Structural Studies of the Proton-Translocating NADH-Quinone Oxidoreductase (NDH-1) of *Paracoccus denitrificans*: Identity, Property, and Stoichiometry of the Peripheral Subunits†

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ABSTRACT: The proton-translocating NADH-quinone oxidoreductase (NDH-1) of *Paracoccus denitrificans* is composed of at least 14 unlike subunits and contains one FMN and at least five EPR-detectable iron−sulfur clusters. The 14 subunits are designated NQO1 through NQO14. The expression and partial characterization of the NQO4, -5, and -6 subunits have been performed. The NQO4, -5, and -6 subunits were individually expressed in *Escherichia coli*. The NQO4 subunit was expressed in both the cytoplasmic phase and membrane fraction, the NQO5 subunit in the cytoplasmic phase only, and the NQO6 subunit in the membrane fraction only. The NQO4 and NQO5 subunits were purified from cytoplasmic phase. Neither subunit contains non-heme iron or acid-labile sulfide, suggesting that the NQO4 or NQO5 subunit is not an iron−sulfur subunit. The antibodies against the NQO4, -5, and -6 subunits cross-reacted with their counterpart subunits in bovine heart complex I. The NQO4, -5, and -6 subunits in membrane-bound *P. denitrificans* NDH-1 were extracted by treatment at alkaline pH (≥10) or with chaotropes (NaBr, NaI, and urea), suggesting that these subunits are localized in the peripheral part (not in the membrane sector) of the enzyme complex similar to the NQO1, -2, and -3 subunits. In addition, the subunit stoichiometry of NQO1 through -6 of the membrane-bound *P. denitrificans* NDH-1 has been determined by radioimmunoassays. There is 1 mol each of the NQO1 through -6 subunits per mol of the *P. denitrificans* NDH-1.

Paracoccus denitrificans is a Gram-negative soil bacterium and has been called "a free-living mitochondrion" (Stouthamer, 1992; Steinrücke & Ludwig, 1993; Roise & Maduke, 1994). Aerobically grown *P. denitrificans* expresses a mammalian mitochondrial-type respiratory chain which appears to bear only the proton-translocating NADH-quinone oxidoreductase (NDH-1)[1] (Yagi et al., 1988, 1993; Yagi, 1991, 1993). *P. denitrificans* NDH-1 is composed of at least 14 unlike subunits and bears one noncovalently bound FMN and at least five EPR-detectable iron−sulfur clusters (two binuclear and three tetranuclear clusters) as prosthetic groups (Yagi, 1986, 1993; Meinhardt et al., 1987; Yagi et al., 1992, 1993). These iron−sulfur clusters have been designated center N1a and N1b (for [2Fe-2S] clusters) and N2, N3, and N4 (for [4Fe-4S] clusters) (Meinhardt et al., 1987). Recently, the gene cluster encoding the *P. denitrificans* NDH-1 has been cloned and sequenced (Xu et al., 1991a,b, 1992a,b, 1993). The gene cluster is composed of 14 structural genes and six URFs. These structural genes encoding 14 subunits have been designated *NQO1* through *NQO14* genes. Direct photoaffinity labeling with [^{32}P]NAD(H) indicates that the NQO1 subunit contains the NADH-binding site (Yagi & Dinh, 1990). In addition, this subunit is believed to bear FMN and to ligate center N3 (Yano et al., 1996; Xu et al., 1991a; Hatefi et al., 1985; Hatefi, 1985; Fecke et al., 1994). Furthermore, the individual expression experiments of the NQO2 and -3 subunits may suggest that NQO2 subunit carries center N1a and NQO3 subunit bears centers N1b and N4 and probably another [4Fe-4S] cluster (Yano et al., 1994a, 1995; Crouse et al., 1994). Utilizing site-directed mutagenesis techniques, we have shown that the four conserved cysteine residues (C96, C101, C137, and C141) of the NQO2 subunit coordinate the [2Fe-2S] cluster (N1a) (Yano et al., 1994b). On the basis of comparison of the deduced primary structures of the *P. denitrificans* NDH-1 subunits to known iron−sulfur binding proteins, we anticipated that the NQO4 or -5 subunit may not ligate iron−sulfur clusters but the NQO6 subunit may coordinate an iron−sulfur cluster (Yano et al., 1993, 1995).

Determination of subunit stoichiometry and subunit topology of the *P. denitrificans* NDH-1 is a prerequisite to study structure and mechanism of action of this enzyme complex. The use of subunit-specific antibodies together with membrane preparations is a reliable method for determining the subunit stoichiometry of a membrane-bound enzyme complex (Matsuno-Yagi & Hatefi, 1984; Hekman et al., 1991).

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[1] Abbreviations: NDH-1, bacterial proton-translocating NADH-quinone oxidoreductases; complex I, mitochondrial proton-translocating NADH-quinone oxidoreductases; FP, IP, and HP, flavoprotein, iron−sulfur protein, and hydrophobic protein fractions of complex I, respectively; SDS, sodium dodecylsulfate; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; EPR, electron paramagnetic resonance; EDTA, ethylenediaminetetraacetate; PMSF, phenylmethylsulfonyl fluoride; IPTG, isopropyl β-D-thiogalactopyranoside; DTT, 1,3-dithiothreitol; CHES, 2-[*N*-cyclohexylamino]ethanesulfonic acid.

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This procedure has been used successfully for determination of the subunit stoichiometry of the stalk portion of bovine heart ATP synthase (Hekman et al., 1991). Recently, Belogrudov and Hatefi (1994) have determined stoichiometries of subunits constituting the water-soluble fraction (designated FP + IP subcomplexes, peripheral sector, or catalytic sector) of the isolated bovine complex I, utilizing this procedure. Bovine complex I is composed of at least 41 dissimilar subunits (Walker, 1992). FP + IP fractions contain at least 10 different subunits. FP contains three subunits with molecular masses of 51, 24, and 9 kDa. The bovine 51- and 24-kDa subunits are homologues to the *P. denitrificans* NQO1 and -2 subunits, respectively (Yagi et al., 1992; Yagi, 1993). The *P. denitrificans* NDH-1 lacks a counterpart of the bovine 9-kDa subunit. IP contains seven subunits with molecular masses of 75, 49, 30, 18, 15, 13, and 11 kDa. The bovine 75-, 49-, and 30-kDa subunits are counterparts of the NQO3, -4, and -5 subunits, respectively (Yagi et al., 1992; Yagi, 1993). Homologues to other IP small subunits are absent in the *P. denitrificans* NDH-1. According to their results, per mole of bovine heart complex I, there are 2 mol of the IP 15-kDa subunit and 1 mol each of FP and other IP subunits. However, nothing is known about the subunit stoichiometry of the bacterial NDH-1. The homologue of the NQO6 subunit is known to be present in bovine heart complex I (Masui et al., 1991). The bovine homologue of the NQO6 subunit is designated the 20-kDa or PSST subunit (Masui et al., 1991; Walker, 1992). Although the PSST (20 kDa) subunit is often detected in IP (Masui et al., 1991), its stoichiometry or localization in situ has not yet been determined. At the present time, subunit-specific antibodies to the *P. denitrificans* NQO1 through 6 subunits are available in this laboratory. Therefore, it was of interest to elucidate the stoichiometry of the NQO1 through -6 subunits of the *P. denitrificans* NDH-1 and to clarify the localization of the NQO4 through -6 subunits in the *P. denitrificans* NDH-1 in situ.

This paper describes the expression and partial characterization of the NQO4, -5, and -6 subunits of the *P. denitrificans* NDH-1. Under a co-expression system with thioredoxin, the NQO4 subunit has been expressed in both the cytoplasmic phase and the membrane fraction in *E. coli*, whereas the NQO5 and NQO6 subunits have been expressed, respectively, in the cytoplasmic phase and the membrane fraction. Subunit-specific antibodies directed against the NQO4, -5, and -6 subunits cross-reacted with their bovine counterparts. The NQO4, -5, and -6 subunits have been extracted from the *P. denitrificans* membranes by treatment at alkaline pH or with chaotropic agents, suggesting that these subunits are localized in the peripheral part of the *P. denitrificans* NDH-1 in situ. In addition, subunit stoichiometries of the NQO1 through -6 have been determined by radioimmunoassay. The results show that there is one copy each of the NQO1 through -6 subunits in the *P. denitrificans* NDH-1.

MATERIALS AND METHODS

Materials. Acrylamide, *N,N*′-methylenebis(acrylamide), SDS, low-range marker proteins, Coomassie Brilliant Blue R-250, and the in vitro mutagenesis kit were from Bio-Rad; the DNA sequencing kit was from U.S. Biochemical Corp; the [α-^{35}S]thio-dATP, [^{125}I]protein A, and enhanced chemiluminescence (ECL) kit were from Amersham; alkaline phosphatase-conjugated affinity-purified antibodies to rabbit IgG were from Calbiochem; expression vectors pET11a and pET16b, competent *E. coli* strains BL21(DE3) and BL21-(DE3)pLysS, and His:Bind metal chelation resin were from Novagen; expression vector pTrx was from Invitrogen; carbenicillin was from ICN. Bovine heart complex I was kindly provided by Drs. Y. Hatefi and A. Matsuno-Yagi (The Scripps Research Institute).

Preparation of Cholate-Treated P. denitrificans Membranes. *P. denitrificans* membranes were prepared as described in Yagi (1986). The membranes were suspended and homogenized in 10 mM Tris-acetate buffer (pH 7.5) containing 1% (w/v) potassium cholate and 0.15 M KCl. The membrane suspension was incubated at 4 °C for 30 min. The suspensions were centrifuged at 50 000 rpm in the 60Ti rotor. After the pellet was washed twice with 10 mM Tris-acetate buffer (pH 7.5) containing 1 mM EDTA and 0.1 mM PMSF, the membrane pellet was resuspended and homogenized in the same buffer. The membranes prepared as described above are designated cholate-treated *P. denitrificans* membranes. The cholate-treated membranes have approximately 5 times as much NADH oxidase and NADH-ubiquinone-1 reductase activities as the original membrane fragments. Concerning rotenone sensitivity, there is no difference between the cholate-treated membranes and the original membranes (the NADH oxidase activities of both membrane preparations are inhibited to higher than 95% by addition of 10 μM rotenone).

Construction of Expression Vector

NQO4. The *NQO4* gene was constructed from two plasmids, pXT-3 (Xu et al., 1992a) and pXT-1 (Xu et al., 1991a,b, 1992b), as follows. The *Hin*dIII/*Sph*I fragment encoding the C-terminal region of the NQO4 subunit was isolated from pXT-1 and ligated into *Hin*dIII/*Sph*I-digested pTZ18U. The plasmid thus obtained was designated pTZ18U-(NQO4c). In addition, the *Hin*dIII fragment encoding the remaining region of the NQO4 subunit was cleaved from pXT-3 and ligated into *Hin*dIII site of pTZ18U(NQO4c). The proper construct was confirmed by cleavage with several restriction enzymes and by DNA sequencing around the *Hin*dIII ligation site (Sanger et al., 1977). The resulting construct was designated pTZ18U(NQO7654). The oligonucleotide 5′-TGAGGGCTG*CAT*A*TG*GACGGC-3′ (the underlined bases were altered from the *P. denitrificans* DNA for the mutation, and the italic bases indicate the *Nde*I site) was designed in order to generate an *Nde*I site at the NQO4 translation initiation codon. The mutagenesis was performed using the Bio-Rad in vitro mutagenesis kit based on the method of Kunkel et al. (1987). The mutation was confirmed by DNA sequence analysis of the region surrounding the initiation site. In order to avoid DNA sequencing of the entire region, the *Pst*I fragment of the mutated plasmid was replaced with the *Pst*I fragment from the original plasmid pTZ18U(NQO7654). The resulting plasmid is designated pTZ18U(NQO4-NdeI). The *Nde*I/*Bam*HI fragment from pTZ18U(NQO4-NdeI) were ligated in expression vectors pET11a (designed for expression of nonfused protein) and pET16b (designed for expression of N-terminal His_{10}-tag fused protein). The resulting expression plasmids are designated pET11a(NQO4) and pET16b(NQO4).

NQO5 and 6. The *NQO5* and *6* genes were constructed from pXT-3 plasmid utilizing PCR. The primers were

synthesized in order to generate the *Nde*I site at the initiation codon of the subunits of interest and the *Bam*HI site at the stop codon of the subunits of interest as described below.

NQO5:

sense primer 5′-GGTGA*CATATG*TCCGAAG-3′

antisense primer 5′-GCGC*GGATCC*GGTCACTT-3′

NQO6:

sense primer 5′-GGGC*CATATG*ACCGGCCTG-3′

antisense primer 5′-CTTC*GGATCC*GGCTCACCTC-3′

The underlined bases were altered from *P. denitrificans* DNA for the mutations. The italicized bases exhibit the *Nde*I or *Bam*HI site.

PCR amplification of the *NQO5* and *-6* genes was performed with a thermocycler. DNA sequences were amplified with Taq polymerase in a 100 μL reaction mixture containing 700 ng of plasmid DNA, 100 mM Tris-HCl (pH 8.3), 20 mM $MgCl_2$, 500 mM KCl, 1% Triton X-100, 200 μM each dNTPs (dATP, dCTP, dGTP, and dTTP), 1 μM of both oligonucleotide primers, and 5 units of Taq polymerase. The samples were subjected to 35 amplification cycles each involving denaturation at 94 °C for 1 min, annealing at 55 °C for 2 min, and extension at 72 °C for 1.5 min except that denaturation at the initial cycle and extension at the final cycle were prolonged to 5 and 10 min, respectively. The amplified DNA were isolated by chloroform extraction and ethanol precipitation. The isolated DNA fragments were digested with *Nde*I and *Bam*HI. The digested fragments were purified by 1% agarose gel electrophoresis and Qiagen Gene Clean kit and then ligated into pET11a and pET16b. These plasmids are designated pET11a(NQO5), pET16b(NQO5), pET11a(NQO6), and pET16b(NQO6).

Thioredoxin. Thioredoxin expression vector was constructed according to Yasukawa et al. (1995). The *Nde*I/*Hin*dIII fragment (450 bp) containing the thioredoxin gene was cleaved from pTrx vector (Invitrogen) and cloned into *Nde*I/*Hin*dIII site of pET11a expression vector. The construct was designated pET11a(Trx). The *Bgl*II/*Hin*dIII fragments (560 bp) encompassing the thioredoxin gene and T7 transcription/expression region was isolated from pET11a-(Trx) and ligated into *Bam*HI/*Hin*dIII sites of pACYC184 plasmid. The final construct was designated as pACYC-(Trx). The constructions were verified by DNA sequencing of both strands.

Expression of Subunits NQO4 through NQO6. The expression of the NQO4 through -6 subunits was performed as described previously (Yano et al., 1995). Competent *E. coli* strains BL21(DE3) and BL21(DE3)pLys were transformed with pET11a(NQO4), pET11a(NQO5), pET11a-(NQO6), pET16b(NQO4), pET16b(NQO5), or pET16b-(NQO6) and spread onto the 2× YT agar plate containing 100 μg of carbenicillin/mL. A well-isolated colony was selected and used to inoculate 10 mL of 2× YT medium containing 100 μg of ampicillin/mL and cultivated at 37 °C to the stationary phase. The culture was used to inoculate 500 mL of 2× YT or TB medium containing 100 μg of ampicillin/mL. When required, 100 μg of ammonium Fe(II) citrate/mL and 100 μM sodium sulfide were added to the culture medium. Cells were grown at 37 and 25 °C until absorbance (at 600 nm) of the culture reached approximately 0.4. IPTG was then added to 0.4 mM final concentration, and the cells were further cultured for 5 h at 37 °C or for 18 h at 25 °C. The cells were harvested by centrifugation at 6000 rpm for 10 min in a GSA rotor. The cell precipitates were suspended in 50 mM Tris-HCl buffer (pH 8.5) containing 1.0 mM EDTA, 1 mM PMSF, and 1 mM DTT (buffer A). The cell suspensions were rapidly frozen in liquid nitrogen and stored at −20 °C until use.

Expression of the NQO4 through NQO6 Subunits with Thioredoxin. The co-expression of the NQO4, -5, or -6 subunit with thioredoxin was performed principally according to Yasukawa et al. (1995). Competent *E. coli* strain BL21-(DE3) was transformed with pACYC(Trx) and either pET11a-(NQO4), pET11a(NQO5), pET11a(NQO6), pET16b(NQO4), pET16b(NQO5), or pET16b(NQO6) and was spread onto 2× YT agar plate containing 50 μg of ampicillin/mL and 50 μg of chloramphenicol/mL. A well-isolated colony was retrieved and inoculated into 10 mL of 2× YT medium in the presence of 100 μg of ampicillin/mL and 50 μg of chloramphenicol/mL. The suspension was cultivated aerobically at 37 °C to the stationary phase. The culture was inoculated into 500 mL of TB medium bearing the above two antibiotics. When required, ammonium Fe(II) citrate (100 μg/mL) and sodium sulfide (100 μM) were added to the culture medium. IPTG was added to 0.4 mM final concentration for induction. Cells were grown at 25 °C overnight, harvested by centrifugation at 6000 rpm for 10 min in a GSA rotor, and then suspended in 50 mM Tris-HCl buffer (pH 8.0) containing 0.1 mM PMSF and 1 mM DTT. The cell suspension was rapidly frozen in liquid nitrogen and stored at −20 °C until use.

Preparation of Cytoplasmic, Membrane, and Inclusion Body Fractions. The cell suspensions were freeze−thawed twice using liquid nitrogen and a water bath at 30 °C and then sonicated briefly until their viscosity was reduced. Afterward, all procedures were performed at 4 °C. The suspensions were further treated twice with a Parr cell disruption bomb at >1000 kg/cm^2. The resultant suspension was centrifuged at 10 000 rpm for 10 min in an SS34 rotor. Inclusion body fractions were usually collected as precipitates at this step. The supernatant was carefully separated from the pellet and ultracentrifuged at 48 000 rpm in a Beckman 60Ti rotor. The supernatant was used as the cytoplasmic fraction. The pellet was suspended in 10 mM Tris-HCl buffer (pH 8.0) containing 0.1 mM PMSF and 1 mM DTT and was used as the membrane fraction for further analyses.

Isolation of the NQO1 through NQO6 Subunits from the Aggregate Forms (So-Called Inclusion Bodies). The crude inclusion body pellet described above was resuspended in buffer A containing 10% sucrose and homogenized. The suspension was again centrifuged at 12 000 rpm for 20 min in the SS34 rotor. The pellet was resuspended in buffer A containing 2% Triton X-100 and homogenized. The suspension was centrifuged at 12 000 rpm for 20 min in the same rotor. This washing step was repeated three times. The final pellet was resuspended in buffer A. As seen in Figure 2, purity of each subunit appears to be higher than 90% on the basis of the SDS−polyacrylamide gel patterns. These subunit preparations were used to raise subunit-specific antibodies and for antibody purification and quantitative immunoblotting experiments.

Purification of the Soluble His-Tag Fused NQO4 and NQO5 Subunits. Water-soluble His-tag fused NQO4 and NQO5 subunits were prepared from cells harboring pET16b-

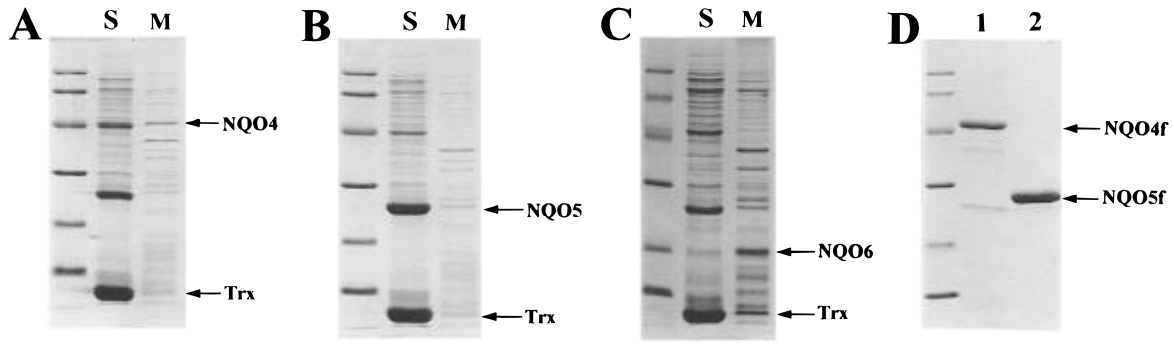

FIGURE 1: Polyacrylamide SDS gel patterns of the expressed NQO4 subunit (panel A), NQO5 subunit (panel B), and NQO6 subunit (panel C) with co-production of thioredoxin, and the purified soluble His-tagged NQO4 and NQO5 subunits (panel D). S, cytoplasmic fraction; M, membrane fraction; Trx, thioredoxin; NQO4f, His-tagged NQO4; NQO5f, His-tagged NQO5. The preparation of cytoplasmic and membrane fractions is described in Materials and Methods. The cytoplasmic fractions were mixed with an equal volume of 2× Laemmli sample buffer [160 mM Tris-HCl (pH 6.8), 12% (w/v) SDS, 0.01% (w/v) bromophenol blue, 2% (v/v) β-mercaptoethanol] and boiled for 5 min. The membrane pellet was suspended in 10 mM Tris-HCl buffer (pH 8.0) containing 1.0 mM EDTA, 0.1 mM PMSF, and 1.0 mM DTT, and then the final volumes were adjusted to those of supernatant. The membrane suspension was prepared for SDS−PAGE as described in the supernatant. 5 μL each of the cytoplasmic fraction (S) and membrane fraction (M) was loaded on 13% polyacrylamide SDS gel (panels A−C). The purified His-tagged NQO4 (panel D, lane 1) and NQO5 (panel D, lane 2) were denatured as described above, and 1.25 μg of each subunit was loaded on 13% polyacrylamide SDS gel. The molecular markers used were phospholipase B (97 kDa), bovine serum albumin (64 kDa), ovalbumin (45 kDa), carbonic anhydorase (31 kDa), trypsin inhibitor (22 kDa), and lysozyme (14 kDa). Electrophoresis, staining, and destaining were performed as described previously (Yagi, 1987).

(NQO4) and pET16b(NQO5), respectively, in addition to pACYC(Trx). The His-tagged NQO4 and NQO5 subunits were purified with His:Bind metal chelation column according to Yano et al. (1994a) except that imidazole concentration in the washing buffer was increased to 100 mM.

Raising Antibodies to the NQO1 through NQO6 Subunits. Antisera to purified NQO1 through NQO6 subunits were raised in New Zealand white female rabbits. In all cases, an initial injection of approximately 1 mg of the subunits into lymph nodes and under the skin was followed by a second injection of the same amounts under the skin 2 weeks later. The rabbits were then boosted with approximately 500 μg of the subunits at monthly intervals, and blood was collected 10 days after each boost.

Sequence Analysis. GCG software programs were used to analyze the amino acid sequence (Devereux et al., 1984). Sequence comparison of the polypeptides were conducted with BESTFIT and PILEUP programs. The FASTA and PROFILESEARCH programs were used to search for homology with the GenBank/EMBL sequence databases. Homology search was also carried out by using the BLAST program running at the National Center for Biotechnology Information (Altschul et al., 1990).

Other Analytical Procedures. Protein was estimated by the method of Perterson (1977), by the method of Lowry et al. (1951), or by the biuret method in the presence of sodium deoxycholate at 1 mg/mL (Gornall et al., 1949). SDS−polyacrylamide gel electrophoresis was carried out by modified methods of Laemmli (1970). Non-heme Fe and acid-labile sulfide were determined according to Doeg and Ziegler (1962) and Fogo and Popowski (1949), respectively. Amino acid composition (Yagi & Dinh, 1990), amino acid sequences (Matsudaira, 1987), DNA sequences (Sanger et al., 1977), and immunoblotting (Hekman & Hatefi, 1991) were done according to the references cited. Quantitative immunoblotting was carried out according to Hekman et al. (1991). The binding amounts of primary antibodies were detected with [^{125}I]protein A (250 000 cpm/mL) dissolved in Tris-buffered saline buffer (25 mM Tris-HCl and 150 mM NaCl, pH 7.4) after incubation with nitrocellulose blots for 1 h at 25 °C. The nitrocellulose membranes were washed with Tris-buffered saline containing 0.5% Tween-20, dried in air, and exposed to Fuji medical X-ray film overnight at room temperature. Radioactive bands were then excised from the nitrocellulose membranes, and the radioactivity associated with each band was determined by Beckman γ-counter model 550. Any variations from the procedures and other details are described in the figure legends.

RESULTS

Expression of the NQO4, -5, and -6 Subunits of the P. denitrificans NDH-1 in E. coli. When the *NQO4*, *-5*, and *-6* genes were expressed individually as full-length nonfused or fused proteins in *E. coli*, the subunits were found exclusively as insoluble aggregates (inclusion bodies) under any conditions we tested except that the NQO5 subunit was expressed into cytoplasmic phase to a small extent (data not shown). As described previously (Yano et al., 1994a, 1995, 1996), subunits in inclusion bodies are not suitable for characterization of the polypeptides. In order to circumvent the problems, we employed the native expression system which has been recently developed by Yasukawa et al. (1995). According to the reference, the co-production of thioredoxin increases solubility of recombinant proteins mainly by facilitating proper polypeptide folding in situ. Construction of plasmids that produce thioredoxin under T7 promoter was described in Materials and Methods. The standard expression procedures were principally the same as procedures of Yasukawa et al. (1995). We examined temperature effects on the expression of our target proteins in this expression system because lower temperature is effective for proper folding of the expressed polypeptides as described previously (Yano et al., 1994a, 1995, 1996). When the expression of the NQO4, -5, and -6 subunits was induced at lower temperature (25 °C), no production of inclusion bodies of the subunits expressed was observed. When the NQO4 subunit was co-expressed with thioredoxin, the subunit was produced in both cytoplasmic and membrane fractions (Figure 1, panel A). In the case of the NQO5 subunit, the protein was expressed as soluble form in the cytoplasmic fraction (Figure 1, panel B). Meanwhile, the NQO6 subunit was detected only in the membrane fraction

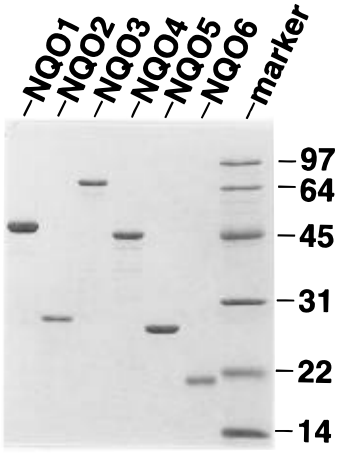

FIGURE 2: SDS−polyacrylamide gel showing the purified *P. denitrificans* NQO1 through -6 subunits stained with Coomassie Brilliant Blue. The purified NQO1 through -6 subunits were denatured in the Laemmli sample buffer, containing 80 mM Tris-HCl (pH 6.8), 6% (w/v) SDS, 0.005% (w/v) bromophenol blue, 1% (v/v) β-mercaptoethanol, and 2 μg of each subunit was loaded on a Laemmli-type 12.5% polyacrylamide SDS gel. Electrophoresis, staining, and destaining were performed as described previously (Yagi, 1987).

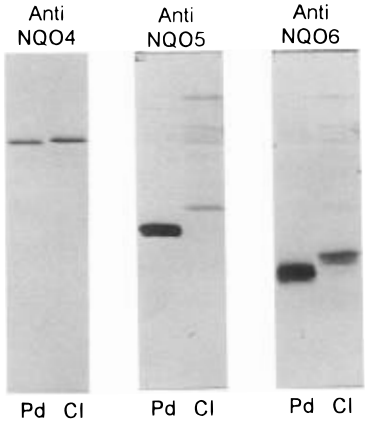

FIGURE 3: Cross-immunoreactivity between the *P. denitrificans* NQO4 through -6 subunits and the bovine 49-, 30-, and 20-kDa subunits, respectively. The 12% SDS−polyacrylamide gels were prepared and loaded as follows: lanes (Pd) and (CI) were loaded with 2 μg of *P. denitrificans* membranes and 20 μg of bovine complex I, respectively. Subsequent to electrophoresis and transfer of the proteins to nitrocellulose membranes, the membranes were blotted with affinity-purified antibodies against the *P. denitrificans* NQO4, -5, and -6 subunits as previously described (Han et al., 1988, 1989; Hekman et al., 1991). The detecting antibody used was an alkaline phosphatase conjugated anti-rabbit IgG.

(Figure 1, panel C). When a His-tag was attached to the N-terminal end of the subunits, the soluble NQO4 and NQO5 subunits could be purified with Ni-chelation column (see Figure 1, panel D). The difference of localization of the expressed NQO4, -5, and -6 subunits is in agreement with their hydrophobicity calculated by deduced primary structures (NQO6 > NQO4 > NQO5). Therefore, the NQO4, -5, and -6 subunits expressed under co-expression system with thioredoxin were considered suitable materials for investigation of their molecular properties. Taken together, the co-production system of recombinant protein with thioredoxin seems to be also effective for native expression of the *P. denitrificans* NDH-1 subunits in *E. coli*.

The expressed nonfused NQO4, -5, and -6 subunits purified from inclusion bodies were used to raise subunit-specific antibodies directed against these subunits (see Figure 2). As shown in Figure 3, the subunit-specific antibodies raised to the NQO4, -5, and -6 subunits, when tested against the *P. denitrificans* membranes, reacted with single bands which migrated in the SDS−polyacrylamide gels with molecular masses of 48, 28, and 20 kDa, respectively. The apparent molecular masses of the bands reacted by subunit-specific antibodies to the NQO4, -5, and -6 are consistent with the relative molecular mass of the overexpressed subunits, respectively. The expressed NQO4 through -6 subunits were subjected to the amino acid analysis and gas-phase amino acid sequencing. The amino acid compositions of the expressed NQO4, -5, and -6 subunits reasonably correspond with those deduced from the *NQO4, -5, and -6* genes, respectively (see Table 1). In addition, the expressed NQO4 and -5 subunits were similar, respectively, to the NQO4 and -5 subunits isolated from the *P. denitrificans* NADH dehydrogenase complex regarding amino acid compositions (Xu et al., 1992a). The N-terminal partial amino acid sequences of the expressed NQO4 through -6 subunits were MDGDIR for the NQO4 subunit, SEALS for the NQO5 subunit, and TGLNT for the NQO6 subunit, indicating that methionines of the N-termini of the NQO5 and -6 subunits are cleaved by post-translational processing. Similar post-translational processing was observed in mature NQO3 and -9 subunits (Yano et al., 1995; T. Yano and T. Yagi, unpublished results). The sequences perfectly match the deduced primary structures of these subunits. Taken together, we conclude that the NQO4, -5, and -6 subunits were correctly expressed in *E. coli*.

Do the Expressed NQO4, -5, and -6 Subunits Ligate the Iron−Sulfur Cluster? The NQO4, -5, and -6 subunits contain seven (C99, C165, C195, C257, C273, C279, C288), one (C57), and five (C53, C54, C118, C135, C148) cysteine residues, respectively. Comparison of the deduced primary structures of the NQO4, -5, and -6 subunits with their homologues suggests that the NQO4 and -5 subunits bear one (C273) and no conserved cysteine residues, respectively, while the NQO6 subunit contains four conserved cysteine residues (C53, C54, C118, C148). As described above, the soluble His-tag fused NQO4 and NQO5 subunits were purified nearly homogeneously by the Ni-chelation column chromatography (Figure 1, panel D). The purified subunits were colorless and contained neither non-heme iron nor acid-labile sulfide (data not shown), suggesting that the NQO4 or NQO5 subunit does not ligate an iron−sulfur cluster as expected. Masui et al. (1991) and Albracht (1993) suggested that the NQO6 and its homologues contain sequence motifs of the small subunit of the Ni-Fe hydrogenases. The three-dimensional structure of the Ni-Fe hydrogenase from *Desulfovibrio gigas* (Volbeda et al., 1995) may suggest that the three cysteines, C54, C118, and C148 (*Paracoccus* numbering), may be involved in coordination of a [4Fe-4S] cluster. As described above, the NQO6 subunit was expressed in the membrane fraction without forming inclusion bodies in the co-expression system with thioredoxin. In addition, the NQO6 purified from the inclusion body contains small amounts of non-heme iron and acid-labile sulfide (data not shown). Therefore, it is of interest to purify the NQO6 subunit from the membrane fraction and then to investigate the purified subunit by chemical analyses and physicochemical measurements such as EPR spectroscopy in order to verify whether the subunit bears an iron−sulfur cluster. Such attempt is currently under way in collaboration with Dr. Tomoko Ohnishi's group.

Table 1: Amino Acid Compositions of the Expressed NQO4, -5, and -6 Subunits of the *Paracoccus denitrificans* NDH-1

	NQO4		NQO5		NQO6	
amino acid	by amino acid analysis	from DNA sequence	by amino acid analysis	from DNA sequence	by amino acid analysis	from DNA sequence
alanine	36.5	34	14.1	13	15.7	15
arginine	35.2	35	17.2	17	15.0	16
aspartic acid	43.3	42	22.0	22	15.9	15
glutamic acid	41.4	42	23.0	24	14.2	13
glycine	34.5	35	12.6	12	15.4	15
histidine	11.7	12	4.1	4	2.5	2
isoleucine	21.3	22	10.8	11	7.6	8
leucine	46.1	44	23.6	22	18.4	17
lysine	13.5	13	7.6	7	4.2	3
methionine	11.7	16	1.1	1	5.4	8
phenylalanine	13.6	14	11.9	12	3.7	3
proline	22.2	22	10.7	11	9.7	10
serine	11.2	12	9.4	11	7.0	8
threonine	16.6	16	7.8	8	13.1	14
tyrosine	14.8	15	7.9	8	8.2	9
valine	24.6	24	19.1	20	10.1	10

Cross-Immunoreactivities of the NQO4, -5, and -6 Subunits. Figure 3 shows cross-immunoreactivities of the subunit-specific antibodies directed against the *P. denitrificans* NQO4, -5, and -6 subunits with the bovine heart complex I. As anticipated, the subunit-specific antibodies reacted with single bands of the *P. denitrificans* membranes (M_r = 48 000, 28 000, and 20 000, respectively). These antibodies also cross-reacted with single bands of the bovine complex I (M_r = 49 000, 30 000, and 20 000). The M_r of these bands are consistent with the M_r of the bovine homologues reported previously (Hatefi et al., 1985; Hatefi, 1985). The subunit-specific antibody to the NQO5 subunit also cross-reacted with the band (M_r = 60 000) of bovine heart complex I. It remains to be seen whether this band is a dimeric form of the IP30-kDa subunit or contains a different polypeptide with similar epitopes. The *P. denitrificans* NQO6 subunit antibody did not cross-react with the *Thermus thermophilus* HB-8 membranes, which are known to bear an NDH-1 (Yagi et al., 1988; Meinhardt et al., 1990) (data not shown). Similar results have been reported concerning the NQO1 subunit (Xu & Yagi, 1991). The subunit-specific antibodies to the bovine 49- and 30-kDa subunit cross-reacted with the isolated *P. denitrificans* NQO4 and -5 subunits, respectively (Xu et al., 1992a). It should be noted that the *P. denitrificans* NQO4, -5, and -6 subunits exhibit significant amino acid sequence identities to their bovine homologues (58.6% for the NQO4; 47.9% for the NQO5; 67.4% for the NQO6) (Xu et al., 1992a). Therefore, the *P. denitrificans* NQO4, -5, and -6 subunits are akin to their bovine homologues, respectively, in terms not only of amino acid sequences but also of antigenic sites. Similar trends were observed with the NQO1, -2, -3, and -9 subunits (Yagi & Dinh, 1990; Xu et al., 1991b, 1992a,b, 1993; T. Yano and T. Yagi, unpublished results).

Topology of the NQO4, -5, and -6 Subunits in the P. denitrificans NDH-1. Figure 4 illustrates the effects of alkaline or salt treatment on the subunits NQO4, -5, and -6 extraction into soluble phase from the *P. denitrificans* membranes. Treatment at pH 10 partially extracted the NQO4, -5, and -6 subunits from the *P. denitrificans* membranes. When pH of the treatment was raised to 11, these subunits were almost completely extracted. NaCl is not effective for extraction of these subunits from the *P. denitrificans* membranes. These subunits were extracted only partially by NaBr treatment but were completely extracted by NaI treatment. In the case of urea, the NQO6 subunit was only partially extracted but the NQO4 and -5 subunits were almost completely extracted. The treatments under alkaline conditions or with chaotropes are believed to extract the peripheral part of the membrane-bound enzyme complexes (Fujiki et al., 1982; Hatefi et al., 1985; Azevedo et al., 1992). Taking these into consideration, we may conclude that the NQO4, -5, and -6 subunits are located in the peripheral part of the *P. denitrificans* NDH-1 but not in the membrane-sector. Similar trends are observed in the isolated *E. coli* NDH-1 (Lief et al., 1995). These results are consistent with the results obtained from resolution of bovine heart complex I (Han et al., 1989; Masui et al., 1991). Taken together, it is clear that the PSST (20-kDa) subunit, the bovine homologue of the NQO6 subunit, is present in the peripheral part of complex I. If the NQO6 subunit turned out to carry an iron−sulfur cluster, it would be indicative of

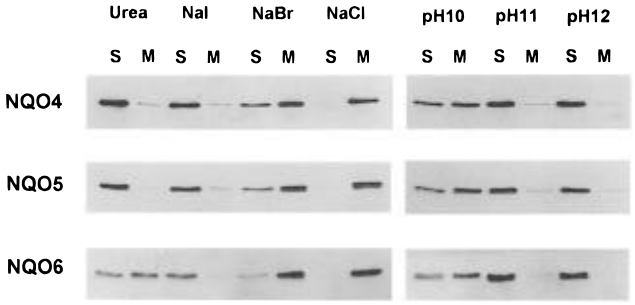

FIGURE 4: Effects of salt and alkaline pH on extraction of *P. denitrificans* NDH-1 subunits NQO4, -5, and -6 from the cholate-treated *P. denitrificans* membranes. The cholate-treated membrane suspension (1.0 mg/mL, final volume, 120 μL) was incubated for 10 min at 30 °C in the presence of 6 M urea, 2 M NaI, 2 M NaBr, or 2 M NaCl. The other cholate-treated membrane suspension (the same concentration, the same volume) was incubated in 100 mM CHES buffer at pH 10, 11, or 12 for 10 min at 30 °C. The membrane suspension was freeze−thawed twice using liquid nitrogen and a water bath at 30 °C followed by centrifugation in Airfuge at 30 psi for 10 min. 90 μL of the supernatant was carefully transferred into microtubes. After the remaining supernatant was completely removed by the aspirator, the pellet was resuspended in 120 μL of 10 mM Tris-acetate buffer (pH 7.5). The resulting supernatant (S) and membrane suspensions (M) were mixed with equal volume of 2× Laemmli's sample buffer. 5 μL of each sample was loaded on 13% polyacrylamide SDS gels. Immunoblotting analyses were performed with affinity-purified antibodies against each subunit as descibed in Figure 3 except that ECL kit was used for detection of immunoblotting.

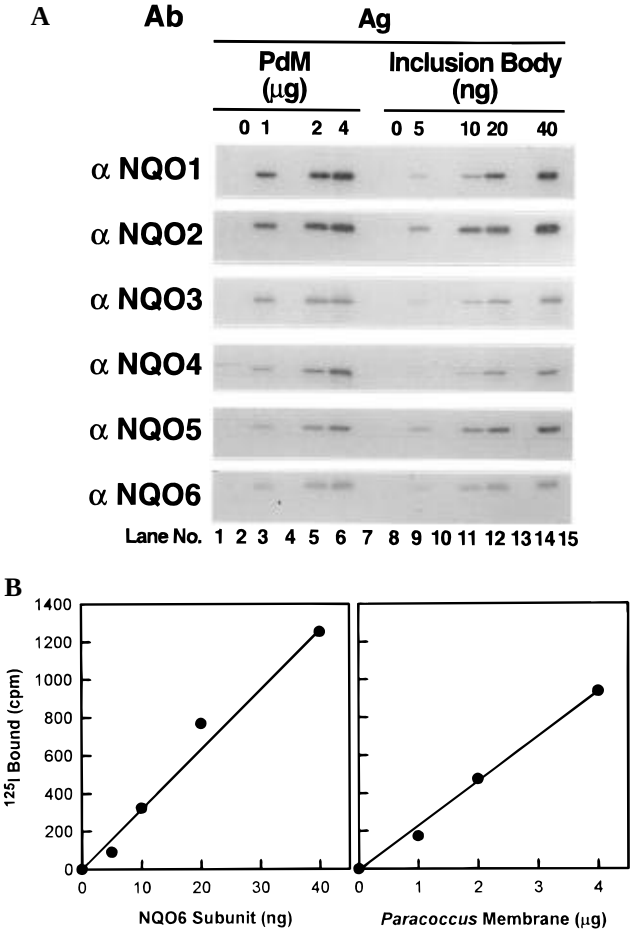

FIGURE 5: (A) Autoradiograms of immunoblotting of the purified *P. denitrificans* NQO1 through -6 subunits and the cholate-treated *P. denitrificans* membranes, using subunit-specific antisera to the NQO1 through -6 subunits. (B) Standard curves (left) and experimental curves (right) for quantitative immunoblotting for the NQO6 subunit. Purified NQO1 through 6 subunits and cholate-treated *P. denitrificans* membranes were electrophoresed on a 12% polyacrylamide SDS gels and transferred to nitrocellulose membranes. The nitrocellulose membranes were incubated with subunit-specific antisera to the NQO1 through -6 subunits followed by [^{125}I]protein A as described under Materials and Methods. After radioactive bands of the membranes were located, they were excised from the membranes and counted in a γ-counter.

the clusters being present in the peripheral part of the *P. denitrificans* NDH-1.

Stoichiometry of the NQO1 through NQO6 Subunits. Availability of the subunit-specific antibodies made it possible to determine the stoichiometries of subunits NQO1 through -6 in the *P. denitrificans* NDH-1 using radioimmunoassays and *P. denitrificans* membranes. Using purified expressed subunits, a standard curve was constructed for each subunit, in which the [^{125}I]protein A bound to the nitrocellulose membrane was plotted as a function of the amount of purified subunit placed on the gels (Figure 5A). An example is shown in Figure 5B for the NQO6 subunit. In the range 0−40 ng for the purified NQO6 subunit and 0−4 μg for the cholate-treated *P. denitrificans* membranes the bound radioactivity is proportional to the amounts of the NQO6 subunit and the membranes. In order to determine the concentration of each subunit in the cholate-treated *P. denitrificans* membranes, as shown in Figure 5B, the correlation between the [^{125}I]protein A bound and the amounts of the *P. denitrificans* membranes was determined in each set of experiments. Table 2 shows the stoichiometry of subunits NQO1 through -6 in the *P. denitrificans* NDH-1. It is clear

Table 2: Stoichiometry of Subunits NQO1−6 in the *P. denitrificans* NDH-1

subunit	n^a	pmol/μg	SD^b	mol/mol of NQO1
NQO1	8	0.371	0.057	
NQO2	4	0.393	0.050	1.06
NQO3	6	0.299	0.034	0.81
NQO4	4	0.368	0.039	0.99
NQO5	5	0.292	0.026	0.79
NQO6	6	0.378	0.022	1.02

[a] *n*, number of determinations. [b] SD, standard deviation of the mean.

that these subunits exist as a single copy, suggesting that the *P. denitrificans* NDH-1 is identical to the bovine complex I in terms of subunit stoichiometry of the "catalytic sector" (composed of bovine homologues of the NQO1 through -5 subunits). Therefore, it is conceivable that the PSST (20-kDa) subunit, which is a bovine counterpart of the NQO6 subunit, is present in complex I as a single copy. The proton-translocating NADH-Q oxidoreductases are believed to be present in situ as monomers (Weiss et al., 1991; Walker, 1992). Therefore, the *P. denitrificans* NDH-1 probably contains one mole each of the NQO1 through -6 subunits. According to a series of the expression experiments of the iron−sulfur subunits of the *P. denitrificans* NDH-1, the [2Fe-2S] centers N1a and N1b appear to be ligated by the NQO2 and NQO3 subunits, respectively (Yano et al., 1994a, 1995). The [4Fe-4S] centers N3 and N4 are believed to be coordinated by the NQO1 and NQO3 subunits, respectively (Yano et al., 1995, 1996). Therefore, it is conceivable that centers N1a, N1b, N3, and N4 are present at 1 mol each per mol of the *P. denitrificans* NDH-1. If that is the case, it seems unlikely that the bacterial NDH-1 is a functional heterodimer as has been proposed for mitochondrial complex I (De Jong & Albracht, 1994).

DISCUSSION

The NDH-1 and complex I can both be divided into a water-soluble fraction (peripheral fraction for NDH-1 and FP + IP fractions for complex I) and a water-insoluble fraction (membrane-bound fraction for NDH-1 and HP fraction for complex I) (Hatefi et al., 1985; Hatefi, 1985) just as the ATP synthase complex from *E. coli* can be divided into a water-soluble F_1 and a hydrophobic F_0 segment (Fillingame, 1990). The peripheral fraction of the *P. denitrificans* NDH-1, like the F_1 segment of the ATP synthase, is believed to be completely extramembranous. This is supported by the deduced primary structures of the *P. denitrificans* NDH-1 peripheral subunits (NQO1 through -6 and -9) which indicate that these subunits lack the hydrophobic transmembrane spans generally found in membrane bound proteins. Further evidence is provided by data from Finel et al. (1992, 1994) which illustrate that, although the soluble fraction (so-called complex Iλ) isolated from bovine heart complex I houses FMN and all the EPR-visible iron−sulfur clusters, it lacks the seven ND products which constitute a part of the membrane bound fraction of complex I. As shown in this paper, the NQO4, -5, and -6 subunits are present in the peripheral part of the NDH-1. Furthermore, the NQO1, -2, -3, and -9 subunits are also located in the peripheral segment on the basis of the extraction experiments of these subunits from the *P. denitrificans* membranes with chaotropes or at alkaline pH (T. Yano, and T. Yagi, unpublished results). These indicate that all putative cofactor

binding subunits are also present in the peripheral sector of the *P. denitrificans* NDH-1. Thus, the two structurally distinct domains of NDH-1/complex I might represent separate functional roles as is the case for the ATP synthase where the F_1 sector is responsible for the catalytic (scalar) reaction and the F_0 sector is more involved in proton translocation (vectorial reaction) (Belogrudov & Hatefi, 1994). As a further point of comparison between the two systems, DCCD, which is a specific inhibitor of the proton translocation of ATP synthase, is also believed to inhibit the proton translocation of complex I and NDH-1 (Yagi, 1987; Yagi & Hatefi, 1988; Hassinen & Vuokila, 1993; Vuokila & Hassinen, 1988, 1989). This inhibition is correlated with modification of the hydrophobic ND1 subunit (a homologue of the NQO8 subunit) by DCCD (Yagi, 1987; Yagi & Hatefi, 1988). Since the water-soluble fractions of both NDH-1 and complex I appear to be not only extramembranous but also house all the necessary cofactors for catalysis, it may be conceivable that the analogy to the ATP synthase is extended such that the peripheral segment of NDH-1 would catalyze the scalar reaction of the enzyme and the membrane-bound segment would provide a proton channel through the membrane (Yagi, 1987; Belogrudov & Hatefi, 1994). Demonstration of the validity of the proposed analogy between the NDH-1/complex I and the ATP synthase clearly requires an in-depth understanding of structure of the NDH-1/complex I.

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