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Characteristics of the Energy-Transducing NADH-Quinone Oxidoreductase of *Paracoccus denitrificans* as Revealed by Biochemical, Biophysical, and Molecular Biological Approaches

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A comparison of the mitochondrial NADH-ubiquinone oxidoreductase and the energy-transducing NADH-quinone oxidoreductase (NDH-1) of *Paracoccus denitrificans* revealed that both systems have similar electron-transfer and energy-transduction pathways. In addition, both complexes are sensitive to the same inhibitors and contain similar electron carriers, suggesting that the *Paracoccus* NDH-1 may serve as a useful model system for the study of the human enzyme complex. The gene cluster encoding the *Paracoccus* NDH-1 has been cloned and sequenced. It is composed of 18,106 base pairs and contains 14 structural genes and six unidentified reading frames (URFs). The structural genes, URFs, and their polypeptides have been characterized. We also discuss nucleotide sequences which are believed to play a role in the regulation of the NDH-1 gene cluster and *Paracoccus* NDH-1 subunits which may contain the binding sites of substrates and/or electron carriers.

KEY WORDS: NADH-quinone oxidoreductase; *Paracoccus denitrificans*; gene cluster; H^+ pump; gene expression; FMN; iron-sulfur cluster.

INTRODUCTION

The NADH-quinone oxidoreductases of the bacterial respiratory chain can be divided into two groups depending on whether or not they bear an energy-coupling site (Yagi, 1989, 1991, 1993). The enzyme complexes lacking an energy-coupling site are known as NDH-2. These complexes contain noncovalently bound FAD as a cofactor and consist of a single polypeptide (Yagi *et al.*, 1988; Yagi, 1989, 1991, 1993). On the other hand, those enzymes which bear an energy coupling-site can be further divided into two groups (Bourne and Rich, 1992). The enzymes pumping Na ions are designated Na-NDH. These complexes contain FMN and FAD as cofactors but lack iron-sulfur (FeS) clusters (Unemoto and Hayashi, 1989; Bourne and Rich, 1992). The Na-NDH isolated from *Vibrio alginolyticus* has been reported to be composed of three subunits (Hayashi and Unemoto, 1987). Those enzymes associated with an energy-coupling site which function as H^+ pumps are designated as NDH-1 (Yagi *et al.*, 1988; Yagi, 1989, 1991, 1993). All members of the NDH-1 group analyzed to date are multiple polypeptide enzymes (probably 14 subunits) and contain noncovalently bound FMN and FeS clusters as prosthetic groups (Yagi, 1993). In terms of polypeptide composition, cofactors, and specific inhibitors, the bacterial NDH-1 type enzymes appear to be counterparts of the mitochondrial NADH-ubiquinone oxidoreductase (complex I) (Yagi, 1987, 1988, 1990, 1993).

Paracoccus denitrificans is a soil bacterium and has been called "a free-living mitochondrion" (John and Whatley, 1975, 1977). As has been described in previous reviews (Yagi, 1991, 1993), the NDH-1 of *P. denitrificans* is more akin to the mitochondrial complex I in terms of EPR-visible FeS clusters than is the

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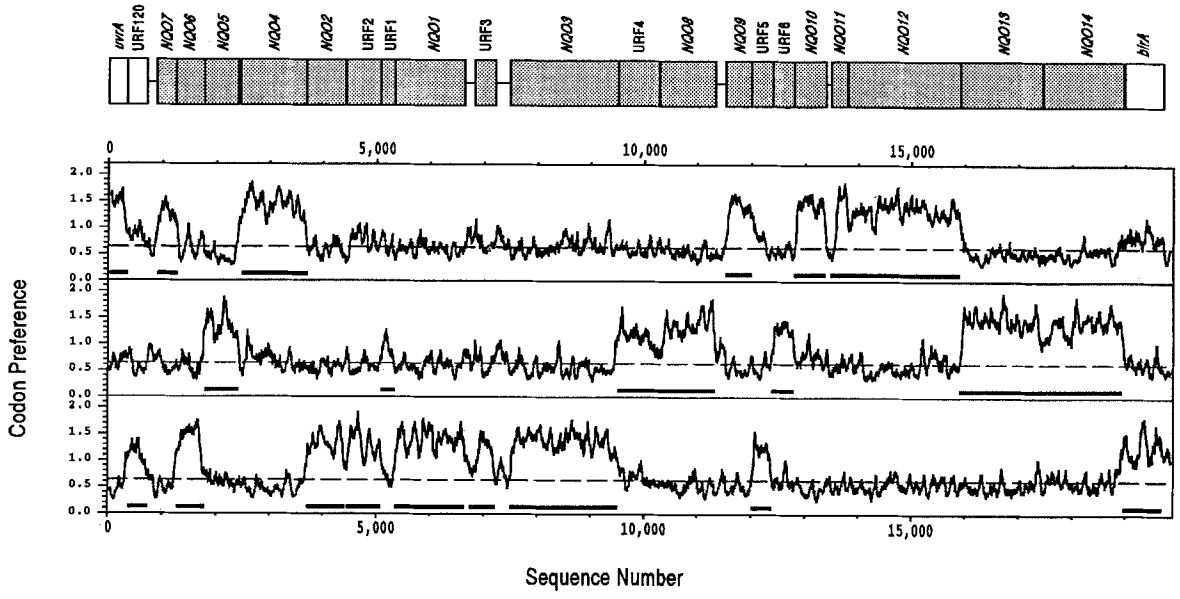

Fig. 1. Predictions of structural genes and URFs from the *P. denitrificans* DNA sequences. The CODONPREFERENCE program of the University of Wisconsin Genetic Computer Group was employed to analyze the deduced DNA sequence and predict the presence of open reading frames. The three boxes represent the translational reading frames of the DNA which are indicated by bold bars. The scale on each ordinate represents the relative probability of coding. The positions of the NDH-1 subunit genes and URFs are marked as *NQO1–14* and URF1–6.

NDH-1 of *Thermus thermophilus* HB-8 or *Escherichia coli* (Meinhardt *et al.*, 1987a, 1989, 1990). Because the characteristics of the polypeptides encoded by the *Paracoccus* NDH-1 gene cluster have recently been discussed in a separate review (Yagi, 1993), this article will focus on the regulation of the *Paracoccus* NDH-1 gene cluster and on our recent structural studies on the NDH-1 of *P. denitrificans*.

THE *PARACOCCUS* NDH-1 GENE CLUSTER

Identification and Sequencing of the NDH-1 Gene Cluster. In 1986, the NADH dehydrogenase complex was isolated from *P. denitrificans* membranes in this laboratory (Yagi, 1986). The NADH-binding (M_r 50 kDa) subunit of this enzyme complex was identified by a direct photoaffinity labeling procedure using [^{32}P]NADH (Yagi and Dinh, 1990). This subunit was subsequently isolated by electroelution from the SDS-polyacrylamide gels (Xu and Yagi, 1991; Xu *et al.*, 1991a). Partial amino acid sequences of this subunit were determined (Xu *et al.*, 1991a). Utilizing information on the partial primary structure of this subunit, we screened the *Paracoccus* genomic library and then cloned and sequenced the structural gene (*NQO1*) encoding the *Paracoccus* NADH-binding subunit (Xu *et al.*, 1991a). It was discovered at that time that the structural genes encoding the other subunits of the *Paracoccus* NDH-1 lay on either side of the *NQO1* gene and appear to constitute a gene cluster (Xu *et al.*, 1991a). Subsequently complete DNA sequencing of the *Paracoccus* NDH-1 gene cluster has been carried out (Xu *et al.*, 1991a,b; 1992a,b; 1993). This *Paracoccus* NDH-1 gene cluster is composed of 18,106 base pairs (bp) of DNA. Based on the analysis by the CODONPREFERENCE program (Devereaux *et al.*, 1984) (Fig. 1), it was predicted that the NDH-1 gene cluster contains 14 structural genes (*NQO1–14*) and six unidentified reading frames (URFs) (Yagi *et al.*, 1992; Yagi, 1993). Purification and partial *N*-terminal sequence analysis of the *Paracoccus* 50 kDa, 25 kDa, 66 kDa, 48 kDa, and 21 kDa subunits revealed a perfect match to the primary structures deduced from the *NQO1*, *2*, *3*, *4*, and *5* genes, respectively. In addition, when these five subunits of the *Paracoccus* NDH-1 were characterized biochemically, it was found that the subunits all cross-reacted with the monospecific antisera to their bovine counterparts (Xu and Yagi, 1991). Similarly the bovine subunits cross-reacted with antisera raised against the *Paracoccus* subunits (Xu and Yagi, 1991; Yano and Yagi, unpublished results). Table I summarizes the characteristics of the structural genes

Table I. Characteristics of the *NQO1-14* and URF1-6 Genes and Their Products

Gene	DNA length (bp)	GC content (%)	Initiation codon	Termination codon	Characteristics of polypeptide product[a]			
					Number of amino acids	MW	pI	Bovine homologue
NQO1	1296	65.7	ATG	TAA	431	47191	6.5	51 kDa (FP)[b]
NQO2	720	67.1	ATG	TAA	239	26122	4.8	24 kDa (FP)[b]
NQO3	2022	67.2	ATG	TGA	673	73159	5.3	75 kDa (IP)[b]
NQO4	1242	65.6	ATG	TGA	413	46674	5.4	49 kDa (IP)[b]
NQO5	627	62.5	ATG	TGA	208	23731	5.0	30 kDa (IP)[b]
NQO6	525	66.7	ATG	TGA	174	19117	7.7	20 kDa (IP)[b]
NQO7	369	59.2	GTG	TGA TGA	122	13601	4.2	ND3 product (HP)[b]
NQO8	1038	61.8	ATG	TAA	345	38751	5.4	ND1 product (HP)[b]
NQO9	492	62.4	ATG	TGA	163	18959	6.5	23 kDa (HP)[b]
NQO10	603	65.0	ATG	TGA	200	21819	6.5	ND6 product (HP?)[b]
NQO11	306	57.2	ATG	TAA	101	10856	7.7	ND4L product (HP)[b]
NGO12	2112	63.8	ATG	TGA	703	77705	6.7	ND5 product (HP?)[b]
NQO13	1542	63.7	ATG	TAG	513	56417	7.6	ND4 product (HP?)[b]
NQO14	1500	66.1	ATG	TGA	499	52536	5.4	ND2 product (HP)[b]
URF1	264	69.0	ATG	TAA	87	9277	12.2	
URF2	645	70.6	ATG	TGA	214	22906	5.0	
URF3	399	65.2	ATG	TGA	132	14670	10.4	
URF4	768	67.7	ATG	TGA	255	26869	9.7	
URF5	393	68.2	ATG	TGA	130	13765	6.3	
URF6	405	63.7	ATG	TGA TGA	134	15002	4.7	

[a] Predicted from nucleotide sequence.

[b] Bovine complex I can be resolved into a water-soluble fraction and a water-insoluble fraction (hydrophobic protein fraction, HP) by treatment with $NaClO_4$ (Hatefi, 1985; Hatefi *et al.*, 1985). The water-soluble fraction can be further resolved into two fractions by ammonium sulfate fractionation (Hatefi, 1985; Hatefi *et al.*, 1985). The two water-soluble fractions have been designated the flavoprotein fraction (FP) and the iron-sulfur protein fraction (IP).

and their products. The bovine homologue of each structural gene product has also been listed for the readers' reference. The data reveal that the GC content of these NDH-1 structural genes is in the range of 57–67% which is comparable to that of other *Paracoccus* genes (Stouthamer, 1992). Concurrent with our work on the *Paracoccus* NDH-1, cloning of NDH-1 gene clusters from *Synechocystis* (Steinmüller *et al.*, 1989; Steinmüller, 1992), *Escherichia coli* (Weidner *et al.*, 1992), and *Rhodobacter capsulatus* (Dupuis, 1992) has been conducted in other laboratories, and the DNA sequencing of these gene clusters is now in progress.

Characterization of NQO Genes. The translation initiation codon of the *NQO7* gene, which is the 5′-terminal structural gene of the *Paracoccus* NDH-1 gene cluster, is GTG. This is in contrast to the other structural genes and URFs of this gene cluster which are initiated by ATG (see Table I). The initiation codon for the majority of *Paracoccus* genes reported to date is ATG (36 of 38 in the GenBank). A similar situation has been reported for *E. coli* genes (Gold *et al.*, 1981). However, whereas TTG, ATA, and ATT are also employed as initiation codons in *E. coli* (Gold, 1988), these codons have not been found in the initiation codons of the *Paracoccus* genes. The only other *Paracoccus* structural gene reported to use GTG as the initiation codon is the 5′-terminal gene (*fbcF*) of the *fbc* operon which encodes the quinol–cytochrome *c* oxidoreductase (bc_1 complex or complex III) (Kurowski and Ludwig, 1987). It is interesting to note that these *Paracoccus* enzyme complexes appear to be synthesized constitutively (Trumpower, 1991; Van der Oost *et al.*, 1991), suggesting that NDH-1 and complex III may be expressed in a coordinated fashion. Should this prove to be the case, it is likely that the *Paracoccus* NDH-1 gene cluster acts as a single operon and that the promoters for both enzyme complex operons are similar to each other. In support of this hypothesis, examination of the region surrounding the *NQO7* gene revealed that the regions located 1–17 bp and 103–115 bp upstream from the *NQO7* gene are highly homologous to the regions located 1–17 bp and 98–108 bp upstream

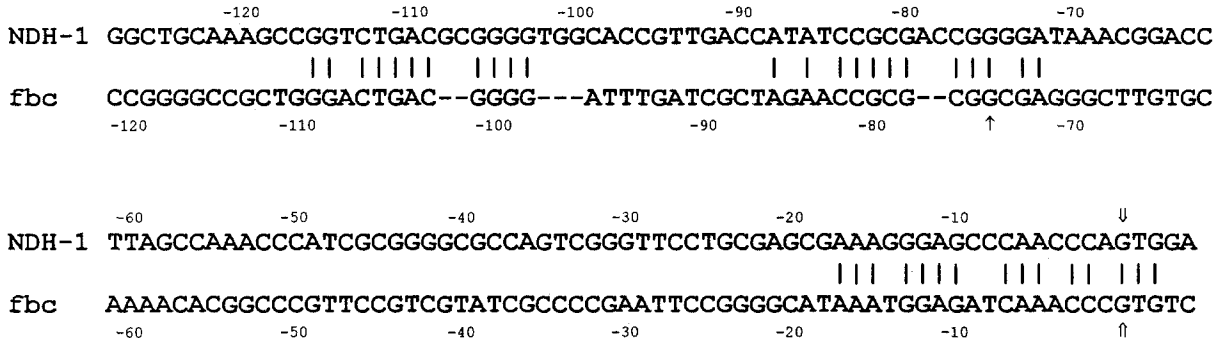

Fig. 2. Alignment of the DNA sequences located upstream from the translational initiation sites of the *Paracoccus* NDH-1 gene cluster (NDH-1) and the *Paracoccus fbc* operon (fbc). The arrow (↑) marks the probable 5′-start of the *fbc* transcript. The open arrows (⇓, ⇑) indicate translation initiation sites of the *Paracoccus* NDH-1 gene cluster and the *Paracoccus fbc* operon, respectively.

from the *fbcF* gene of the *Paracoccus* complex III operon, respectively (see Fig. 2). This is particularly significant when considered in the light of the fact that the region located 1–17 bp upstream from the initiation codon is generally considered to be a ribosome-binding site (Shine and Dalgarno, 1975). The similarity in the regions located approximately 100 bp upstream from these structural genes is also not without relevance since studies conducted on *lacZ* fusions have indicated that internal promoter sequences appear to be located at approximately 100 bp upstream from the start of the coding region (Walker *et al.*, 1984). Furthermore, the location of this region, approximately 100 bp upstream from the *fbcF* gene and 23–33 bp upstream of the probable 5′-start of the *fbc* operon transcript (Kurowski and Ludwig, 1987), is similar to the location of the promoter of *E. coli*. On the basis of this informaton, it might be speculated that the region located 103–115 bp upstream of the *NQO7* gene may be a promoter-binding region. Together these data support the hypothesis that the gene cluster encoding the *Paracoccus* NDH-1 functions as an operon. In addition, the data also suggest that the homologous DNA sequences found in the *Paracoccus* NDH-1 gene cluster and the *fbc* operon may play a role in the regulation of the *Paracoccus* NDH-1 gene expression.

Regarding termination codon usage in the *Paracoccus* NDH-1 genes, nine genes employ TGA, four genes utilize TAA, and one gene uses TAG (see Table I). The *NQO7* gene ends with tandem termination codons TGATGA. This is the first report of the tandem termination triplets in the *Paracoccus* genes, although approximately 13% of *E. coli* genes contain termination signals TAG or TAA in tandem with a second nonsense codon (Lu and Rich, 1971). According to Walker *et al*, (1984), the tandem termination codons are believed to ensure that a readthrough does not occur. If this is, in fact, the purpose of these tandem termination codons, their rare presence in the *Paracoccus* genes may indicate a much lower probability of readthrough in the *Paracoccus* genes than in the *E. coli* genes.

SUBSTRATES AND COFACTORS OF THE *PARACOCCUS* NDH-1

NADH: As described above, we have identified the NADH-binding subunit (the 50 kDa subunit encoded by the *NQO1* gene) of the *Paracoccus* NDH-1 by utilizing direct photoaffinity labeling with [^{32}P]NAD(H) (Yagi and Dinh, 1990). Prior to our work, Chen and Guillory (1984) demonstrated that both the M_r 51,000 and 42,000 polypeptides of the bovine complex I could be labeled with tritiated A3′-*O*-{3-[*N*-(4-azido-2-nitrophenyl)amino]-propionyl}NAD$^+$. It was subsequently found that the bovine 51 kDa polypeptide is the counterpart of the *Paracoccus* NADH-binding subunit. This determination was made on the basis of sequence similarity and immunocross-reactivity (Yagi and Dinh, 1990; Xu and Yagi, 1991). However, a search through the determined sequences for the *Paracoccus* NDH-1 genes did not reveal any polypeptide which might be the counterpart of the labeled 42 kDa bovine subunit (i.e., GxGxxGxxxG) (Walker *et al.*, 1992). Therefore, the function of the bovine 42 kDa polypeptide labeled with [^{3}H]-A3′-*O*-{3-[*N*-(4-azido-2-nitrophenyl)amino]propionyl}NAD$^+$ remains to be determined.

FMN: The *Paracoccus*, *T. thermophilus*, and *E.*

Table II. Putative FeS Cluster-Binding Sites of the *NQO1, 2, 3,* and *9* Subunits

Gene	FeS Cluster	Putative FeS cluster-binding sequence
NQO1	(N3)	$C_{347}xxC_{350}xxC_{353}(x)_{39}C_{393}$
NQO2	(N1b)	$H_{92}xxxC_{96}xxxxC_{101}(x)_{35}C_{137}xxxC_{141}$
NQO3	(N4)	$C_{158}xxC_{161}xxC_{164}(x)_{43}C_{208}P_{209}$
	(N1a?)	$C_{26}(x)_{10}C_{37}xH_{39}(x)_8C_{48}xxC_{51}(x)_{14}C_{66}$
		$H_{105}xxxC_{109}xxC_{112}(x)_5C_{118}$
NQO9	(N2)	$C_{64}xxC_{67}xx_{70}xxxC_{74}P_{75}(x)_{27}C_{103}xxC_{106}xxC_{109}xxxC_{113}P_{114}$

coli NDH-1 and mitochondrial complex I have all been reported to bear noncovalently bound FMN (Yagi, 1986; George and Ferguson, 1987; Yagi *et al.*, 1988; Hayashi *et al.*, 1989). Although there is no direct evidence that the FMN moiety is associated with the *NQO1* product (NADH-binding subunit), FMN can be provisionally assigned to this subunit (Hatefi *et al.*, 1985), because the FMN appears to be the primary oxidant of NADH. However, this assignment must be more thoroughly investigated in the future. The fact that consensus sequences for FMN-binding sites have not yet been established (Fukuyama *et al.*, 1992) further complicates this issue and precludes any discussion regarding the location of the FMN-binding site in the *NQO1* (NADH-binding) subunit.

FeS Clusters: Albracht *et al.* (1980) reported that four EPR-visible FeS clusters (one binuclear and three tetranuclear clusters) are present in the *Paracoccus* NDH-1. These have been designated cluster N1b, N2, N3, and N4 according to the terminology of Ohnishi (Ohnishi and Salerno, 1982). Recently, Ohnishi and her colleagues (Meinhardt *et al.*, 1987a) have detected the presence of another binuclear cluster, cluster N1a, present in the *Paracoccus* NDH-1. This cluster is labile and may give rise to variable spin concentrations and extremely low E_m values due to the facile modifications of the microenvironment of the cluster (Meinhardt *et al.*, 1987a). Both cluster N1a and N2 have E_m values which appear to be pH dependent, suggesting that these two FeS clusters may play a role in the H^+ pumping activity of the NDH-1. It should be noted, however, that fully rotenone-sensitive electron transfer can be observed, in the *in situ Paracoccus* NDH-1, even when the cluster N1a signal is not detected. Thus, it remains to be determined whether or not the cluster N1a is an essential component of the *Paracoccus* NDH-1. Taken together, at least four, possibly five, EPR-visible FeS clusters appear to be associated with the *Paracoccus* NDH-1.

The consensus sequence for tetranuclear FeS clusters has been established (Matsubara and Saeki, 1992). Comparing the deduced primary structures of the *Paracoccus* NDH-1 subunits against the consensus sequence has made it possible to speculate as to which NDH-1 subunits bind to FeS clusters. The *NQO1*, *NQO3*, and *NQO9* subunits all contain at least one consensus sequence for tetranuclear FeS cluster-binding sites (Yasunobu and Tanaka, 1980). In fact, the *NQO9* subunit bears two putative tetranuclear-binding sites. In addition, the *NQO2* and *NQO3* subunits bear possible binuclear FeS cluster binding sites, although the consensus sequence for binuclear-binding sites is not as well established as that for tetranuclear binding sites. Based on both the presence of FeS cluster consensus sequences and on the proposed subunit location of FeS clusters in bovine complex I (Hatefi *et al.*, 1985; Hatefi, 1985; Ragan, 1987), a tentative assignment for the EPR-visible FeS clusters, in terms of both subunits and putative FeS cluster-binding sequences, is presented in Table II. In an attempt to identify whether the *NQO1*, *NQO2*, *NQO3*, and *NQO9* subunits actually do ligate FeS clusters, expression of these four genes in *E.coli* has been carried out (Yano, T., and Yagi, T., unpublished results). The results revealed that all four expressed subunits bear at least one FeS cluster(s) which can be reduced by addition of sodium dithionite, thereby confirming the speculation described above. These expressed subunits will provide useful material for future studies regarding the characteristics of FeS cluster(s)-bound subunits and of the residues ligating the FeS cluster.

Recently, FeS cluster proteins in which the FeS cluster has a nonredox function have been reported (Beinert and Kennedy, 1989; Woods *et al.*, 1988; Yumoto and Tokushige, 1988; Bell *et al.*, 1989; Vollmer *et al.*, 1983; Nelson *et al.*, 1991; Cammack, 1992). It has been postulated that some of these FeS clusters serve to maintain the structure of the polypeptide (Kuo *et al.*, 1992). Bovine complex I has been

reported to contain both EPR-visible and EPR-invisible FeS clusters (Hatefi *et al.*, 1985; Hatefi, 1985). Although the EPR-visible FeS clusters in the bovine and *Paracoccus* sysems are generally believed to play a role in the oxidation of substrates and subsequent electron transfer to ubiquinone, the role of the EPR-invisible clusters in the bovine complex I has remained a mystery. In light of these reports, it seems possible that these EPR-invisible FeS clusters may not play a role in the redox reaction at all but may be involved in maintaining the structure of the enzyme complex. It is not clear at this point whether EPR-invisible FeS clusters are also present in the bacterial NDH-1.

Quinones. Suzuki and Ozawa (1986) have reported that the 14 kDa polypeptide of the bovine complex I iron-sulfur protein fragments is a quinone-binding subunit on the basis of the presence of ubiquinone in the isolated polypeptide. In contrast, Werner and coworkers (Heinrich and Werner, 1992; Heinrich *et al.*, 1992) have recently claimed that in the *N. crassa* complex I the 9.5 kDa polypeptide is a quinone-binding polypeptide. The latter claim was made on the basis of photoaffinity labeling experiments using a quinone analogue (2,3-dimethoxy-5-methyl-6-[7-(4-azido-3-[^{125}I]iodophenyl)-heptanyl]-*p*-benzoquinone). Although a homologue of the *N. crassa* 9.5 kDa polypeptide has been detected in the bovine complex I (Walker *et al.*, 1992), none has been detected in the polypeptides encoded by the structural genes of the *Paracoccus* NDH-1 gene cluster (Yagi, 1993). Furthermore, no significant sequence similarity has been observed between the polypeptides of the *Paracoccus* NDH-1 gene cluster and the *N. crassa* 9.5 kDa polypeptide. Assuming that the reaction mechanism of the *Paracoccus* NDH-1 is similar to that of its mitochondrial counterparts, these data suggest one of the following possibilities: (1) The *Paracoccus* NDH-1 structural gene encoding the 9.5 kDa homologue of the *N. crassa* quinone-binding subunit is located in a region separate from the NDH-1 gene cluster and has not yet been cloned. (2) These "apparent quinone-binding polypeptides" are not required for the binding of substrate quinone *in situ* to the NDH-1. This issue remains to be resolved. However, it should be noted that the bacterial complex III lacks a homologue of the "apparent quinone-binding subunit" of bovine complex III (Steinrücke *et al.*, 1991). Nevertheless, a highly stabilized antimycin-sensitive Q_i^- has been demonstrated in the *Paracoccus* complex III (Meinhardt *et al.*, 1987b). Therefore, the reaction mechanism of bacterial complex III appears to be similar, if not identical, to that of its mitochondrial counterparts. These results lend support to the latter possibility.

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