi, 1986; Yagi *et al.*, 1988), one of which (NDH-1) is sensitive to rotenone and piericidin A (see Table II) and exhibits formation of the membrane potential coupled with the electron-transfer (Meinhardt *et al.*, 1990).

Like the mitochondrial system, membrane particles from *T. thermophilus* HB-8 contain five low-potential EPR detectable iron-sulfur clusters (see Table I) (Meinhardt *et al.*, 1990). Two binuclear clusters correspond to mitochondrial N1a and N1b centers according to spectra, midpoint redox potentials, and their pH dependences. At the same time, distinctly no N2 equivalent (i.e., high-potential [4Fe–4S] cluster with pH-dependent E_m) was found in *T. thermophilus* membranes. The presence of N2 in the mitochondrial complex has been correlated with sensitivity to rotenone and with the appearance of the coupling site (Cobley *et al.*, 1975). In the cases where no N2 equivalent cluster is seen, the H^+/e^- stoichiometry determination may be important in discerning if the complexes function with the same mechanism. *T. thermophilus* membrane, in contrast to the other aforementioned systems, contains as a pool component only menaquinone, which possesses a much lower midpoint potential of the two-electron reduction step than ubiquinone [$E_{m,7} = -75$ mV vs. $+100$ mV (Kroger and Unden, 1985)]. Thus it exhibits a smaller free-energy gap between the Site I reductant and oxidant, and one can speculate about the lack of one of the coupling subsites (Vinogradov, this volume) in thermophile NDH-1.

Attempts to isolate NDH-1 from *T. thermophilus* HB-8 membranes (Yagi *et al.*, 1988; Meinhardt *et al.*, 1990; Yumoto *et al.*, 1992) lead to a significant modification of the quinone-binding site(s) although EPR spectra of the FeS clusters remains unaltered. This modification is clearly seen by comparing the affinities of low-molecular-weight quinone homologues (Table III) and Q-site inhibitors (Table II) of the membrane-bound and reconstituted with phospholipid purified forms of NDH-1. Water-soluble low-molecular-weight quinone derivatives are capable of accepting electrons from both isolated Complex I and submitochondrial particles in two ways: completely rotenone-insensitive and rotenone-sensitive. The contribution of the rotenone-insensitive path depends on the preparation and lipophilicity of quinones (Schatz

and Racker, 1966; Ragan, 1978; Sled' *et al.*, 1990). This peculiarity of water-soluble quinones' interaction with NADH dehydrogenase is also applicable to bacterial systems, including *T. thermophilus*. Detailed kinetic analysis of the inhibitory effect of rotenone, piericidin A, and annonin VI (Lounder-shausen *et al.*, 1991) on the NADH-DB reductase activity of isolated NDH-1 from *T. thermophilus* (Sled' *et al.*, manuscript in preparation) revealed that solubilization of NDH-1, even using very mild detergents (CHAPS), leads not to the *complete loss* of the former rotenone-sensitive acceptance site and to the manifestation of only the rotenone-insensitive one but, in fact, to a change of the inhibitor's (and quinone's) affinity to the *same* rotenone-sensitive site, so that electron transfer from NADH to quinone in isolated enzyme occurs via the same iron-sulfur centers as in the NDH-1 *in situ*.

The same reasoning can probably be applied to some other preparations of isolated NDH-1 from *P. denitrificans* (Yagi, 1986), *N. crassa* (Ise *et al.*, 1985), *Rb. sphaeroides* (Fukumori, unpublished data), and a number of high-molecular-weight (type I) NADH dehydrogenase preparations from mammalian mitochondria (Ringler *et al.*, 1963; Cremona and Kearney, 1964, Kaniuga and Gardas, 1967; Huang and Pharo, 1971; Baugh and King, 1972, Ragan and Racker, 1973; Paech *et al.*, 1982; Finel *et al.*, 1992). To date there are no reported purified NDH-1 with unaltered inhibitor (rotenone and piericidin) sensitivity,[9] which have been supposed to be obligatorily related to the presence of coupling Site I (Cobley *et al.*, 1975) and claimed to be the criterion of an intact enzyme. The question is whether this fundamental failure of intact rotenone-sensitive NADH dehydrogenase purification is a result of the modification of protein–lipid interaction during the course of detergent treatment or is a result of the loss of a hydrophobic "rotenone-sensitivity conferring protein" after solubilization. The mitochondrial ND1 gene product could be a pretender to the role of such a protein since it has been shown to be specifically labeled with [H^3]-dehydrorotenone (Earley *et al.*, 1987) and was not found in any of the subcomplexes obtained after bovine Complex I splitting (Finel *et al.*, 1992; Finel, this volume). Having great imagination, the inability of isolated NDH-1 preparation to reconstitute the proton-translocating function (Ragan and Racker, 1973) can also be attributed to the loss of this polypeptide, since this subunit was found to bind DCCD (Yagi and Hatefi, 1988). Taking into account the hypothetical existence of two coupling subsites in Site I (Ragan, 1990; Kotlyar *et al.*, 1990), the $\Delta\mu H^+$ generating function of the first FMN-containing coupling subsite is likely to be preserved in isolated NDH-1; therefore any attempt to estimate H^+/e^- stoichiometry in the reconstituted system seems quite worthwhile.

EPR and thermodynamic anaysis of the FeS cluster composition of NDH-1 from different bacteria (see Table I) confirms their genetic and evolutional homology with eukaryotic counterparts. The most conspicuous similarity between these enzymes is the general thermodynamic profile of the set of iron-sulfur clusters: (1) a very-low-potential binuclear cluster, which has a pH-dependent E_m and is located, most probably, at the electron entry part of the peripheral arm of the enzyme. This center is not reducible with NADH, but is thermodynamically equilibrated with bound FMN, at least in the case of mammalian Complex I (Sled' *et al.*, 1993); thus, it probably directly participates in the second flavin oxidation step and is involved in the proton-pumping mechanism at the first coupling subsite (Ohnishi, 1973; Ragan, 1990; Vinogradov, this volume); (2) A pool of almost equipotential clusters with intermediate and pH-independent E_m values which could function as an electron-conducting wire between peripheral and membrane-bound catalytic parts of Complex I; (3) A tetranuclear cluster which is immersed in the hydrophobic domain of the membrane arm of the enzyme. Its midpoint redox potential is relatively high, pH-dependent, and sensitive to lipid environment (Ohnishi *et al.*, 1974). For bovine heart Complex I this component was shown to magnetically interact with one of two ubisemiquinone free radicals (Burbaev *et al.*, 1989; Kotlyar *et al.*, 1990). This iron-sulfur center most probably is the direct reductant of the bound ubiquinone species and could function as an essential part of the proton-pumping machinery in this catalytic domain (Ohnishi, 1979, Vinogradov, this volume).

[9] Except bovine heart Complex I preparation (Hatefi *et al.*, 1962), which probably can be accepted only as partially purified enzyme.

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