Characterization of the Membrane Domain Nqo11 Subunit of the Proton-Translocating NADH-Quinone Oxidoreductase of *Paracoccus denitrificans*†

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ABSTRACT: The proton-translocating NADH-quinone oxidoreductase (NDH-1) of *Paracoccus denitrificans* consists of at least 14 unlike subunits (designated Nqo1−14). The NDH-1 is composed of two segments (the peripheral and membrane segments). The membrane domain segment appears to be made up of seven subunits (Nqo7, -8, -10−14). In this report, the characterization of the *Paracoccus* Nqo11 subunit has been investigated. An antibody against the C-terminal 12 amino acid residues of the *Paracoccus* Nqo11 subunit (Nqo11c) has been raised. The Nqo11c antibody reacted with a single band (11 kDa) of the *Paracoccus* membranes and cross-reacted with *Rhodobactor capsulatus* membranes. The Nqo11 subunit was not able to be extracted from the *Paracoccus* membranes by NaI or alkaline treatment, unlike the peripheral subunits (Nqo1 and Nqo6). The C-terminal region of the *Paracoccus* Nqo11 is exposed to the cytoplasmic phase. For further characterization of the *Paracoccus* Nqo11 subunit, the subunit was overexpressed in *Escherichia coli* by using the maltose-binding protein (MBP) fusion system. The MBP-fused Nqo11 subunit was expressed in the *E. coli* membranes (but not in soluble phase) and was extracted by Triton X-100. The isolated MBP-fused Nqo11 subunit interacted with the phospholipid vesicles and suppressed their membrane fluidity. Topological studies of the Nqo11 subunit expressed in *E. coli* membranes have been performed by using cysteine mapping and immunochemical analyses. The data suggest that the Nqo11 subunit has three transmembrane segments and its C-terminus protrudes into the cytoplasmic phase.

Paracoccus denitrificans is a Gram-negative soil bacterium with a respiratory chain highly similar to that of mammalian mitochondria when grown in aerobic conditions (*1*). This mitochondrial-type respiratory chain in *Paracoccus* contains only the proton-translocating NADH-quinone oxidoreductase (NDH-1)[1] as the NADH dehydrogenases (*2*). The *Paracoccus* NDH-1 is known as a multiple subunit enzyme complex which is composed of at least 14 unlike subunits, named Nqo1−14 (*3*, *4*), and bears 1 noncovalently bound FMN and 8 iron−sulfur clusters as cofactors (*2*). The membrane extraction studies suggest that the *Paracoccus* NDH-1 can be divided into two sectors, the peripheral segment and the membrane segment (*5*, *6*). The peripheral segment in the *Paracoccus* NDH-1 houses all putative cofactors (*2*, *7*, *8*), and is made up of seven subunits (Nqo1−6 and Nqo9 subunits) (*5*, *6*). Among these subunits, the Nqo6 and Nqo9 subunits are considered to act as connectors between the peripheral and the membrane segments (*2*, *6*, *8*).

The membrane segment of the *Paracoccus* NDH-1 also appears to consist of seven subunits (Nqo7, Nqo8, and Nqo10−14) (*9*, *10*), which are the homologues of the mitochondrially encoded subunits of the mammalian complex I (*11*, *12*). In contrast to the peripheral segment, this membrane-bound segment does not contain any known cofactor. Although the exact function of the membrane segment is still not clear, it is most likely involved in the proton translocation and Q-binding of the *Paracoccus* NDH-1 (*2*, *13*). To clarify the mechanism of proton translocation, the first step is to understand the structural properties of the individual subunits in the membrane segment (*2*). However, it is generally recognized that native isolation of the hydrophobic subunits from the enzyme complexes remains challenging. In addition, it seems difficult that ample materials for characterization will be accumulated by this strategy. Therefore, expression of individual subunits appears to be superior. In a previous study, we have reported to express the *Paracoccus* membrane domain Nqo7 subunit with the GST fusion system in *E. coli* (*14*). The success in expressing this subunit in the membrane fraction of host cells

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[1] Abbreviations: AIAS, 4-acetamido-4′-[(iodoacetyl)amino]stilbene-2,2′-disulfonic acid; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; complex I, mitochondrial proton-translocating NADH-quinone oxidoreductase; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; FMN, flavin mononucleotide; GST, glutathione-*S*-transferase; IPTG, isopropyl β-D-thiogalactopyranoside; ISO, inside-out; MBP, maltose-binding protein; NDH-1, bacterial proton-translocating NADH-quinone oxidoreductase; NEM, *N*-ethylmaleimide; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PMSF, phenylmethanesulfonyl fluoride; Q, quinone; RSO, right-side-out; SMP, submitochondrial particles; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TES buffer, 0.2 M Tris-HCl (pH 8.0) containing 5 mM EDTA and 0.5 M sucrose.

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          ---------I---------      ----------II----------          ---------
Pd  ~MIGLTHYLVVGAILFVTGIFGIFVNRKNVIVILMSIELMLLAVNINFVAFSTHLGDLAGQVFTMFVLTV
Rc  MTIGLEHYLAVAAILFVTGIFGIFVNRKNVIVILMSIELMLLAVNINMVAFSTHLGDLVGQVFTMFVLTV
Tt  ~~~~~MSYLLTSALLFALGVYGV.LTRRTAILVFLSIELMLNAANLSLVGFARAYG.LDGQVAALMVIAV
Ec  ~MIPLQHGLILAAILFVLGLTGLVI.RRNLLFMLIGLEIMINASALAFVVAGSYWGQTDGQVMYILAISL
Bt  ~~~~MSMVYMNIMMAFTVSLVGLLMYRSHLMSSLLCLEGMMLSLFVMAALTILNSHFTLASMMPIILLVF
                    *     *    *          * *

    --III----------
Pd  AAAEAAIGLAILVVFFRNRGTIAVEDVNVMKG
Rc  AAAEAAIGLAILVVFFRNRGTIAVEDVNVMKG
Tt  AAAEVAVGLGLIVAIFRHRESTAVDDLSELRG
Ec  AAAEASIGLALLLQLHRRRQNLNIDSVSEMRG
Bt  AACEAALGLSLLVMVSNTYGTDYVQNLNLLQC
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FIGURE 1: Comparison of the deduced primary structure of the *Paracoccus* Nqo11 subunit with its homologues from various organisms. The alignment was conducted using the PILEUP program of the GCG package (*29*). Pd, *Paracoccus denitrificans* (P29923) (*10*); Rc, *Rhodobacter capsulatus* (P50940) (*49*); Tt, *Thermus thermophilus* HB-8 (Q56226) (*23*); Bt, bovine heart mitochondrial complex I (NP_008103) (*50*); Ec, *Escherichia coli* K-12 (AAC75339) (*51*). Conserved amino acid residues are marked by asterisks. Predicted transmembrane helices of the *Paracoccus* Nqo11 subunit are indicated with Roman numerals above the sequences.

has proven that overexpression strategy may be useful for structural studies of other hydrophobic subunits. Therefore, to further explore other subunits in the membrane segment, we selected the *Paracoccus* Nqo11 (a counterpart of the mitochondrial ND4L subunit) to study its structural properties. It has been reported that some mutations of the mitochondrial ND4L subunit are possibly related to diseases (see http://www.gen.emory.edu/cgi-bin/MITOMAP). It has also been reported that deletion of the Nqo11 subunit in *Rhodobacter capsulatus* causes inactivation of NDH-1 (*15*). Other than that, our knowledge about properties of the ND4L subunit is limited. Hydropathy profile analyses of the deducted amino acid sequences of the *Paracoccus* Nqo11 subunit and its homologues reveal three stretches of hydrophobic residues that might constitute three transmembrane domains (Figure 1).

This paper describes partial characterization of the *Paracoccus* Nqo11 subunit. By using the MBP fusion system, the Nqo11 subunit has been successfully overexpressed in the membrane fraction of *E. coli*. The topology of the Nqo11 subunit in the *E. coli* membrane has also been determined by cysteine accessibility analyses and immunochemical methods. The data suggest that the N-terminus of the Nqo11 is located on the periplasmic side of the membrane whereas the C-terminus faces the cytoplasm. As a prediction, the Nqo11 subunit is composed of three transmembrane spans.

EXPERIMENTAL PROCEDURES

Materials. Anti-MBP serum, expression vector pMAL-p2G, amylose resin, and Genenase I were from New England Biolabs (Beverly, MA). The pCR-Script Amp Cloning kit was from Stratagene (La Jolla, CA). Materials for PCR product purification, gel extraction, and plasmid preparation were obtained from Qiagen (Valencia, CA). SM2 Bio-Beads were from Bio-Rad (Hercules, CA). *N*-Ethyl-[1,2-^{3}H]maleimide was from NEN (Boston, MA). 4-Acetamido-4′-[(iodoacetyl)amino]stilbene-2,2′-disulfonic acid sodium salt was from Molecular Probes (Eugene, OR). The BCA protein assay kit and SuperSignal West Pico chemiluminescent substrate were from Pierce (Rockford, IL).

Antibody Production. Two oligopeptides, H-MIGLTHYLVVGAC-OH (Nqo11n) and H-CTIAVEDVNVMKG-OH (Nqo11c), derived from the N- and C-terminal regions, respectively, of the Nqo11 subunit were conjugated to maleimide-activated bovine serum albumin (Pierce, Rockford, IL) as immunogens according to the manufacturer's protocol. Antibodies were raised in rabbits and were affinity-purified according to references (*16*−*18*).

Preparation of ISO and RSO Membrane Vesicles of Paracoccus and E. coli. French press treatment of *E. coli* cells mostly produces inside-out (ISO) membrane vesicles (*19*, *20*). We showed that the same method can be applied to *Paracoccus* cells (*14*). Therefore, ISO membrane vesicles were prepared as before (*14*) with some minor modifications as follows: the cells were suspended in TES buffer at approximately 15 mg/mL (wet weight) and passed through a French press at 15 000 psi. Unbroken cells and large cell debris were removed by repeated centrifugation. The supernatant was centrifuged at 50 000 rpm for 30 min in a 60Ti rotor. The resulting vesicles were then resuspended in 50 mM Tris-HCl (pH 8.0) containing 5 mM $MgCl_2$, 1 mM DTT, and 0.1 mM PMSF. For the preparation of RSO membrane vesicles (spheroplasts), an established method of lysozyme−EDTA treatment was used (*21*, *22*). To preserve the orientation and intactness of the membrane vesicles during incubation and washing steps, an isotonic solution [TES buffer containing 0.2 M Tris-HCl (pH 8.0), 0.5 M sucrose, and 0.5 mM EDTA] was employed.

Construction of the MBP-Fused Nqo11 Expression Vector. The *nqo11* gene used for expression and mutagenesis was constructed as follows. Two oligonucleotides, 5′-CCCG-GAGAATTCAGGACGATGATCGG-3′ and 5′-CCATG-CAAGCTTATCCCTTCATCACG-3′, where the underlined bases were altered from *Paracoccus* DNA for the mutations, were synthesized to generate a *Eco*RI site and a *Hin*dIII site near the initiation and the stop codon, respectively, of the *nqo11* gene. PCR amplification of the *nqo11* gene was conducted using the oligonucleotides with pXT-2b plasmid (*10*) as a template as described previously (*23*). The amplified DNA was subcloned into pCR-Script Amp SK(+), and its sequence was verified by sequencing. The plasmid thus obtained was named pCR(Nqo11). The pCR(Nqo11) was digested with *Eco*RI/*Hin*dIII, and the DNA fragment containing the *nqo11* gene was ligated in the *Eco*RI/*Hin*dIII sites of the pMAL-p2G vector. The resulting plasmid was designated pMAL-p2G(Nqo11) which encodes a fusion protein with the MBP moiety located at the N-terminus of Nqo11.

Table 1: Primers for Introduction of a Unique Cysteine Residue into MBP-Fused *Paracoccus* Nqo11 Subunit

mutation	mutagenic primer sequence[a]	codon change
I2C	5′-GGAGAATTCAGGACGATGT*G*CGGATTGACTCATTATCTTG-3′	ATC → TGC
F23C	5′-GGCATTTTCGGGATCTG*CGTGAACCGAAAGAAC-3′	TTC → TGC
N25C	5′-ATTTCGGGATCTTCGTCT*G*CCCGAAAGAACGTCATCG-3′	GTG → TGC
G55C	5′-TCGACCCATCTGT*GCGATCTGGCCGG-3′	GGC → TGC
I91C	5′-CAACCGCGGCACCT*G*CGCGGTGGAAGAC-3′	ATC → TGC

[a] Asterisks indicate mismatches.

Expression of the MBP-Fused Nqo11 Subunit. Derivatives of plasmid pMAL-p2G bearing wild-type *nqo11* or various *nqo11* mutations were transformed into the *E. coli* strain BLR(DE3)pLysS. A well-isolated colony selected from the LB agar plate containing 100 μg/mL ampicillin was inoculated into 5 mL of LB medium with 100 μg/mL ampicillin and incubated overnight at 37 °C. One milliliter of the above starter culture was then inoculated into 500 mL of TB medium supplemented with 0.2% glucose and 100 μg/mL ampicillin. Cells were grown at 37 °C with shaking to $A_{600} \cong 1.5$. IPTG was then added to a final concentration of 0.01 mM, and the culture was incubated for an additional 5 h at 20 °C with shaking at 250 rpm. The cells were then harvested by centrifugation and either stored at −80 °C or immediately used for preparation of membrane vesicles.

Purification of the MBP-Fused Nqo11 Subunit. The cell pellet was suspended (1 g of cells/10 mL) in 20 mM Tris-HCl (pH 8.0) containing 1 mM EDTA, 1 mM DTT, 0.3 M NaCl, and 0.1 mM PMSF (buffer A). The cells were disrupted by freeze−thawing twice, followed by passing through a French press at 15 000 psi. The resulting suspension was centrifuged in a Sorvall GSA rotor at 12 000 rpm for 20 min, and the supernatant was further ultracentrifuged at 50 000 rpm for 30 min in a Beckman Spinco 60Ti rotor to separate soluble and membrane fractions. The collected membrane fractions were resuspended to 10 mg/mL protein in buffer A containing 5% (v/v) Triton X-100. The suspension was placed in an ice−water bath and sonicated with a Branson sonifier attached to a narrow tip at an amplitude of 6 with 50% pulse for 5 min 3 times. After sonication, the sample was incubated on ice for 1 h and then ultracentrifuged at 50 000 rpm for 1 h in the 60Ti rotor. The supernatant was loaded onto an amylose column (3 mL of bed volume) equilibrated with buffer A containing 3% Triton X-100. After the column was washed with 20 column volumes of the column buffer, the MBP-fused *Paracoccus* Nqo11 subunit was eluted with 10 mM maltose, concentrated in an Amicon Centriprep-30, and stored at − 20 °C.

To cleave the fusion protein, the purified MBP-fused *Paracoccus* Nqo11 subunit (2 mg/mL) in buffer A containing 0.05% (w/v) SDS was treated with Genenase I at 0.04 mg/mL at room temperature for 48 h. The cleavage of the MBP-fused *Paracoccus* Nqo11 subunit was partial but not complete. Prolonged incubation with larger amounts of Genenase I did not yield more cleaved products. In contrast, when a water-soluble MBP-fused β-galactosidase was treated with Genenase I, only 2 h incubation cleaved more than 90% of the fused protein into MBP and β-galactosidase domains.

Reconstitution of the MBP-Fused Nqo11 Subunit. The reconstitution procedure was performed as described previously (*14*) with some modifications. Briefly, phospholipid vesicles were first prepared by mixing a chloroform/methanol (2:1) solution of phosphatidylcholine (type XVI-E) with 1-(4-trimethylammonium-phenyl)-6-phenyl-1,3,5-hexatriene in a 200:1 molar ratio. The solvent was evaporated under argon flux, and the lipid film was then hydrated in 10 mM Tris-HCl (pH 8.0) to a phospholipid concentration of 10 mg/mL with agitation. The resultant suspension was sonicated on ice with 50% pulse mode for 45 min, followed by the addition of dodecyl maltoside until its turbidity disappeared (approximately 2.25 mol of detergent/mol of phospholipid). Aliquots of the purified MBP-fused Nqo11 subunit were added to the detergent−phospholipid suspension to give the desired protein-to-lipid ratio, and the resultant mixture was incubated at room temperature for 1 h. To remove the detergent, the suspension was mixed with SM2 Bio-Beads (10 mg/mg of phospholipid) by the method of Holloway (*24*), and incubated at room temperature with gentle agitation for 2 h. The SM2 beads were then removed by centrifugation. The phospholipid suspension was dialyzed against 10 mM Tris-HCl (pH 8.0) overnight. The reconstituted MBP-fused Nqo11 phospholipid vesicles were subjected to the fluorescence anisotropy analyses according to references (*25*) and (*26*).

Cysteine Scanning Mutagenesis. Single cysteine substitution mutations were introduced into the Nqo11 subunit using the GeneEditor (Promega) according to the manufacturer's instructions. Starting with pCR(Nqo11) as a template, a synthetic oligonucleotide listed in Table 1, which contained the desired codon change, could replace a specific amino acid residue with cysteine. The single cysteine mutations were verified by DNA sequencing. The mutated *nqo11* genes were ligated to pMAL-p2G vector.

Chemical Labeling and Blocking. ISO and RSO membrane vesicles were collected by centrifugation, washed with the labeling buffer containing 50 mM Tris-HCl, pH 8.0, and 5 mM $MgCl_2$ (for RSO membrane vesicles, 0.5 M sucrose was also included), and resuspended in the same buffer to give a final protein concentration of 15 mg/mL. Approximately 6 mg of membrane protein was used for the labeling reaction. Six microliters of the membrane-impermeable SH modifier, AIAS (40 mM), or the same volume of distilled water was added to the membrane vesicles, followed by incubation at room temperature for 2 h. Subsequently, the mixture was treated with 3 μL of 1 mCi/mL [^{3}H]NEM and incubated at room temperature for 2 h. After [^{3}H]NEM labeling, membrane vesicles were solubilized with 2% SDS for 2 h, and then immunoprecipitated with anti-Nqo11c antiserum according to a protocol previously described (*27*, *28*). The immunoprecipitate was suspended in 60 μL of 80 mM Tris-HCl (pH 6.8) containing 6% SDS and 20% glycerol and incubated for 2 h at room temperature. The sample was mixed with 5 mL of scintillation cocktail and subjected to radioactive analyses.

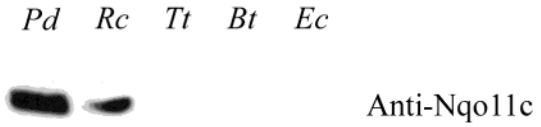

FIGURE 2: Cross-immunoreactivity of the *Paracoccus* membrane (*Pd*), *R. capsulatus* chromatophores (*Rc*), *T. thermophilus* HB-8 membranes (*Tt*), bovine heart SMP (*Bt*), and *E. coli* membranes (*Ec*) with affinity-purified antibodies directed against the *Paracoccus* Nqo11c. Ten micrograms each of the *Paracoccus* membranes, *R. capsulatus* chromatophores, *T. thermophilus* HB-8 membranes, bovine heart SMP, and *E. coli* membranes was subjected to SDS−PAGE (15% polyacrylamide gel). Immunoblotting was performed with the SuperSignal West Pico system (Pierce).

Sequence Analysis. The GCG software package was used to analyze the amino acid sequence (*29*). Sequence comparison of the polypeptides was conducted with the BESTFIT and PILEUP programs. Homology search was carried out using the BLAST program running at the National Center for Biotechnology Information (*30*). Transmembrane prediction was carried out using web servers TMHMM, TMpred, TopPred2, and HMMTOP2.

Other Analytical Procedures. Protein concentrations were determined by the BCA protein assay kit (Pierce) according to the manufacturer's protocol. SDS−PAGE was performed according to Laemmli (*31*). Membrane, cytoplasmic, and inclusion body fractions were prepared as described previously (*8*). Protein samples were incubated at room temperature for 1 h before being loaded onto the gel. Any variations from the procedures and other detail are described in the figure legends.

RESULTS

Antibodies against the Paracoccus Nqo11 Subunit. To use as a tool to probe the Nqo11 subunit in the NDH-1/complex I in situ, we attempted to raise antibodies to this subunit using oligopeptides from the N- and C-terminus (designated Nqo11n and Nqo11c, respectively). Of these, only the antibody against Nqo11c was successfully produced. The Nqo11c antibody reacted with a single band at approximately 11 kDa in the *Paracoccus* membranes (Figure 2). The molecular mass agrees with the MW = 10 856 deduced from its primary structure. Furthermore, the antibody against the *Paracoccus* Nqo11c cross-reacted with a 11 kDa band in the *Rhodobacter capsulatus* membranes but did not react with *T. thermophilus* HB-8 membranes, *E. coli* membranes, or bovine heart SMP. These results are consistent with the fact that the Nqo11 homologue of *R. capsulatus* has a molecular size (MW = 10 983) similar to the *Paracoccus* Nqo11 and exhibits 100% sequence identity with the *Paracoccus* Nqo11 in the C-terminal 43 residues. In contrast, the sequence identity of the C-terminal 12 residues, between *Paracoccus* and *Thermus*, bovine, and *E. coli* is much smaller at 33%, 25%, and 25%, respectively (see Figure 1).

Characterization of the Paracoccus Nqo11 subunit. As reported earlier (*5*, *6*), the peripheral subunits of the *Paracoccus* NDH-1 (Nqo1−6 and -9) can be extracted from the membranes by a chaotropic agent such as NaI or by incubation at high pH, whereas membrane subunits resist these treatments. Figure 3 illustrates the results of such

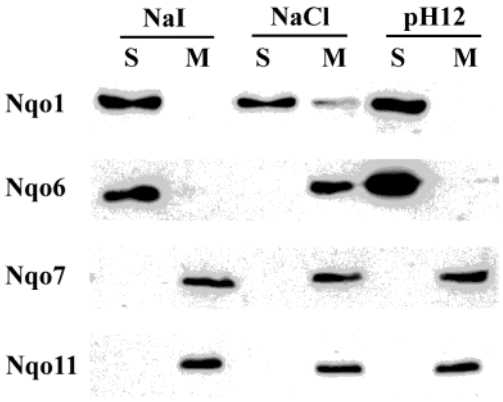

FIGURE 3: Effects of chaotropic reagents and alkaline buffer on extraction of the Nqo1, Nqo6, Nqo7, and Nqo11 subunits from the cholate-treated *P. denitrificans* membranes. For chaotrope treatment, 120 μL of the cholate-treated membrane suspension (1.0 mg/mL) was incubated for 10 min at 30 °C in the presence of 1.5 M NaI or NaCl. For alkaline treatment, the same amount of the cholate-treated membranes was suspended in 100 mM 2-(*N*-cyclohexylamino)-ethanesulfonic acid buffer (pH 12) to a final concentration of 1 mg/mL. The membrane suspension was then freeze−thawed twice using liquid nitrogen and a water bath at 30 °C followed by centrifugation in an Airfuge at 30 psi for 10 min. Ninety microliters of the supernatant was carefully transferred into microtubes and used as the supernatant fraction (S). After the remaining supernatant was completely removed by aspiration, the pellet was resuspended in 120 μL of 10 mM Tris−acetate buffer (pH 7.5). This suspension was used as the membrane fraction (M). The supernatant (S) and membrane suspensions (M) were mixed with equal volumes of 2 × Laemmli's sample buffer. Seven microliters of each sample was loaded on Laemmli SDS−13% polyacrylamide gels. Immunoblotting was carried out with affinity-purified antibodies against Nqo1, Nqo6, Nqo7c, and Nqo11c as described in Figure 2.

treatments of the *Paracoccus* membranes. Nqo1 (the NADH-binding subunit) and Nqo6 (the connector subunit) represent the peripheral subunits and were indeed extracted from the membranes. The *Paracoccus* Nqo1 subunit was partially extracted by NaCl. In contrast, subunits Nqo11 and Nqo7 remained in the membranes under the same conditions. The data confirmed that Nqo11 and Nqo7 subunits belong to the intrinsic membrane segment of the *Paracoccus* NDH-1.

As a first step to study the membrane domain subunit, we investigated to determine its topology. For this purpose, the inside-out (ISO) and right-side-out (RSO) membrane vesicles were prepared as described under Experimental Procedures. Based on the results of previous studies (*14*), the Nqo1 (NADH-binding) subunit and the C-terminal region of the Nqo7 subunit (Nqo7c) of the *Paracoccus* NDH-1 were chosen as markers of the cytoplasmic and periplasmic sides of the membranes, respectively. As shown in Figure 4, the anti-Nqo1 antibody reacted with ISO membrane vesicles but did not react with the RSO membrane vesicles. The anti-Nqo7c antibody recognized the RSO membrane vesicles but did not recognize the ISO membrane vesicles. These results signify the correct sidedness of the prepared membrane vesicles. The anti-Nqo11c antibody reacted with the ISO, but not the ROS, membrane vesicles, indicating that the C-terminus of the Nqo11 subunit is located on the cytoplasmic side. In addition, since the antibody was able to interact with the C-terminal region of the Nqo11 subunit in the native *Paracoccus* membranes, it is conceivable that the C-terminal region is freely accessible and is not covered by other subunits.

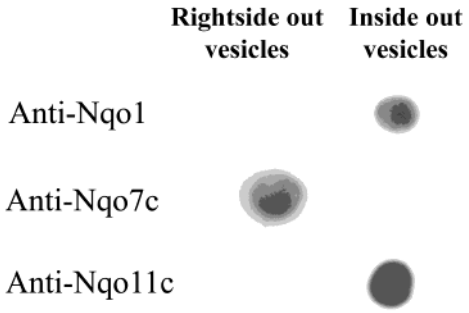

FIGURE 4: Localization of the C-terminal region of the Nqo11 subunit in the membranes of *P. denitrificans*. Aliquots (100 μL) of RSO and ISO membrane vesicles from *Paracoccus* were bound directly to nitrocellulose membranes by incubation of the membranes with the membrane vesicles in a DOT Blot apparatus for 2 h at room temperature. The wells were washed 3 times with TES buffer and incubated with 2% skim milk in PBS buffer for 1 h to block nonspecific binding sites. The nitrocellulose membranes were then thoroughly washed and incubated with the affinity-purified primary antibodies followed by detection of antibodies as described previously (*5*, *6*). The primary antibodies employed included antibodies directed against the Nqo1 (positive control for RSO membrane vesicles), the Nqo7c (positive control for ISO membrane vesicles), and the Nqo11c.

Expression of the Paracoccus Nqo11 Subunit. To conduct further characterization of the *Paracoccus* Nqo11 subunit, native overexpression of this subunit was indispensable. A few techniques have been developed to facilitate overexpression of membrane proteins in correct folding (*32*). One such example, which is fairly commonly used, is the GST-fused expression system. However, the use of the GST expression system for the *Paracoccus* Nqo11 subunit was not suitable, because the fusion protein was exclusively accumulated in inclusion bodies and could not be recovered as a soluble, renatured protein. Recently, a maltose-binding protein (MBP) fusion system has also been developed for expression of membrane proteins (*33*, *34*). Because it can be made either secretable or nonsecretable (without signal sequence), the system is potentially useful for assigning the correct location of the N-terminus of a target protein. We constructed both secretable and nonsecretable MBP-fused *Paracoccus* Nqo11 expression plasmid and investigated their heterologous expression. The expression of nonsecretable MBP-fused Nqo11 subunit was apparently unsuccessful; the antibody directed against Nqo11c did not react with host cells, and the antibody against MBP mainly recognized one band (43 kDa) which is consistent with the molecular size of MBP. In contrast, use of the secretable MBP-fused expression system gave rise to successful expression of the Nqo11 subunit. As shown in Figure 5A, the MBP-fused Nqo7 subunit was expressed both in the membrane fraction and in the inclusion body fraction. The optimal procedure for expression of MBP-fused Nqo11 subunit in the membranes is detailed under Experimental Procedures and was employed for the entire study. As in the case of the GST-fused Nqo11 subunit, the MBP-fused Nqo11 subunit accumulated in the inclusion bodies could not be recovered in a native form. On the other hand, the MBP-fused Nqo11 subunit expressed in the membrane fraction was extracted with Triton X-100. After extraction, the MBP-fused Nqo11 subunit was purified by amylose affinity chromatography. The identity and purity of the isolated material were verified by SDS−PAGE (Figure 5B, lane 5). The purified MBP-fused

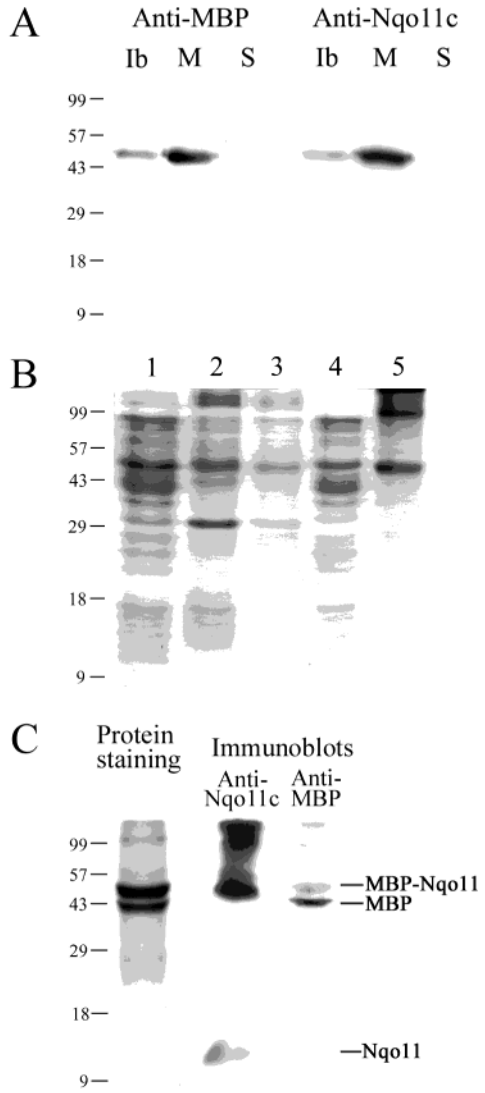

FIGURE 5: (A) Immunoblotting of the inclusion body (Ib), membrane (M), and soluble (S) fractions of *E. coli* bearing the pMAL-p2G(Nqo11) expression vector. Left panel, anti-MBP serum; right panel, affinity-purified anti-Nqo11c antibodies. Ten micrograms of protein was applied to each lane of a 15% Laemmli gel. The electrophoresis and electronic transfer were carried out according to references (*16*−*18*). Immunoblotting was performed with the SuperSignal West Pico system (Pierce). (B) SDS−polyacrylamide gel pattern of the MBP-fused *Paracoccus* Nqo11 subunit expressed in *E. coli*. Lane 1, cell lysate of *E. coli* expressing MBP-fused *Paracoccus* Nqo11 subunit; lane 2, inclusion body fraction; lane 3, membrane fraction; lane 4, supernatant fraction; lane 5, MBP-fused Nqo11 subunit isolated by amylose affinity column chromatography. The numbers on the left side indicate the molecular mass (kDa) of the marker proteins. Ten micrograms of protein was loaded per lane on a 15% Laemmli SDS gel. After electrophoresis, the gel was stained with GelCode blue. (C) Identification of the cleaved fragments derived from the Genenase I-treated MBP-fused Nqo11 subunit by use of affinity-purified anti-*Paracoccus* Nqo11c antibody and anti-MBP serum. Left panel, SDS gel pattern with GelCode blue staining; center panel, immunoblotting with affinity-purified anti-Nqo11c antibodies; right panel, immunoblotting with anti-MBP serum. Aliquots (50 μg, left panel; 10 μg, center and right panels) of the protein were loaded on a Laemmli 15% SDS gel. The electrophoresis and immunoblotting were performed as in panel A.

Nqo11 subunit migrated with M_r = 53 000, which reasonably agreed with the molecular size of this fusion product (MW = 54 000) deduced from the primary structure. A minor band at approximately 100 kDa was also recognized by the

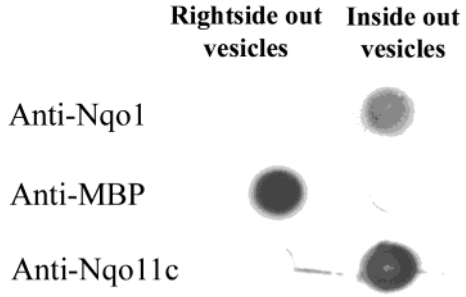

FIGURE 6: Localization of the C- and N-terminal regions of the MBP-fused Nqo11 subunit expressed in the *E. coli* membranes. Aliquots (100 μL) of RSO and ISO membrane vesicles from *E. coli* were bound directly to nitrocellulose membranes by incubation of the membranes with the membrane vesicles in a DOT Blot apparatus for 2 h at room temperature. Dot-blotting has been performed as described in Figure 4. Primary antibodies employed included antibodies directed against the *E. coli* Nqo1 homologue, the Nqo11c, and MBP.

antibodies against MBP and the Nqo11c (data not shown). It might be a dimer of the MBP-fused Nqo11 subunit.

The purified MBP-fused Nqo11 subunit was treated with Genenase I to separate the fusion protein into its constituent parts, MBP and Nqo11. As seen in Figure 5C, when the partially cleaved sample was subjected to SDS−PAGE, two new bands appeared. The lower band (11 kDa) was diffuse and was poorly stained with Coomassie Brilliant Blue or GelCode blue, indicative of a hydrophobic protein. This band reacted with the Nqo11c antibody. The upper band (42 kDa) reacted only with antibodies to MBP. These results indicate that the MBP-fused *Paracoccus* Nqo11 subunit was correctly expressed in *E. coli*.

The fact that the isolated MBP-fused *Paracoccus* Nqo11 subunit binds to the amylose column suggests that the intactness of the MBP domain is conserved in the fused protein. It is of interest to examine whether the *Paracoccus* Nqo11 subunit domain is also intact in the MBP-fused Nqo11 subunit. To pursue this objective, the effect of MBP-fused Nqo11 subunit on the membrane fluidity of phospholipid vesicles was examined by using fluorescence anisotropy. The results indicated that the Nqo11 domain of the MBP-fused Nqo11 subunit interacted with the phospholipid vesicles and suppressed their membrane fluidity, while the MBP-β-galactosidase, being a water-soluble polypeptide, did not interact with phospholipid vesicles (data not shown). Together with the result that the MBP-fused Nqo11 subunit could be partially cleaved into its MBP and Nqo11 parts, it is likely that the MBP and Nqo11 domains of the MBP-fused Nqo11 subunit behave independently.

Topographical Studies of the MBP-Fused Paracoccus Nqo11 Subunit. To assess the topology of the Nqo11 subunit expressed in *E. coli*, the ISO and RSO vesicles were prepared from *E. coli* membranes by the established procedure (*19*, *21*). As seen in Figure 6, the antibody to the *E. coli* homologue of the Nqo1 subunit reacted with the ISO, but not RSO, vesicles, confirming that the sidedness of the *E. coli* membrane vesicles is correctly prepared. The antibody against MBP reacted with the RSO vesicles but did not react with the ISO vesicles, confirming the presence of MBP on the periplasmic side of the *E. coli* membranes. In contrast, the anti-Nqo11c antibody reacted only with the ISO vesicles, not with the RSO vesicles. These results indicate that the location of the C-terminus of the MBP-fused Nqo11 in *E. coli* membranes is consistent with that in the *Paracoccus* membranes. It seems likely that the MBP-fused *Paracoccus* Nqo11 expressed in *E. coli* is similar to the Nqo11 subunit in the native *Paracoccus* membranes in terms of topology. The *E. coli* membranes expressing the MBP-fused Nqo11 made further topological studies possible as described below.

Hydropathy plots of the deduced primary structure of the Nqo11 subunit give three distinct transmembrane segments as marked in Figure 1. Furthermore, when the deduced Nqo11 sequence was analyzed by available software programs developed for prediction of the orientation of membrane proteins [TMHMM (*35*), TMpred (*36*), TopPred2 (*37*), and HMMTOP2 (*38*)],[2] all dictated that the N-terminus and a connecting segment between transmembrane segment 2 (TM2) and TM3 are "outside" and the C-terminus and a connecting segment between TM1 and TM2 are "inside". A few approaches have been applied to resolve the issues of how many transmembrane segments and how they are oriented. Vik's group and Fillingame's group have reported that the substituted cysteine scanning analyses are superior to other approaches [including the alkaline phosphatase (*PhoA*) fusion procedure (*39*)] in their topological studies of subunit A in ATP synthase (*19*, *20*, *40*). Since the *Paracoccus* Nqo11 subunit or MBP does not contain any cysteine residue, we could readily generate a collection of Nqo11 subunit mutants, each having a single unique cysteine residue, and express them in *E. coli*. A unique cysteine residue was introduced into the MBP-fused *Paracoccus* Nqo11 subunit at positions 2, 23, 25, 55, and 91 (the *Paracoccus* Nqo11 numbering). I2 and I91 are presumably located in the N- and C-terminal regions, respectively. Likewise, F23 and N25 are predicted to be in a loop between TM1 and TM2 (designated loop 1−2) and G55 in a loop between TM2 and TM3 (designated loop 2−3). These mutants grew as well as wild type. The expression level of mutated MBP-fused *Paracoccus* Nqo11 subunit is not different from that of the wild type.

To determine the surface accessibility of mutated MBP-fused Nqo11 subunit, the ISO and RSO *E. coli* membrane vesicles were treated with or without the membrane-impermeable SH modifier AIAS and labeled with [^{3}H]NEM. The labeled subunit was immunoprecipitated by anti-Nqo11c antiserum and was subjected to radioactive analyses. Table 2 illustrates the results of radiolabeling experiments and the ratio of radioactivity between the absence and the presence of AIAS (designated R_r for the RSO vesicles and R_i for the ISO vesicles). That is because the protection level of NEM-labeling by AIAS is the most critical factor to access whether the introduced cysteine residues are exposed to the periplasmic or cytoplasmic phase. As expected, the wild-type MBP-fused Nqo11 subunit showed low susceptibility in both the ISO and RSO vesicles. The mutants F23C and N25C were preferably labeled in the ISO vesicles, suggesting that F23 and N25 face the cytoplasmic phase. In contrast, the mutant G55C was preferably labeled in the RSO vesicles, implying

[2] Prediction methods are based on local properties of amino acid sequences (TMpred), global approaches which determine the statistically most probable topology for the whole protein (HMMTOP and TMHMM), or combined forms in which results on a local level are evaluated by global heuristics such as the positive-inside rule (Toppred2).

Table 2: Effect of the Membrane-Impermeable SH Modifier AIAS on [^{3}H]NEM-Labeling in the MBP-Fused *Paracoccus* Nqo11 Mutants

	right-side-out vesicle			inside-out vesicle			
mutation	+AIAS (cpm)[a]	−AIAS (cpm)[a]	R_r[b]	+AIAS (cpm)[a]	−AIAS (cpm)[a]	R_i[b]	location
wild type	53	78	1.5	80	115	1.4	−
I2C	46	79	1.7	77	129	1.7	−
F23C	46	52	1.1	82	215	2.6	C[c]
N25C	53	62	1.2	87	253	2.9	C
G55C	71	161	2.3	144	164	1.1	P
I91C	72	84	1.2	215	752	3.5	C

[a] Values are the labeling collected from ^{3}H radioactivity counting. [b] Values are derived by dividing the ^{3}H radioactivity counting from the samples with AIAS preincubation by the corresponding value from the samples without AIAS preincubation. R_r is the value for right-side-out membrane vesicles. R_i is the value for inside-out membrane vesicles. [c] C, cytoplasmic location; P, periplasmic location; −, no location assigned.

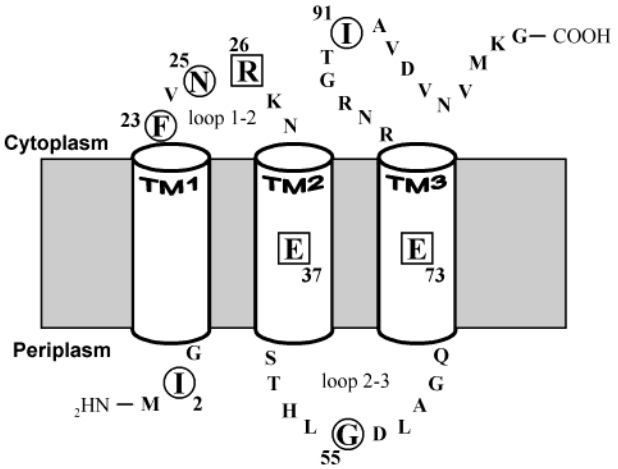

FIGURE 7: Topological model of the *Paracocus* Nqo11 subunit. Three transmembrane segments of the *Paracoccus* Nqo11 subunit from the N-terminus to the C-terminus are tentatively designated TM1, TM2, and TM3, respectively. The N-terminus and C-terminus of the subunit are exposed to the periplasmic and the cytoplasmic side of the membrane, respectively. The residues replaced by a cysteine are depicted in large boldface letters enclosed in circles with numbers representing the positions. Charged residues conserved among the homologues of *Paracoccus* Nqo11 subunit in the transmembrane segments are shown in squares.

that G55 is exposed to the periplasmic phase. The I91C mutant was more significantly labeled in the ISO vesicles than in the RSO vesicles, in good agreement with the immunochemical results that the C-terminal region of the Nqo11 subunit is directed to the cytoplasmic space. Labeling of the I2C mutant was poor, giving almost the same numbers as the wild-type. The location of I2, therefore, could not be assigned to either phase. These results might be interpreted by at least two speculations. One is that I2 may be embedded in the membranes or is located too close to the membrane. The other is as follows. The poor labeling of the I2C mutant may be related to the results that digestion of the MBP-fused Nqo11 subunit by Genenase I was only partial. If so, it seems likely that the N-terminal region of the Nqo11 in the MBP-fused protein might be covered. Nevertheless, as described above, on the basis of the topological studies with anti-MBP antiserum, it is clear that the N-terminus of the MBP-fused subunit is directed to the periplasmic phase. Considering the available data together and assuming that the MBP-fused *Paracoccus* Nqo11 subunit in *E. coli* is akin to the Nqo11 subunit in *Paracoccus* with respect to orientation, the speculated topology of the *Paracoccus* Nqo11 subunit is illustrated in Figure 7. It should be noted that our picture of the *Paracoccus* Nqo11 subunit in Figure 7 is consistent with the results given by computer programs for prediction of transmembrane regions and orientation.

DISCUSSION

As described in the introduction, the membrane segment of the *Paracoccus* NDH-1 appears to contain seven subunits (Nqo7, -8, -10−14). To elucidate functional roles of the membrane segment, it is a prerequisite to understand the architecture of individual membrane domain subunits and the interaction between these subunits. In a previous paper (*14*), we reported that the *Paracoccus* Nqo7 (ND3 homologue) subunit has been successfully overexpressed in the membrane fraction of *E. coli* as a GST-fused protein. Furthermore, we provided evidence that the Nqo7 subunit directly interacts with the Nqo6 (PSST homologue) subunit by means of heterologous coexpression and cross-linking experiments (*41*). In this paper, we have shown that the *Paracoccus* Nqo11 subunit has also been successfully overexpressed in the membrane fraction of *E. coli* by using the secretable MBP fusion system. The expressed MBP-fused *Paracoccus* Nqo11 subunit appears to have retained the same native topology as in the *Paracoccus* membrane. These materials (including the antibody directed against the *Paracoccus* Nqo11c) will be useful not only for further characterization of the Nqo11 subunit by various chemical and physicochemical analyses but also for investigating in vitro/in vivo interactions of the Nqo11 with other subunits in the *Paracoccus* NDH-1.

Hydropathy profiles seem to indicate that the three membrane-spanning sections exist in all homologues of the *Paracoccus* Nqo11 subunit. However, primary sequences of these homologues are not well conserved among species. Amino acid sequence comparison from bacteria to human revealed a few well-conserved residues which include F15, G21, R26, E37, E73, and L78 (*Paracoccus* numbering). Of these, three amino acid residues, R26, E37, and E73, have a charged group. The proposed topology of the *Paracoccus* Nqo11 subunit places R26 in a short loop 1−2 facing the cytoplasmic phase. It is possible that the conserved R26 of the Nqo11 subunit interacts with a subunit(s) in the peripheral segment and is essential for structure and function of NDH-1/complex I (*42*). In this regard, there is a report on an arginine residue that is crucial to the function of complex I. In the MWFE subunit, a small membrane fraction subunit in mammalian complex I, a point mutation of R50 (hamster numbering) to K caused complete complex I deficiency (*43*). This R is well-conserved among species and is located outside the membrane. Concerning E37 and E73, these residues are predicted to be located in the middle of the transmembrane spans (TM2 and TM3, respectively). E37 is perfectly conserved among all homologues reported to date, whereas E73 is almost completely conserved except in some parasites where it is replaced by S. It is well-known that the DCCD-binding subunit of the ATP synthase houses one exclusively conserved carboxyl residue centered in its transmembrane helix and this carboxyl residue participates

in the proton translocation of the membrane sector of the ATP synthase (*44*, *45*). DCCD is also known to inhibit energy-coupling activity of complex I/NDH-1 (*46*−*48*). Therefore, it is of interest to search for conserved carboxyl residues possibly located in the transmembrane segments of the seven hydrophobic subunits (Nqo7, -8, -10−14). First, by using the TMpred and TMHMM software, all D and E predicted to be in transmembrane helices were identified. Then, they were checked against known sequences of homologues of their corresponding subunits. This left only eight conserved carboxyl residues that are predicted to be located in the transmembrane segments (assuming that the stoichiometry of these subunits is 1 mol each/mol of NDH-1). It may be hypothesized that these residues are candidates involved in proton translocation in NDH-1/complex I. Two of them belong to the Nqo11 subunit as determined in current work. The Nqo7 subunit contains another two as reported earlier (*14*). In other words, a good portion of conserved carboxyl groups are distributed in these two subunits despite the fact that they are significantly smaller than other hydrophobic subunits. Therefore, we may hypothesize that the small hydrophobic subunits may play functionally important roles in coupling site 1. These speculations should be tested by utilizing various approaches in the future.

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