H⁺-translocating NADH-Quinone Oxidoreductase (NDH-1) of Paracoccus denitrificans

STUDIES ON TOPOLOGY AND STOICHIOMETRY OF THE PERIPHERAL SUBUNITS

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Takahiro Yano‡ and Takao Yagi§

From the Division of Biochemistry, Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, California 92037

The proton-translocating NADH-quinone oxidoreductase (NDH-1) of Paracoccus denitrificans is composed of at least 14 subunits (NQO1−14) and is located in the cytoplasmic membrane. In the present study, topological properties and stoichiometry of the 7 subunits (NQO1–6 and NQO9) of the P. denitrificans NDH-1 in the membranes were investigated using immunological techniques. Treatments with chaotropic reagents (urea, NaI, or NaBr) or with alkaline buffer (pH 10–12) resulted in partial or complete extraction of all the subunits from the membranes. Of interest is that when NaBr or urea were used, the NQO6 and NQO9 subunits remained in the membranes, whereas the other subunits were completely extracted, suggesting their direct association with the membrane part of the enzyme complex. Both deletion study and homologous expression study of the NQO9 subunit provided a clue that its hydrophobic N-terminal stretch plays an important role in such an association. In light of this observation and others, topological properties of the subunits in the NDH-1 enzyme complex are discussed. In addition, determination of stoichiometry of the peripheral subunits of the P. denitrificans NDH-1 was completed by radioimmunological methods. All the peripheral subunits are present as one molecule each in the enzyme complex. These results estimated the total number of cofactors in the P. denitrificans NDH-1; the enzyme complex contains one molecule of FMN and up to eight iron-sulfur clusters, 2×[2Fe-2S] and 6×[4Fe-4S], provided that the NQO6 subunit bears one [4Fe-4S] cluster.

Paracoccus denitrificans is a Gram-negative soil bacterium and has been called “a free-living mitochondrion” (1, 2). P. denitrificans expresses a mammalian mitochondrial type respiratory chain that bears only the proton-translocating NADH-quinone oxidoreductase (NDH-1)2 NADH dehydrogenases (3, 4). The P. denitrificans NDH-1 is composed of at least 14 different subunits and bears one noncovalently bound FMN and at least five EPR-detectable iron-sulfur clusters as prosthetic groups (5, 6). These iron-sulfur clusters are designated cluster N1a and N1b (for [2Fe-2S] clusters) and N2, N3, and N4 (for [4Fe-4S] clusters) (6). The gene cluster encoding the P. denitrificans NDH-1 has been cloned and sequenced (7–11). The gene cluster is composed of 14 structural genes and 6 unidentified reading frames (12). These 14 structural genes have been designated nqo1 through nqo14. The NQO1 subunit contains the NDH-binding site (13), ligates cluster N3 (14), and probably bears an FMN (14). Based on the expression experiments from the putative cofactor-binding subunits, it has been suggested that the NQO2 subunit carries cluster N1a (15–17) and NQO3 subunit bears cluster N1b and N4, and probably another [4Fe-4S] cluster (18). The accompanying paper has revealed that NQO9 subunit ligates two [4Fe-4S] clusters. It is hypothesized that the NQO6 subunit may coordinate a [4Fe-4S] cluster (19). The NQO9 and NQO6 subunits are candidates for the cluster N2-binding subunit (20, 21).

Determination of subunit topology and subunit stoichiometry 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 these purposes (22, 23). Previously the topological properties of the NQO4, -5, and -6 subunits of the P. denitrificans NDH-1 have been investigated (24). The NQO4, -5, and -6 subunits in membrane-bound P. denitrificans NDH-1 were extracted by treatment at alkaline pH or with chaotropes. In addition to antibodies specific to the NQO1–6 subunits (24), the antibody directed to the NQO9 subunit is available in this laboratory as shown in the accompanying paper. Therefore, it is of interest to thoroughly investigate the localization of the 7 subunits, particularly that of the NQO9 subunit, in the P. denitrificans NDH-1 in situ. In the previous study (24), the subunit stoichiometry of the NQO1–6 of the membrane-bound P. denitrificans NDH-1 has be determined to be 1 mol each per mol of the enzyme complex using radioimmunological techniques. However, there is no such information available regarding the NQO9 subunit and its homologues of other organisms. Therefore, it is of interest to elucidate the stoichiometry of the NQO9 subunit of the P. denitrificans NDH-1 because this subunit contains 2×[4Fe-4S] clusters.

In this paper we describe subunit topology and the subunit stoichiometry of the P. denitrificans NDH-1. The NQO1–6 and 9 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. Meanwhile, we conducted the homologous expression of the NQO9 subunit of the P. denitrificans NDH-1. The N-terminal truncated and

‡ Present address: Dept. of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, PA 19104.
§ To whom correspondence should be addressed. E-mail: yagi@scripps.edu.

1 The abbreviations used are: NDH-1, bacterial proton-translocating NADH-quinone oxidoreductase; complex I, mitochondrial proton-translocating NADH-quinone oxidoreductase; DTT, dithiothreitol; FMN, flavin mononucleotide; PMSF, phenylmethanesulfonyl fluoride; PVD, polyvinylidene difluoride; BHI, brain heart infusion; CHES, 2-cyclohexylamino)ethanesulfonic acid.
full-length NQO9 subunits were expressed, respectively, in the cytoplasm and in the cytoplasmic membrane of P. denitrificans. Based on these results the location of the NQO9 subunit as well as NQO6 subunit is discussed in this paper. In addition, subunit stoichiometry of the NQO1,-2, -4, and -9 have been determined by radioimmunoassay. The results show that there is one copy each of the NQO1–6 and -9 subunits in the P. denitrificans NDH-1.

EXPERIMENTAL PROCEDURES

Preparation of the Sonicated and Cholate-treated P. denitrificans Membranes—P. denitrificans membrane fraction was prepared from cells grown on glucose by lysozyme osmosis method (3). The membranes were sonicated on ice with a Brownson sonifier attached to a narrow tip at an amplitude of 5 with 50% pulse mode for 3 min. The cholate-treated P. denitrificans membranes were prepared according to Ref. 24.

Expression of the NQO1, NQO2, NQO4, and NQO9 Subunits in Escherichia coli—The individual subunits were expressed in E. coli according to Ref. 24. pET11a(NQO1), pET11a(NQO2), pET11a(NQO4), and pET11a(NQO9), which produce full-length forms of the corresponding subunits, were used to transform E. coli strain BL21(DE3). A single colony was picked up from the plate and inoculated into 30 ml of 2YT medium containing 100 μg/ml ampicillin and was grown at 37 °C. When A600 reached 0.5, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.5 mM and the cells were cultured at 37 °C for 4 h. The cells were harvested in a GSA rotor at 6000 rpm for 10 min.

Preparation of Inclusion Body Fractions—Inclusion body fractions were prepared according to Ref. 24. The cells were suspended in 10 mM Tris-HCl buffer (pH 8.0) containing 0.3 M NaCl, 1.0 mM DTT, 1.0 mM EDTA, and 0.1 mM PMSF. The cell suspension was freeze-thawed twice with liquid nitrogen and twice with a 30 °C water bath. The resulting cell suspension was sonicated on ice with a Brownson sonifier attached to a narrow tip at an amplitude of 7 with 50% pulse mode for 10 min three times. The suspension was centrifuged, and the inclusion bodies were recovered. The inclusion bodies were washed with 50 mM Tris-HCl buffer (pH 8.0) containing 10% (w/v) sucrose, 1.0 mM DTT, 1.0 mM EDTA, and 0.1 mM PMSF by repeating homogenization and centrifugation three times. The inclusion bodies were further washed with 50 mM Tris-HCl buffer (pH 8.0) containing 2% (w/v) Triton X-100, 1.0 mM EDTA, and 0.1 mM PMSF in the same way. After these treatments, purity of the inclusion bodies was assessed to be more than 90% on the basis of SDS-PAGE analysis.

Purification of the Inclusion Bodies by Chromatography—The inclusion bodies obtained were solubilized in 6 M urea buffer containing 10 mM Tris-HCl (pH 8.0), 1.0 mM DTT, 1.0 mM EDTA, and 0.1 mM PMSF and stirred at 4 °C overnight. The protein solutions were cleared by centrifugation in an SS34 rotor at 12,000 rpm for 15 min, and the supernatants were recovered. The solubilized subunits were applied onto a DEAE-Gel column (3 × 10 cm) equilibrated with the same buffer. The column was extensively washed with the equilibration buffer, and the absorbed proteins were eluted with a linear gradient of NaCl (0–0.3 M) in the same buffer. Typically, the subunits were eluted as a single peak. The fractions were pooled, and the purity was checked by SDS-PAGE. Usually the subunits thus obtained were electrophoretically homogeneous. If further purification was needed, the following chromatography was carried out. The protein fractions were dialyzed against 1 liter of 6 M urea buffer containing 10 mM Tris-HCl (pH 8.0), 1.0 mM DTT, 1.0 mM EDTA, and 0.1 mM PMSF overnight. The solutions were applied onto a DEAE-Bio-Gel column (1.5 × 10 cm) equilibrated with the same buffer. The column was washed with the equilibration buffer, and the absorbed proteins were eluted by a linear gradient of NaCl (0–0.3 M) in the same buffer. The fractions were collected and used for the experiments.

Construction of the Plasmid of the Truncated Form of the NQO9 Subunit for Expression in P. denitrificans—For the expression in P. denitrificans a broad range host plasmid pEG400 was used (25). In order to express the nqo9 gene, we used a promoter region of cycA gene that encodes cyctochrome c550 (26). The promoter is known to be active in a regulative manner depending upon energy sources on which the cells grow (27). The promoter region (~240 base pairs) was amplified by polymerase chain reaction method with the following oligonucleotides: cycAF, 5'-GGA TCC TCT ATG AGC GAC AGG GGC CGT CC-3'; cycAR, 5'-CAT ACA TAT CAT CTT CAT CCG GAT TCC TC-3'. The forward primer, cycAF, was designed to have BamHI (GGATCC) and XbaI (TCTAGA) in addition to Sall (GTCGAC) at the 5' end for the convenience of DNA manipulation (underlined letters). Italic letters indicated altered sequences from the original. The reverse primer, cycAR, contains an NdeI site (CATATG, underlined letters) 6 base pairs downstream from the translation initiation codon keeping the first methionine and alanine in frame. Altered sequences were indicated in italic. In other words, a gene is expressed. under the cycA promoter of an amino acid sequence, Met-Ala, at its N-terminus. The polymerase chain reaction-amplified DNA segment was subcloned in pCR-script II (Stratagene), and its sequences were verified by sequencing of both strands. A clone containing the cycA promoter region in a particular direction was chosen and designated pCR(PycycA). The pCR(PycycA) was digested with Sall and BamHI and re-cloned into Sall and NdeI sites of pTZ18U and named pTZ18(PycycA). Meanwhile, one of the NQO9 plasmids such as pCR(NQO9Q1–3) was digested with NdeI and BamHI and the DNA fragments containing the nqo9 gene were ligated at EcoRI and PstI sites of pEG400.

In this way, three plasmids were constructed and designated pEG400(PycycA-NQO9 Δ1–30), pEG400(PycycA-NQO9 Δ1–30, Δ151–163), and pEG400(PycycA-NQO9 Δ1–30, Δ141–163) and used for expression study.

Expression of the Truncated Form of the NQO9 Subunit in P. denitrificans—For the expression of the Truncated Form of the NQO9 Subunit in P. denitrificans strain Pd1222 by conjugation through E. coli strain SM10 according to Ref. 26. The transformed P. denitrificans cells were selected on a brain heart infusion (BHI) plate containing 40 μg/ml rifampicin and 25 μg/ml streptomycin. A single and well isolated colony was picked up and spread onto a new plate containing the same antibiotics. The transformants were grown in liquid BHI medium to late exponential phase. The cell pellets were suspended in 10 mM Tris-HCl buffer (pH 8.0) containing 1.0 mM DTT, 1.0 mM EDTA, and 0.1 mM PMSF to be approximately 20% (w/v). The cell suspensions were freeze-thawed twice with liquid nitrogen and a 30 °C water bath and sonicated with a Brownson sonifier attached to a narrow tip at an amplitude of 5 with 50% pulse mode for 5 min. The sonicated suspensions were centrifuged at 10,000 rpm for 10 min in an SS34 rotor to separate from unbroken cells. The resultant cell-free extracts were then ultracentrifuged at 50,000 rpm for 60 min in 60Ti rotor. The supernatants were carefully recovered and subjected to Western analysis.

Quantitative Immunoblotting—Quantitative immunoblotting was carried out according to Hekman et al. (25). The amounts of bound antibodies were determined by scanning autoradiograms and expressed as a relative optical density by using a g-counter. The relative optical density was calculated as the ratio of the net optical density to the maximum optical density of the standards.

Other Analytical Procedures—Protein was estimated by the method of Lowry et al. (28) in the presence of 1 mg/ml potassium deoxycholate (29). SDS-polyacrylamide gel electrophoresis was carried out by the method of Laemmli (30). Amino acid composition analysis (13), electrophoretic analysis of PVDF membranes (31), and immunoblotting (32–34) were performed according to the references cited. Any variations from the procedures and other details are described in the figure legends.

RESULTS AND DISCUSSION

Localization of the Peripheral Subunits in the P. denitrificans Cytoplasmic Membrane—P. denitrificans was grown on glucose by lysozyme osmosis method (3). The membranes were sonicated on ice with a Brownson sonifier attached to a narrow tip at an amplitude of 5 with 50% pulse mode for 3 min. The cholate-treated P. denitrificans membranes were prepared according to Ref. 24. The cell suspensions were freeze-thawed twice with liquid nitrogen and a 30 °C water bath and sonicated with a Brownson sonifier attached to a narrow tip at an amplitude of 5 with 50% pulse mode for 5 min. The sonicated suspensions were centrifuged at 10,000 rpm for 10 min in an SS34 rotor to separate from unbroken cells. The resultant cell-free extracts were then ultracentrifuged at 50,000 rpm for 60 min in 60Ti rotor. The supernatants were carefully recovered and subjected to Western analysis.
NDH-1. Although function of the individual subunits is not yet fully understood, it seems likely that the electron transfer reaction from NADH takes place through these subunits since the redox components, FMN, and all iron-sulfur clusters reside in them. Of particular interest is the localization of the NQ06 and NQ09 subunits in NDH-1. One of these subunits is postulated to bear the cluster N2 that plays a key role in electron transfer to quinone. We conducted extraction experiments of the subunits with several chaotropic reagents or alkaline solutions and subunit-specific antibodies. Treatment with these reagents has been known to disrupt protein-protein interactions to some extent and to extract extrinsic proteins from membranes (35, 36). Although the structure of the enzyme complex at an atomic level is not yet available, information obtained in these experiments is still beneficial to understanding the rough structure of the enzyme complex. Some of the results have been reported previously concerning the NQ04, NQ05, and NQ06 subunits (24). When the membrane-embedded NDH-1 enzyme complex was treated with NaI, all the subunits were extracted into the supernatants (Fig. 1), whereas treatment with a high concentration of NaCl scarcely extracted the subunits. A noticeable observation is that the extent of extraction with NaBr varied among the subunits. The NQ01 and NQ02 were largely extracted, whereas the NQ03 and NQ04 were moderately extracted, and the release of the NQ05, NQ06, and NQ09 subunits from the membranes was slight. Moreover, when urea was used, the NQ06 and NQ09 remained in the membranes, whereas the other subunits were almost completely extracted. When the membranes were incubated in alkaline buffer, all subunits could be extracted from membranes to greater extents as pH increased from 10 to 12 (Fig. 1). These results demonstrate that all the subunits are extractable from the membranes, but the extraction of the NQ06 and NQ09 subunits is less effective than the other subunits. It can be speculated, therefore, that the NQ06 and NQ09 subunits are directly associated with the membrane part of the complex (NQ07, NQ08, and NQ010–14 subunits). Other groups have also expressed a similar view (37, 38). Of interest is that the topological character of the NQ09 subunit found in this study seems to be compatible with the results of expression studies reported in the accompanying paper and also see Ref. 18. The full-length form of NQ09 was expressed in the cytoplasmic membranes in *E. coli*, whereas the deletion of the hydrophobic N-terminal stretches made the subunit completely water-soluble. We attempted to express these truncated forms of the NQ09 subunit in *P. denitrificans* itself in order to see whether the same phenomena could be observed. We utilized a promoter region of the cyaA gene that encoded cytochrome *c*550. The *nqo9* genes encoding truncated subunits NQ09(D1–30), NQ09(D1–30, D151–163), and NQ09(D1–40, D151–163) were linked under a cyaA promoter on a broad host-range plasmid, pEG400, respectively. The constructs were transferred into *P. denitrificans* strain Pd1222 through *E. coli* strain SM10 by conjugation (see “Experimental Procedures”). Transformants were isolated on BHI plates containing streptomycin + rifampicin and were aerobically grown in BHI liquid medium. In order to avoid undesired recombination events, experiments were conducted with freshly transformed cells each time. Cytoplasmic fractions were prepared from individual transformants and subjected to Western blotting analysis. The truncated subunits could be readily distinguished from the native form of the NQ09 subunit by the difference in molecular size. It is clear in Fig. 2 that all truncated forms of the NQ09 subunit were expressed in cytoplasm of *P. denitrificans* in a manner similar to the expression in *E. coli* (see the accompanying paper). It is noteworthy that the hydrophobic N-terminal region of the NQ09 subunit and its homologues (corresponding to 30 amino acid residue long of the *P. denitrificans* NQ09 subunit) is predicted to form α-helix (data not shown). It can be speculated, therefore, that the hydrophobic N-terminal stretches of the NQ09 subunit may play an important role in its association with the membrane part of the enzyme complex in a manner similar to the Rieske iron-sulfur subunit of ubiquinol-cytochrome *c* oxidoreductase complex. Although the Rieske iron-sulfur subunit is extractable from membranes by the same treatment used in this study, its hydrophobic and less conserved N-terminal region has been found to lie in the membrane together with α-helical bundles of the cytochrome *b* subunit as depicted by x-ray structure (39, 40). The proteolytic cleavage or genetic deletion of the N-terminal stretch of the Rieske iron-sulfur cluster subunit of mitochondrial and bacterial ubiquinol-cytochrome *c* oxidoreductase complex have resulted in the recovery or expression of soluble forms of the iron-sulfur cluster domain (41). Further experiments are needed to examine this notion in the future. On the other hand,
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Stoichiometry of the Peripheral Subunits of the *P. denitrificans* NDH-1—It is important to determine the stoichiometric ratios of all the subunits constituting the NDH-1 enzyme complex since such information will provide the structural basis to clarify the mechanism of action of the enzyme complex. Particularly, determination of the copy number of the cofactor-binding subunits in the enzyme complex will make it possible to estimate the number of cofactors at a protein level. Many attempts have been made in the past to determine the number of cofactors in the mitochondrial complex I by analyzing the contents of FMN, non-heme iron, and acid-labile sulfide (42, 43). However, a clear answer has not yet been found mainly due to technical difficulties and some intrinsic problems associated with the materials used. In the previous study, the stoichiometric ratios of the *NDH-1* subunits of *P. denitrificans* NDH-1 have been determined by a radioimmunological method (24). This method is known to be reliable for estimating the quantity of particular polypeptides in crude preparations or multi-subunit enzyme complexes (23, 44). We employed the same approach to determine the stoichiometry of the *NDH-9* subunit in this study. Contents of the *NDH-9* subunit as well as *NDH-1*, *NDH-2*, *NDH-4*, and *NDH-9* subunits in the cholate-treated *P. denitrificans* cytoplasmic membranes were determined. The individual subunits were expressed in *E. coli* and the polypeptides were homogeneously purified and used as standard proteins (Fig. 3). In order to determine the correct stoichiometric ratio of the subunits in the enzyme complex, a painstaking effort was made to determine the protein concentrations as accurately as possible. Two methods were employed. The protein concentrations were determined for each standard subunit solution by Lowry’s method and corrected using values obtained by amino acid composition analysis. Known amounts of the individual standard subunits and *P. denitrificans* membranes were loaded on a Laemmli’s SDS-polyacrylamide gel, and the proteins were transferred onto PVDF membranes. The immobilized proteins were quantitated on the membranes by radioimmunoassay as described under “Experimental Procedures.”

Fig. 4 depicts one of the examples where the *NDH-9* subunits were detected by autoradiography after incubation with anti-*NDH-9* antiserum followed by 125I-protein A as described under “Experimental Procedures.”

where the stoichiometry of bound 125I to the amounts of loaded proteins gave reasonable linear relationships in ranges of 0–20 ng for the standard proteins and 0–1.0 µg for the *P. denitrificans* membranes. By comparing the slopes of the lines between the standard and experimental plots, the contents of the individual subunits in the membranes (nanomoles of subunit/mg of protein) were calculated. The stoichiometric ratios of the subunits relative to *NDH-1* were then obtained (Table I). The stoichiometric ratio of *NDH-9* subunit to *NDH-1* subunit was found to be 0.95, whereas those of the *NDH-2* and *NDH-4* subunits were 0.97 and 1.04, respectively. The results of the *NDH-2* and *NDH-4* subunits are in a good agreement with the previous report (24). Since it is believed that the mitochondrial complex I and bacterial NDH-1 are present as a monomer (20, 45, 46), it seems likely that these 7 peripheral subunits are all present as one molecule each in the enzyme complex (Table I). These results also allow us to estimate the total number of the cofactors, considering the fact that FMN and all iron-sulfur clusters are located in these subunits. It seems likely that the *P. denitrificans* NDH-1 enzyme complex contains one molecule of FMN and up to 8 iron-sulfur clusters, 2×[2Fe-2S] and 6×[4Fe-4S] (Table II). Mitochondrial complex I and *Rhodobacter capsulatus* NDH-1 may contain the same number of cofactors as *P. denitrificans* NDH-1 because they are predicted to have the same number of cofactor-binding sites (21, 46). In the case of *E. coli* and *Thermus thermophilus* NDH-1, the total number of iron-sulfur clusters can be 9 since they seem to contain an additional [2Fe-2S] cluster that is tentatively designated cluster N1 (also see Table II) (47, 48).
Conclusion—In this study, topology and stoichiometry of the 7 peripheral subunits of the *P. denitrificans* NDH-1 were investigated. It seems likely that the *P. denitrificans* NDH-1 enzyme complex contains one FMN and up to 8 iron-sulfur clusters. The extraction experiments have suggested that the NQO6 and NQO9 subunits are directly associated with the membrane part of the enzyme complex, constituting a junction between the peripheral and membrane portions as shown in Fig. 5. In this connection, it has been suggested that the hydrophobic N-terminal stretch of the NQO9 subunit plays a structurally important role. Recently, the NQO6 subunit and its mitochondrial counterpart, PSST subunit, have been identified as a conserved specific binding site for very hydrophobic complex I inhibitors such as pyridaben, rotenone, and piericidin A (49). Our results described here are also consistent with those findings with respect to the fact that the subunit is partially surrounded by a hydrophobic environment. Both NQO6 and NQO9 subunits appear to be important for the energy transduction at site 1. It is conceivable, therefore, that further characterization of these subunits will be a crucial step toward the elucidation of the mechanism of action of the enzyme complex.

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