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Identification of the NADH-Binding Subunit of NADH–Ubiquinone Oxidoreductase of *Paracoccus denitrificans*†

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ABSTRACT: The NADH dehydrogenase complex isolated from *Paracoccus denitrificans* is composed of approximately 10 unlike polypeptides and contains noncovalently bound FMN, non-heme iron, and acid-labile sulfide [Yagi, T. (1986) *Arch. Biochem. Biophys. 250*, 302–311]. When the *Paracoccus* NADH dehydrogenase complex was irradiated by UV light in the presence of [*adenylate*-^{32}P]NAD, radioactivity was incorporated exclusively into one of three polypeptides of M_r ~50 000. 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 [*adenylate*-^{32}P]NAD was performed in the presence of NADH, but not in the presence of NADP(H). The labeled polypeptide was isolated by preparative sodium dodecyl sulfate gel electrophoresis and was shown to cross-react with antiserum to the NADH-binding subunit (M_r = 51 000) of bovine NADH–ubiquinone oxidoreductase. Its amino acid composition 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 an NADH-binding subunit of the *Paracoccus* NADH dehydrogenase complex.

Mitochondrial NADH–ubiquinone oxidoreductase (complex I)[1] bears coupling site 1 of oxidative phosphorylation and is composed of more than 25 unlike polypeptides (Hatefi, 1985; Yagi, 1989; Ragan, 1987). This complexity has hampered progress in the study of many aspects of the structure and function of mitochondrial complex I (Hatefi, 1985; Yagi, 1989; Ragan, 1987). In an effort to find a simpler system with which to clarify the structure and function of NADH–Q oxidoreductase, we have purified the NADH–Q oxidoreductase from *Paracoccus denitrificans* membranes (Yagi, 1986) which bear coupling site 1 (Stouthamer, 1980) and exhibit similar EPR signals to the mammalian complex I (Albracht et al., 1980; Meinhardt et al., 1987). The NADH dehydrogenase complex purified from *Paracoccus* membranes is composed of 10 unlike polypeptides and contains noncovalently bound FMN and multiple iron–sulfur clusters (Yagi, 1986), suggesting that the *Paracoccus* system is structurally simpler than its mammalian counterpart, as has been shown also for other energy-transducing enzyme complexes of the respiratory chain [cytochrome oxidase (Ludwig & Schatz, 1980; Haltia et al., 1988) and ubiquinol–cytochrome *c* oxidoreductase (Yang & Trumpower, 1986)]. Recently, we (Yagi et al., 1988) have isolated the NADH–Q oxidoreductase of *Thermus thermophilus* HB-8 which bears coupling site 1 (Meinhardt et al., 1990). The *Thermus* NADH–Q oxidoreductase, which is partially sensitive to rotenone, is also composed of 10 unlike polypeptides, although EPR signals of the *Thermus* NADH–Q oxidoreductase segment are different from those of mammalian complex I (Meinhardt et al., 1990).

In the case of bovine complex I, some of the subunits have been characterized (Hatefi, 1985; Yagi, 1987, 1989; Ragan, 1987; Yagi & Hatefi, 1988; Fearnley et al., 1989). For example, Chen and Guillory (1981) have shown that the M_r 51 000 polypeptide of bovine complex I is the NADH-binding subunit, using a tritiated photoaffinity NAD analogue. Recently, it was shown by our laboratory (Yagi, 1987; Yagi & Hatefi, 1988) that DCCD inhibits the NADH–Q oxidoreductases of various organisms bearing energy coupling site 1, and the DCCD-binding subunit of bovine complex I was assigned to the mitochondrial ND-1 gene product. Studies of Earley et al. (1987) have suggested that the rotenone-binding subunit of complex I is also the ND-1 gene product. However, nothing is known about the characteristics of the subunits of the NADH–Q oxidoreductases of bacteria bearing coupling site 1. Therefore, it was of interest to elucidate the function of various polypeptides in *Paracoccus* NADH dehydrogenase complex.

This paper describes the identification of the NADH-binding subunit of the NADH dehydrogenase complex from *Paracoccus denitrificans*. UV irradiation of the NADH dehydrogenase complex with [^{32}P]NAD or [^{32}P]NADH resulted in incorporation of radioactivity into a single polypeptide of M_r 50 000. The M_r 50 000 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 complex I cross-reacted with

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[1] Abbreviations: complex I, NADH–quinone oxidoreductase bearing energy coupling site; Q, quinone; EPR, electron paramagnetic resonance; DCCD, *N,N'*-dicyclohexylcarbodiimide; SDS, sodium dodecyl sulfate; IEF, isoelectric focusing; FP, IP, and HP, flavoprotein, iron–sulfur protein, and hydrophobic protein fractions of complex I, respectively; K_i, inhibition constant.

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the M_r 50 000 polypeptide of the *Paracoccus* enzyme complex.

Experimental Procedures

Growth of Paracoccus denitrificans and Isolation of Membranes. Paracoccus denitrificans (ATCC 13543) were grown aerobically in 550-L cultures with glucose as substrate, as described previously (Yagi, 1986), at BIOPURE Fine Chemicals, Inc. *Paracoccus* membranes and *Paracoccus* NADH dehydrogenase complex were prepared according to Yagi (1986).

UV Irradiation. Experiments for binding of [^{32}P]NAD(H) to the *Paracoccus* NADH dehydrogenase complex were carried out as follows. The reaction mixture (32 μL), containing 10 mM potassium phosphate (pH 7.5), 0.6 mM EDTA, 0.96 mg/mL *Paracoccus* enzyme complex, and [^{32}P]NAD at the specified concentration, was placed in small glass test tubes (5 × 15 mm) on ice and irradiated for varying times (0 to 3 h) at a distance of about 3 cm with a 9-W shortwave (254 nm) UV lamp. When several samples were treated at the same time, the positions of tubes were rotated every 10 min in order that UV irradiation of reaction mixtures would be uniform. After irradiation, the reaction mixture was immediately diluted 2-fold with Laemmli's sample buffer containing 160 mM Tris-HCl (pH 6.8), 12% SDS, 40% glycerol, and 10% β-mercaptoethanol. The samples were subjected to SDS gel electrophoresis. In the case of the two-dimensional gel, the *Paracoccus* NADH dehydrogenase complex treated with UV light was directly mixed with the isoelectric focusing mixture (see below).

Gel Electrophoresis. (A) One-Dimensional Gel. SDS gel electrophoresis was carried out by the method of Laemmli (1970) (10% separation gel and 5% stacking gel). Marker proteins of known molecular weight were obtained from Sigma. The gels were autoradiographed with Kodak X-AR5 film as described in Yagi (1987) and Yagi and Hatefi (1988).

(B) Two-Dimensional Gel. Two-dimensional gel electrophoresis was performed by the modified method of O'Farrell (1975). First dimension: The mixture (0.5 mL) containing 77 μg of *Paracoccus* NADH dehydrogenase complex, 0.24 g of urea, 4% acrylamide, 0.13% *N,N'*-methylenebis(acrylamide), 2% Nonidet P-40, and 0.05% Biolyte ampholytes (pH 3–10) was degassed. The polymerization was initiated by addition of 0.8 μL of 10% ammonium persulfate and 0.5 μL of *N,N,N',N'*-tetramethylenediamine. The mixture was then inserted into a glass tube (1.3 × 75 mm) with a 3-mL syringe. The gels were run at 395 V for 3.5 h (1380 V-h). Second dimension: Following electrophoresis, the isoelectric focusing (IEF) gels were loaded on mini Laemmli-type slab SDS gel (10% separation gel and 5% stacking gel) (55 × 95 × 1 mm) and incubated for 10 min with 40 μL of Laemmli's sample buffer containing 80 mM Tris-HCl (pH 6.8), 6% SDS, 20% glycerol, and 5% β-mercaptoethanol. The running buffer was carefully overlaid on the IEF gel, and the gels were electrophoresed at 195 V for 45 min. The gels were stained for 25 min in 0.05% Coomassie brilliant blue R-250, 25% 2-propanol, and 10% acetic acid and destained in 7% acetic acid.

Other Analytical Procedures. Protein was estimated by the methods of Lowry et al. (1951) or by biuret in the presence of 1 mg of sodium deoxycholate/mL (Gornall et al., 1949). Enzymatic assays were carried out essentially according to Yagi (1986) and Yagi et al. (1988). Monospecific antibody to the *Paracoccus* NADH-binding subunit was purified from the antiserum to the *Paracoccus* NADH dehydrogenase complex according to Han et al. (1988, 1989). Immunoblotting experiments were carried out using skim milk as blocker (Yagi & Hatefi, 1988). Any variations from these procedures and other details are described in the figure legends.

Materials. The sources of the chemicals used were as follows: NADH, NAD, NADPH, and NADP were from Calbiochem; [*adenylate*-^{32}P]NAD (250 Ci/mmol) was from ICN; SDS, acrylamide, Biolyte ampholyte (pH 3–10), and Coomassie brilliant blue R-250 were from Bio-Rad; ubiquinone 1 was a generous gift from Eisai Chemical (Tokyo, Japan); AMP, 4-chloro-1-naphthol, and goat anti-rabbit antibody conjugated to horseradish peroxidase were from Sigma. The [*adenylate*-^{32}P]NADH was prepared from [*adenylate*-^{32}P]-NAD, in the presence of ethanol and yeast alcohol dehydrogenase, and was purified with the use of a Waters C_{18} reverse-phase column. The antisera to bovine FP and the M_r 51 000 subunit were kindly provided by Dr. Aili Han of this department. Other chemicals were reagent grade or of the highest quality available.

Results

Labeling of Polypeptides of Paracoccus NADH Dehydrogenase Complex with [adenylate-^{32}P]NAD. Recently, a method for identifying the nucleotide-binding subunit and/or site of enzymes has been developed. This is direct photoaffinity labeling of the enzyme with radioactive nucleotide substrates as originally reported by Yue and Schimmel (1977) for covalent binding of ATP to aminoacyl-tRNA synthetase. The direct labeling has certain advantages. First, natural substrate can be used, circumventing problems concerning difficult and lengthy chemical synthesis of derivatives and the relative low specific radioactivity of the ligand. Second, the procedure is very simple and easy. Therefore, we attempted to determine the NADH-binding subunit of the NADH dehydrogenase complex of *Paracoccus* respiratory chain utilizing this technique. Radiolabeled NADH is not commercially available. Therefore, [^{32}P]NAD, which also binds to this enzyme complex (see below), was used. When the *Paracoccus* NADH dehydrogenase complex was irradiated by UV light in the presence of [^{32}P]NAD on ice for various times, only one major radioactive band was observed in the autoradiograms of 10% SDS–polyacrylamide gels of the enzyme complex (Figure 1). This band migrated with an apparent molecular weight of 50 000. Incorporation of radioactivity in the M_r 50 000 band reached saturation after 2 h of irradiation time (see Figure 1). A small amount of radioactivity was observed on SDS gels immediately behind the M_r 50 000 band (see Figure 1). However, there was no distinct peak in the radioactivity profile. In addition, it was shown that the M_r 50 000 band was also labeled by UV irradiation of *Paracoccus* NADH dehydrogenase complex in the presence of [*adenylate*-^{32}P]-NADH.

Figure 2 shows the effect of [^{32}P]NAD concentration on incorporation of radioactivity in the *Paracoccus* NADH dehydrogenase complex. In the range from 0 to 64 μM [^{32}P]-NAD, the extent of labeling of the M_r 50 000 band was proportional to the [^{32}P]NAD concentration, suggesting that at least up to 64 μM [^{32}P]NAD there is no shielding effect of the nucleotide on the labeling of the M_r 50 000 band. Therefore, total nucleotide concentration was kept below 64 μM in all the following experiments.

Effects of Nucleotides on [^{32}P]NAD Labeling. NAD is a competitive inhibitor for NADH in the *Paracoccus* NADH dehydrogenase (data not shown). The K_i value for NAD obtained from Dixon plots is 600 μM, which is about 40-fold higher than the K_m value for NADH (15 μM; Yagi, 1986). As seen in Figure 3, NADH significantly diminished the labeling of the M_r 50 000 band by [^{32}P]NAD, but NADPH, which is not a substrate for the *Paracoccus* enzyme (Yagi,

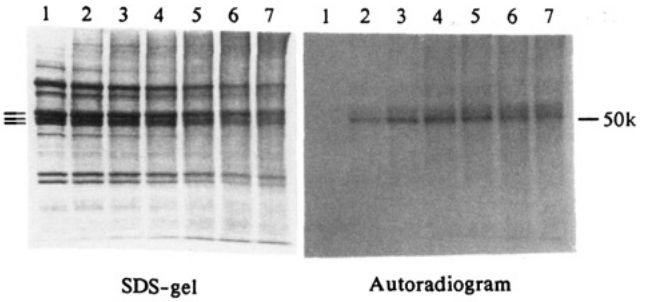

FIGURE 1: Time dependence of [^{32}P]NAD labeling of *Paracoccus* NADH dehydrogenase complex. *Paracoccus* enzyme complex at 1 mg/mL was irradiated by UV light in the presence of 21 μM [^{32}P]NAD, 10 mM potassium phosphate (pH 7.5), and 0.625 mM EDTA for 0 (1), 0.5 (2), 1.0 (3), 1.5 (4), 2.0 (5), 2.5 (6), and 3.0 (7) h on ice. The [^{32}P]NAD-modified *Paracoccus* NADH dehydrogenase complex was immediately diluted 2-fold with Laemmli's sample buffer. Then, 8 μg of *Paracoccus* enzyme complex was loaded on each well of a mini slab gel (55 × 95 × 0.75 cm) composed of 10% acrylamide as described previously (Yagi, 1987). The gel was electrophoresed for 50 min at 195 V. The procedures for staining, destaining, and autoradiography of the gel are described under Experimental Procedures. The bars to the left of the figure indicate three packed protein bands which are designated in order of decreasing molecular weight as the upper, the middle, and the lower bands. When the *Paracoccus* enzyme complex was incubated in Laemmli-type sample buffer lacking β-mercaptoethanol, the mobility of the middle band on the SDS gel increased (data not shown). As a result, the middle band overlapped the lower band [see Yagi (1986)]. Therefore, the sample buffer used in this paper always contained 5% β-mercaptoethanol.

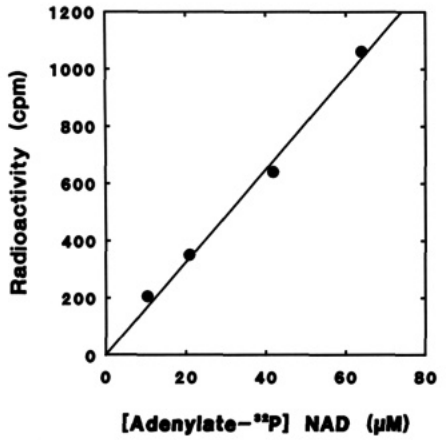

FIGURE 2: Concentration dependence of [^{32}P]NAD labeling of *Paracoccus* NADH dehydrogenase complex. *Paracoccus* NADH dehydrogenase complex (1 mg/mL) was subjected to UV irradiation for 2 h in the presence of various concentrations of [^{32}P]NAD. Electrophoresis, staining, destaining, and measurement of radioactivity in the M_r 50000 band were carried out as described in Yagi (1987).

1986), had no effect. Neither NADP nor AMP decreased this labeling either (data not shown). These results are consistent with the M_r 50000 protein band possessing a binding site that is specific for NAD(H). By comparison, the small degree of labeling that followed the M_r 50000 band (see above) was not affected even by addition of 22 μM NADH, suggesting that radioactivity incorporation in this region is unrelated to the NADH-binding subunit of the *Paracoccus* NADH dehydrogenase complex.

Identification of the NADH-Binding Subunit in the M_r 50000 Region of the SDS Gel. As shown in Figure 1, the M_r 50000 region of the *Paracoccus* NADH dehydrogenase complex contained three closely packed protein bands (identified by markers left of lane 1). These bands will be referred to as the upper, the middle, and the lower bands in order of decreasing apparent molecular weight. Since in this situation

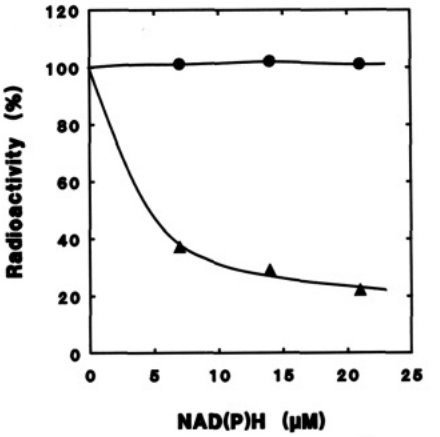

FIGURE 3: Effects of NADH and NADPH on [^{32}P]NAD labeling of the M_r 50000 band of *Paracoccus* NADH dehydrogenase complex. The *Paracoccus* enzyme complex at 1 mg/mL was incubated for 2 h on ice with 21 μM [^{32}P]NAD under UV irradiation in the presence of various concentrations of NADH (▲) or NADPH (●). Other experimental procedures were the same as those in Figure 2.

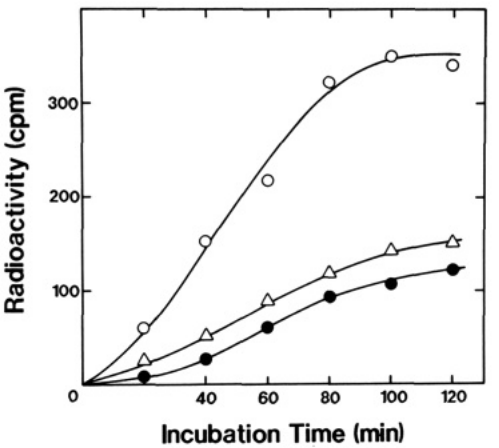

FIGURE 4: Time dependence of UV-induced [^{32}P]NAD labeling of upper, middle, and lower bands of Figure 1. The *Paracoccus* NADH dehydrogenase complex was incubated with 21 μM [^{32}P]NAD under UV light for 0–2 h on ice and diluted 2-fold with Laemmli's sample buffer. Then the preparations were electrophoresed on a (15 × 14 × 0.15 cm) Laemmli-type SDS slab gel at 30 mA for 3.5 h and stained for protein visualization with Coomassie blue. Each well received 20 μg of protein. The upper, middle, and lower bands were excised from the gel and digested overnight with 0.5 mL of 30% H_2O_2 at 50 °C, then mixed with 5 mL of Beta Blend scintillation mixture (ICN Chemicals), and counted for ^{32}P radioactivity in a Beckman LS-1801 scintillation counter.

autoradiography could not allow one to decide which band was the [^{32}P]NAD-binding subunit, each band was carefully excised from gels, and the radioactivity of each band was measured (Figure 4). From Figure 4, it is clear that radioactivity from [^{32}P]NAD was associated mainly with the middle band. The small amount of radioactivity seen in the upper and lower bands is probably due to contamination by the middle band and/or to nonspecific labeling. These results were further confirmed by combining the corresponding slices, removing the proteins by electroelution, and measuring their bound radioactivity after a second SDS gel electrophoresis.

Immunochemical Approach for Identification of the NADH-Binding Subunit. Treatment of bovine NADH–ubiquinone oxidoreductase with chaotropic reagents results in the resolution of the enzyme complex into a water-soluble and a water-insoluble fraction. The water-soluble fraction can be further resolved by $(NH_4)_2SO_4$ fractionation into a flavoprotein fraction (FP) and an iron–sulfur protein fraction (IP). The FP is composed of three unlike subunits (M_r 51000,

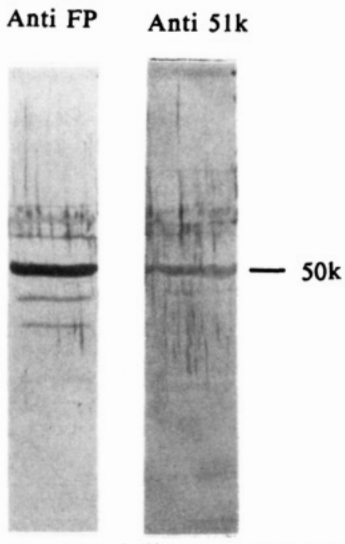

FIGURE 5: Cross-reactivity of the *Paracoccus* NADH dehydrogenase complex with antisera to bovine FP (left) or to its M_r 51 000 subunit (right). Immunoblot analysis was done as described in Yagi (1986) except that the antiserum to bovine FP or to the M_r 51 000 subunit was used at 1:80 dilution.

24 000, and 9000) and displays NADH dehydrogenase activity (Hatefi, 1985). Chen and Guillory (1981) have reported that when FP is irradiated by visible light in the presence of the tritiated photoaffinity NAD analogue arylazido-β-[^{3}H]alanyl-NAD, the main incorporation of radioactivity from this reagent is observed in the M_r 51 000 subunit. In addition, this labeling was prevented by NADH. Therefore, it was concluded that the M_r 51 000 subunit is the NADH-binding subunit of the bovine enzyme complex. Because antisera to bovine FP and the M_r 51 000 polypeptide are available (Han et al., 1988), immunological experiments with the isolated *Paracoccus* NADH dehydrogenase complex were carried out using these antisera. As shown in Figure 5, antisera to total FP and to the M_r 51 000 subunit of bovine enzyme cross-reacted with the M_r 50 000 band of the *Paracoccus* NADH dehydrogenase complex. However, in terms of cross-reactivity, the antiserum to FP was much stronger than the antiserum to the M_r 51 000 subunit. Therefore, immunoblotting experiments with the isolated three bands of Figure 1 were carried out using the antiserum to FP. The isolated middle band strongly cross-reacted with FP antibody, while the isolated upper and lower bands cross-reacted faintly (data not shown). These immunoblotting results indicated that the M_r 50 000 subunit of the *Paracoccus* enzyme complex resembles the M_r 51 000 subunit of the bovine enzyme complex and suggested that the middle band is the NADH-binding subunit of the *Paracoccus* enzyme. The faint cross-reactivity of the upper and lower bands is probably due to contamination with the middle band and related to the difficulty in cleanly separating these bands by excision from SDS gels of the *Paracoccus* enzyme complex.

Does the Middle Band Contain a Single Polypeptide? It was important to investigate whether the M_r 50 000 band (middle band) is composed of only a single NADH-binding subunit. Therefore, the *Paracoccus* NADH dehydrogenase complex was subjected to two-dimensional gel electrophoresis. Figure 6A shows that the middle band appeared as three spots very close to each other. Immunoblotting analysis of two-dimensional gels of the *Paracoccus* NADH dehydrogenase complex with antiserum to FP (Figure 6B) indicated that all three spots cross-reacted with this antiserum, suggesting that immunologically the three spots resemble each other. In addition, it was shown that all three spots had incorporated radioactivity even after 20-min UV irradiation of the dehydrogenase with [^{32}P]NAD (data not shown). Thus, it appears that the M_r 50 000 middle band, which is specifically labeled with [^{32}P]NAD(H) and cross-reacts with antibody to the bovine mitochondrial NADH-binding subunit, is composed of a single subunit which displays some microheterogeneity on two-dimensional gels. That single polypeptides can exhibit several spots on isoelectric focusing gels is well-known (Scheele, 1975). Examples are the α and β subunits of bovine F_1 (Wong et al., 1984; Melese & Boyer, 1985), the M_r 75 000 subunit of bovine IP [T. Yagi, unpublished data; see also Price and Gomer (1989)], and the M_r 30 000 subunit of bovine IP (Ragan, 1987).

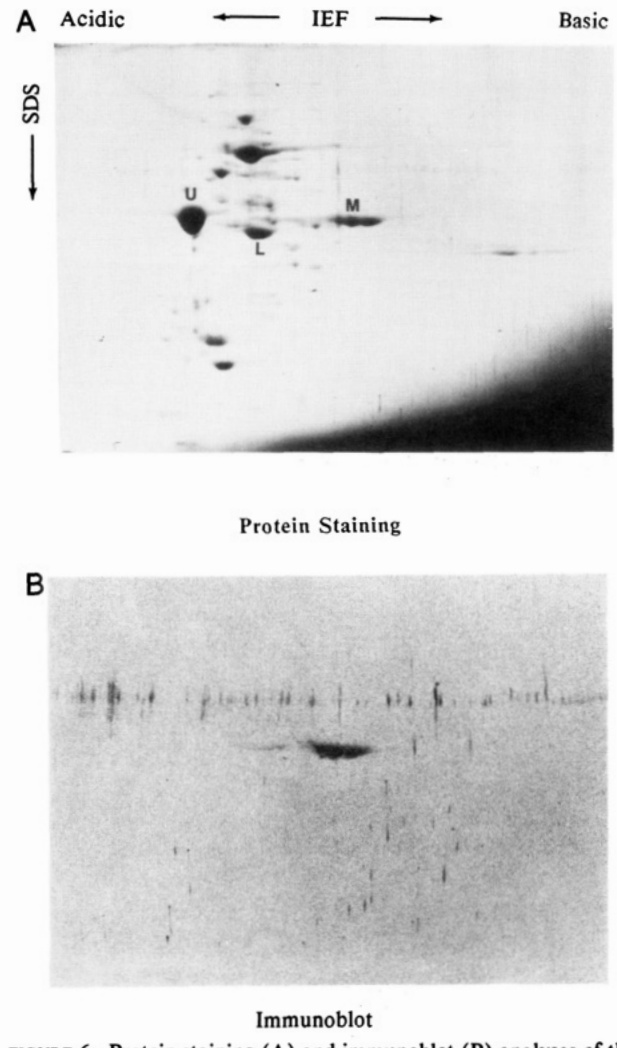

FIGURE 6: Protein staining (A) and immunoblot (B) analyses of the two-dimensional gel of *Paracoccus* NADH dehydrogenase complex. The first-dimensional gel was run in the isoelectric focusing system (pH 3–10), loading 25 μg of NADH dehydrogenase complex. After incubation with 42 μL of Laemmli sample buffer containing 5% β-mercaptoethanol for 10 min, the gels were run in a second-dimension, Laemmli-type SDS–polyacrylamide gel electrophoresis system. The anti-FP antiserum was used in Figure 6B. Details for the immunoblotting experiments are described under Experimental Procedures.

Relationship of the Paracoccus NADH-Binding Subunit to Its Bovine Counterpart. The NADH-binding subunit was purified from SDS–polyacrylamide gels of *Paracoccus* NADH dehydrogenase complex by two successive electroelutions (Yagi & Hatefi, 1988). Its amino acid composition was determined and compared with that of its bovine heart counterpart (Galante & Hatefi, 1979). As seen in Table I, the amino acid compositions of the two polypeptides are very similar. It

Table I: Comparison of the Amino Acid Composition of the NAD(H)-Binding Subunit between *Paracoccus* and Bovine NADH–Q Oxidoreductase Complex

amino acid	% residues	
	Paracoccus[a]	bovine heart[b]
alanine	11.08	10.13
arginine	7.62	6.67
aspartic acid	8.97	7.98
glutamic acid	12.92	10.98
glycine	11.71	13.08
histidine	1.40	2.00
isoleucine	6.50	6.58
leucine	7.96	6.89
lysine	2.83	5.70
methionine	3.30	2.01
phenylalanine	3.53	3.95
proline	5.21	5.53
serine	4.01	4.33
threonine	4.94	5.17
tyrosine	2.38	2.16
valine	5.64	6.98
polarity[c]	42.7	42.8
M_r	50000	51000

[a] The NADH-binding subunit of *Paracoccus* NADH–Q oxidoreductase was isolated according to Yagi and Hatefi (1988). For amino acid analysis, the isolated subunit was reelectrophoresed and reisolated. The purified NADH-binding subunit (20 μg) was hydrolyzed in an evacuated and sealed tube with 6 N HCl at 110 °C for 24 h, 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).

remains to be seen whether this indicates that *Paracoccus* is evolutionally related to mitochondria (John & Whatley, 1977) or that the NADH-binding subunit is a conserved polypeptide, like the α and β subunits of F_1-ATPase (Senior, 1988) and the flavoprotein and iron–sulfur protein subunits of succinate dehydrogenase (Kita et al., 1989). It might be added, however, that the antiserum to the bovine NADH-binding subunit did not cross-react with the energy-transducing NADH–Q oxidoreductase (NDH-1) isolated from *Thermus thermophilus* HB-8 membranes (Xu and Yagi, unpublished results). In addition, antibody to the *Paracoccus* NADH-binding subunit cross-reacted with bovine complex I, but not with the *Thermus* NDH-1 (Xu and Yagi, unpublished results). These results could be a hint in favor of the former possibility.

Discussion

Bacteria contain two types of NADH–quinone oxidoreductase. One is a single-subunit enzyme containing FAD and no iron–sulfur clusters. The other is a multicomponent, energy-transducing enzyme, containing FMN and multiple iron–sulfur clusters. The single-subunit enzyme has been isolated from *Escherichia coli* by Jaworowski et al. (1981), from *Bacillus subtilis* by Bergsma et al. (1982), from *Sulfolobus acidocaldarius* by Wakao et al. (1987), and from *T. thermophilus* in this laboratory (Yagi et al., 1988). The multicomponent energy-transducing enzyme has been isolated from only two sources, *T. thermophilus* and *P. dinitrificans*, both in this laboratory (Yagi et al., 1988; Yagi, 1986). The enzyme from *P. denitrificans* is of particular interest, because it resembles the mammalian NADH–ubiquinone oxidoreductase (complex I) in several important respects. These similarities include FMN and four iron–sulfur clusters as prosthetic groups, ubiquinone as electron acceptor, sensitivity of both systems to inhibition by rotenone (Yagi, 1986) and DCCD (Yagi, 1987), and cross-immunoreactivity of several subunits (Yagi, 1986; George et al., 1986). The *Paracoccus* enzyme has one advantage, however, over its mammalian counterpart, and that is that it contains less than half as many subunits. This relative structural simplicity of the *Paracoccus* enzyme makes it a more useful system for study of the mechanism of electron transfer and energy conservation in this segment of the respiratory chain.

Among the 25–30 subunits of mammalian complex I, at least 9 appear to serve as apoproteins for FMN and the 8 or 9 iron–sulfur clusters of the enzyme complex. A polypeptide of M_r 51 000 contains the NADH-binding site (Chen & Guillory, 1981), and another of M_r 29 000 binds the inhibitors DCCD and rotenone (Yagi & Hatefi, 1988; Earley et al., 1987). There remain in complex I up to 20 polypeptides to which no role has been attributed. By comparison, however, assignment of the NADH-binding site, prosthetic groups (FMN and four EPR-visible iron–sulfur clusters), and the DCCD/rotenone-binding sites should characterize most of the 10 subunits of the *Paracoccus* NADH–Q oxidoreductase.

This paper has shown by direct photolabeling with [^{32}P]-NAD or [^{32}P]NADH that the M_r 50 000 subunit of the *Paracoccus* NADH–Q oxidoreductase bears the NAD(H)-binding site. Inhibition of labeling by NADH, and the lack of effect of NADP(H) and AMP, has further confirmed the specificity of the M_r 50 000 subunit for NAD(H) binding. In addition, it was shown that antibody to the M_r 51 000 NAD(H)-binding subunit of complex I cross-reacted with the M_r 50 000 subunit of the *Paracoccus* enzyme and that the two subunits from bovine mitochondria and *Paracoccus* have very similar amino acid compositions. In the case of complex I, photoaffinity labeling with arylazido-β-[^{3}H]alanyl-NAD (Chen & Guillory, 1981) resulted in the labeling of four polypeptides, thus leaving the identity of the NAD(H)-binding subunit rather ambiguous, and only after application of the technique to the three-subunit flavoprotein fraction of complex I did the assignment of the NAD(H)-binding site to the M_r 51 000 subunit become clearer (see Results). However, even then, significant amounts of radioactivity from arylazido-β-[^{3}H]alanyl-NAD were found in the other two subunits (M_r 24 000 and 9000) of the flavoprotein fraction. By comparison, the direct labeling technique employed here left no room for ambiguity. The only subunit of the *Paracoccus* enzyme photolabeled with [^{32}P]NAD was the M_r 50 000 polypeptide.

The cross-immunoreactivity of corresponding NAD(H)-binding proteins of the mammalian and the *Paracoccus* respiratory chains and their similarity of molecular mass and amino acid composition recall other similarities in the structure and composition of the two redox chains. Not only the redox components of the two systems are nearly identical but also the subunits of bovine succinate–ubiquinone oxidoreductase, ubiquinol–cytochrome *c* oxidoreductase, and cytochrome oxidase cross-react with antisera to the corresponding subunits of the *Paracoccus* enzymes to the extent examined (Pennoyer et al., 1988; Yang & Trumpower, 1986; Steffens et al., 1983). In addition, amino acid sequences of the subunits of *Paracoccus* cytochrome oxidase, the Rieske iron–sulfur protein, and the cytochrome *b* of ubiquinol–cytochrome *c* oxidoreductase are highly homologous to those of the corresponding proteins of mammalian mitochondria (Raitio et al., 1987; Kurowski & Ludwig, 1987). These considerations as well as the similarity of their amino acid compositions suggest that the primary structures of the *Paracoccus* and the mammalian NADH-binding proteins might also show considerable sequence identity.

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