Exploring the Membrane Domain of the Reduced Nicotinamide Adenine Dinucleotide−Quinone Oxidoreductase of *Paracoccus denitrificans*: Characterization of the NQO7 Subunit†

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ABSTRACT: The proton-translocating reduced nicotinamide adenine dinucleotide− (NADH−) quinone oxidoreductase (NDH-1) of *Paracoccus denitrificans* is composed of at least 14 different subunits (NQO1−14). In addition, this enzyme complex houses one flavin mononucleotide (FMN) and 7−8 iron−sulfur clusters as cofactors. The expression and partial characterization of the NQO7 subunit, one of the seven subunits that constitute the hydrophobic sector of the enzyme complex, have been performed and are reported here. Expression of the NQO7 subunit was achieved by use of the glutathione-S-transferase (GST) fusion system together with *Escherichia coli* strains BLR(DE3)pLysS and BL21(DE3)pLysS. The GST-fused NQO7 subunit was expressed in the membrane fraction of the host cells and was extracted from the membranes by nonionic detergents (Triton X-100, dodecyl maltoside). The extracted polypeptide was purified by glutathione affinity column chromatography and characterized. The isolated GST-fused NQO7 subunit (but not the GST alone) was determined to interact with phospholipid vesicles and suppress the membrane fluidity. Antibodies against both the N- and C-terminal regions of the deduced primary structure of the NQO7 subunit reacted with a single band (15 kDa) of the *Paracoccus* membranes. By use of immunochemical and cysteine residue modification techniques, the topology of the *Paracoccus* NQO7 subunit in the membranes has been examined. The data suggest that the *Paracoccus* NQO7 subunit contains three transmembrane segments and that its N- and C-terminal regions are directed toward the cytoplasmic and periplasmic phases of the membrane, respectively. The proposed topology of the GST-fused NQO7 subunit expressed in *E. coli* membranes is consistent with that of the NQO7 subunit in the *Paracoccus* membranes.

Paracoccus denitrificans is a Gram-negative soil bacterium that has been called "a free-living mitochondrion" (*1*−*3*). Aerobically grown *P. denitrificans* expresses a mammalian mitochondrial-type respiratory chain that is believed to bear only one NADH−dehydrogenase (*4*), namely, the proton-translocating NADH-quinone oxidoreductase (NDH-1).[1] The gene cluster encoding the *Paracoccus* NDH-1 is composed of 14 structural genes (designated *nqo1*−*14*) and six unidentified reading frames (URFs) (*5*−*9*). The *Paracoccus* NDH-1 enzyme complex is composed of at least 14 unlike subunits, designated NQO1−NQO14 (*10*, *11*), and bears one noncovalently bound FMN and 7−8 iron−sulfur clusters as prosthetic groups (*12*−*14*). It is generally accepted that mitochondrial complex I and bacterial NDH-1 can be divided into two sectors (*15*−*17*), the peripheral segment and the membrane segment (*18*, *19*). The peripheral segment in the bacterial NDH-1 consists of seven subunits, including the NQO1−6 and NQO9 subunits (*14*, *18*). Various expression and extraction studies reported previously suggest that the NQO6 and NQO9 subunits appear to act as connectors between the peripheral and the membrane segments (*14*, *20*, *21*). Available data also indicate that all cofactors of the *Paracoccus* NDH-1 are located in the peripheral subunits (*20*−*22*).

The membrane-bound segment of the *Paracoccus* NDH-1 also appears to be composed of the seven subunits (NQO7, 8, and 10−14) (*8*, *9*). However, in contrast to the peripheral

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[1] Abbreviations: Q, quinone; NDH-1, bacterial proton-translocating reduced nicotinamide adenine dinucleotide−quinone oxidoreductase; complex I, mitochondrial proton-translocating reduced nicotinamide adenine dinucleotide−quinone oxidoreductase; complex III, ubiquinol−cytochrome *c* oxidoreductase; SMP, submitochondrial particles; DTT, dithiothreitol; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; FMN, flavin mononucleotide; IPTG, isopropyl β-D-thiogalactopyranoside; PBS, phosphate-buffered saline; PMSF, phenylmethanesulfonyl fluoride; AIAS, 4-acetamido-4′-[(iodoacetyl)amino]stilbene-2,2′-disulfonic acid; PCR, polymerase chain reaction; NEM, *N*-ethylmaleimide; GST, glutathione-S-transferase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; TES buffer, 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-Cl) (pH 8.0) containing 0.5 M sucrose and 5 mM ethylenediaminetetraacetic acid.

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Pd  ~~~~~MEYLLQEYLPILVFLGMASALAIVLILAAAVIAVRNPDPEKVSAYECGFNAFDDARMKFDVRFYLVSILFIIFDL
Rc  MQDALTHGMLREYLPILVLLAMAIGLGLILIAAAAIIAYRNPDPEKVSAYECGFNAFDDARMKFDVRFYLVSILFIIFDL
Tt  ~~MEGTLAPIQEYVGTLIYVGVALFIGVAALLVGALLGPKKPGRAKLMPYESGNDPAGEVK.RFPVHFYVVAMLFILFDV
Bt  ~~~~~~~~~~~~~~MNLMLALLTNFTLATLLVIIAFWLPQLNVYSEKTSPYECGFDPMGSARLPFSMKFFLVAITFLLFDL
Ec  MSMSTSTEVIAHHWAFAIFLIVAIGLCCLMLVGGWFLGGRARARSKNVPFESGIDSVGSARLRLSAKFYLVAMFFVIFDV
                                                  *    * *              * *   *   **

Pd EVAFLFPWAVSFASLSDVAFWGMMVFLAV.LTVGFAYEWKKGALEWA~~~ ~~~~~~~~~~ ~~~~~~~~
Rc EVAFLFPWAVAFGDMSMTAFWSMMVFLSV.LTVGFAYEWKKGALEWA~~~ ~~~~~~~~~~ ~~~~~~~~
Tt EVAFLWPYAVSAGGLGLYGFLGVLAFTLL.LFVGFLYEWWKGVMRWH~~~ ~~~~~~~~~~ ~~~~~~~~
Bt EIALLLPLPWASQTANLNTMLTMALFLIILLAVSLAYEWTQKGLEWTE~~ ~~~~~~~~~~ ~~~~~~~~
Ec EALYLFAWSTSIRESGWVGFVEAAIFIFVLLA.GLVYLVRIGALDWTPAR SRRERMNPET NSIANRQR
   *   *                    *    *    *        *
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FIGURE 1: Comparison of the deduced amino acid sequence of the *Paracoccus* NQO7 subunit with its counterparts from various organisms. The comparison was conducted with the PILEUP program. Pd, *Paracoccus denitrificans* (M93015) (*8*); Rc, *Rhodobacter capsulatus* (AF029365) (*54*); Tt, *Thermus thermophilus* HB-8 (U52917) (*31*); Bt, bovine heart mitochondrial complex I (J01394) (*55*); Ec, *Escherichia coli* K-12 (U00096/b2288-b2276) (*56*). Conserved amino acid residues are marked by asterisks. The predicted three transmembrane segments of the *Paracoccus* NQO7 subunit are underlined.

segment, this membrane-bound segment is believed to be involved in proton translocation of the *Paracoccus* NDH-1 (*20*). Therefore, understanding the properties and characteristics of the NQO7, 8, and 10−14 subunits is a prerequisite to understanding the mechanism of proton translocation of *Paracoccus* NDH-1 (*20*). Although to date fragmental information about functional roles of the hydrophobic subunits has been available (*8*, *9*, *20*, *23*−*30*), little is known about structural properties of these subunits. In addition, there has not been any report of successful expression of the hydrophobic subunits that would make studies of their biochemical and physicochemical properties possible. Expression strategy of individual cofactor-binding subunits has been proven to be a powerful approach to determine localization of cofactors in the NDH-1/complex I. Therefore, we first attempted to express the *Paracoccus* NQO7 subunit (a counterpart of mitochondrial ND3 subunit) in *E. coli* in a hope that similar strategy may be useful for structural studies of other hydrophobic subunits. The deduced primary structures of the *Paracoccus* NQO7 subunit and its homologues are not well conserved among species (Figure 1), whereas in terms of hydropathy profiles these homologues are akin to each other.

This paper describes the expression and partial characterization of the NQO7 subunit from the *Paracoccus* NDH-1. By use of a GST-fused expression system, the NQO7 subunit has been expressed in the membrane fraction in *E. coli* and then purified and partially characterized. The topology of the NQO7 subunit in the *Paracoccus* membranes has also been determined by immunochemical and chemical modification methods. The data indicate that the N-terminal region faces the cytoplasmic phase while its C-terminal region is directed toward the periplasmic phase of the bacterium. A loop containing the unique cysteine residue at position 47 is also directed to the periplasmic phase. The data further indicate that the NQO7 subunit comprises three transmembrane segments as predicted from the hydropathy plots. Similar topographical results were obtained for the GST-fused NQO7 subunit expressed in *E. coli* membranes.

EXPERIMENTAL PROCEDURES

Materials. SM2 Bio-Beads were from Bio-Rad (Hercules, CA). Carbenicillin, IPTG, PMSF, AEBSF, leupeptin, and 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (*p*-toluenesulfonate salt) were from Sigma (St. Louis, MO). The enhanced chemiluminescence (ECL) kit and the glutathione−Sepharose 4B gel were from Amersham−Pharmacia Biotech (Arlington Heights, IL). Anti-GST antibody was from Boehringer Mannheim (Indianapolis, IN). *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). Finally, the expression vector pGEX2TKcs was a generous gift from Dr. Masahiko Aoki (The Scripps Research Institute, CA).

Construction of the NQO7 Expression Vector. The *nqo7* gene was constructed by PCR technology together with the pXT-3 plasmid (*8*). Primers were synthesized in order to generate the *Nde*I site and *Bam*HI site at the initiation and stop codons, respectively, of the NQO7 subunit (see below). The sense primer employed had the sequence 5′-CCAAC-*CATATG*GAATACCTGCTGCAAG-3′. The antisense primer had the sequence 5′-ACGGTC*GGATCC*TGCGGTGTT-CAGG-3′, where the underlined bases were altered from *Paracoccus* DNA for the mutations and the italicized bases exhibit the *Nde*I and *Bam*HI sites. PCR amplification of the *nqo7* gene was performed with a thermocycler as described previously (*31*). The amplified DNA was isolated by chloroform extraction followed by ethanol precipitation. The isolated DNA fragments were then digested with *Nde*I and *Bam*HI. The digested DNA fragments were purified by 1% agarose gel electrophoresis with a Qiagen Gene Clean kit and then ligated into pGEX2TK$_{CS}$. The plasmid was designated pGEX2TK$_{CS}$(NQO7).

Expression of the GST-Fused NQO7 Subunit. Competent *E. coli* strains JM109, MV1190, XL1-Blue, BL21(DE3)-pLysS, and BLR(DE3)pLysS were transformed with the pGEX2TK$_{CS}$(NQO7) plasmid, spread onto the 2× YT agar plates containing 400 μg of carbenicillin/mL, and cultivated overnight at 37 °C. A well-isolated colony was then selected and inoculated into 5 mL of 2× YT medium containing 400 μg of carbenicillin/mL and cultivated at 37 °C to the stationary phase. One milliliter of the culture was used to inoculate 500 mL of 2× YT medium containing 400 μg of carbenicillin/mL. Cells were grown at 37 °C until the absorbance (at 600 nm) of the culture reached approximately 1.5. IPTG was then added to a final concentration of 0.1 mM, after which the cells were further cultured on an orbit shaker set to rotate at 150 rpm for 3 h at either 37 or 25 °C. After IPTG induction, the transformed cells were able to grow only in 2× YT or TB medium with BLR(DE3)pLysS or in 2× YT medium with BL21(DE3)pLysS. Therefore, we

routinely used 2× YT medium + competent strain BLR-(DE3)pLysS for expression of the GST-fused *Paracoccus* NQO7 subunit. The cells were harvested by centrifugation at 6000 rpm for 10 min in a Sorvall GSA rotor. The cell pellet was immediately frozen in liquid nitrogen and stored at −80 °C until use.

Purification of the GST-Fused NQO7 Subunit. The GST-fused NQO7 subunit was extracted from the membranes with Triton X-100 as follows: The membranes were suspended (a final protein concentration of 10 mg/mL) in 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA, 0.1 mM PMSF, 0.1 mM AEBSF, 2 μg/mL leupeptin, 1 mM DTT, and 1 M KCl. Triton X-100 was added to a final concentration of 5%. The suspension was sonicated 3 times for 10 min each time. Following sonication, the suspension was incubated on ice for 1 h and then subjected to ultracentrifugation at 50 000 rpm for 30 min in a Beckman 60Ti rotor. The supernatant fraction was applied on the glutathione−Sepharose 4B gel column (1.5 mL of bed volume). The column was washed with 10 column volumes of a buffer (pH 7.3) containing 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 10 mM NaCl, 2.7 mM KCl, 1% dodecyl maltoside, and 0.1 mM PMSF. The GST-fused NQO7 subunit was then eluted with 50 mM Tris-HCl (pH 8.0) buffer containing 10 mM reduced glutathione, 1% dodecyl maltoside, and 0.1 mM PMSF. The eluted GST-fused NQO7 subunit was dialyzed overnight against the above buffer lacking reduced glutathione. Finally, the material was concentrated to 1.3 mg/mL using a Centricon-10, frozen in liquid nitrogen, and stored at −80 °C.

To separate the GST from the NQO7, the purified GST-fused NQO7 subunit (500 μg) was digested with thrombin (1 thrombin cleavage unit/mg of protein) for 1 h at room temperature. The sample was then subjected to the SDS−PAGE. The NQO7 subunit part was recovered from the gel slices by electroelution in the cold room and lyophilized.

Antibody Production. Antibodies directed against the 12 amino acid oligopeptides corresponding to the N- and C-terminal regions of the NQO7 subunit were produced as follows: The peptides H-MEYLLQEYLPILC-OH (N-terminus) and H-CAYEWKKGALEWA-OH (C-terminus) were synthesized. These oligopeptides were designated NQO7n and NQO7c, respectively. The peptides were conjugated to maleimide-activated bovine serum albumin (Pierce, Rockford, IL) according to the manufacturer's protocol. It should be noted that for the purpose of conjugation with bovine serum albumin an additional cysteine is present in the oligopeptides that is not present in the intact NQO7 sequence. New Zealand white female rabbits were immunized with these conjugated peptides and bled on a regular schedule as described previously (*18*). The antibodies were affinity-purified according to refs *32*−*34*.

Reconstitution of the GST-Fused Paracoccus NQO7 Subunit. Phospholipid vesicles for the reconstitution of the GST-fused NQO7 subunit were prepared as follows: A chloroform/methanol (2:1) solution of phosphatidylcholine (type XVI-E) was mixed with 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene at a final ratio of 1/200 (mol/mol). The solution was then dried by argon flux and suspended in 10 mM Tris-HCl (pH 8.0) to a phospholipid concentration of 1 mg/mL. The suspension was sonicated for 20 min on ice, after which dodecyl maltoside was added to the phospholipid suspension until its turbidity disappeared. Reconstitution of GST-fused *Paracoccus* NQO7 subunit was then accomplished by adding the purified subunit to the phospholipid suspension followed by incubation for 30 min on ice. To remove the detergent, the suspension was mixed with SM2 Bio-Beads (5 mg/mg of phospholipid) and gently shaken for 1 h at room temperature. The SM2 Bio-Beads were then removed by centrifugation. The recovered phospholipid suspension was dialyzed against 10 mM Tris-HCl (pH 8.0) overnight and subjected to Sephadex G-150 column chromatography to isolate the phospholipid vesicles from the free phospholipids. The recovered, reconstituted GST-fused NQO7 material was subjected to the GST activity and fluorescence anisotropy analyses. The analyses were performed at 25 °C. For control experiments, the GST-fused NQO7 subunit was replaced by GST.

Preparation of Inside-Out and Right-Side-Out Membrane Vesicles of Paracoccus and E. coli. Inside out membrane vesicles of *Paracoccus* and *E. coli* were prepared according to procedures previously described (*35*, *36*). Briefly, the cells were suspended at a concentration of 10 mg wet weight/mL in TES buffer. The cells were broken open by treatment with a French press at 16 000 psi. The suspension was then centrifuged at 2000 rpm for 30 min in a GSA rotor. The supernatant fraction was collected and centrifuged at 25 000 rpm in a 60Ti rotor for 30 min. The resulting pellet was suspended in 50 mM Tris-HCl (pH 8.0) containing 0.5 M sucrose, 5 mM $MgCl_2$, 1 mM DTT, and 0.1 mM PMSF. The membrane suspension was employed as inside-out membrane vesicles without further purification.

The procedures used to prepare right-side-out membrane vesicles of *Paracoccus* and *E. coli* were based on an established method with lysozyme (*37*, *38*).

Sequence Analysis. GCG software programs were used to analyze the amino acid sequence (*39*). Sequence comparison of the polypeptides was conducted with the 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 using the BLAST program running at the National Center for Biotechnology Information (*40*).

Other Analytical Procedures. Protein was estimated by the method of Lowry et al. (*41*) or by the biuret method in the presence of sodium deoxycholate at 1 mg/mL (*42*). SDS−PAGE was conducted by the methods of Laemmli (*43*) and Schägger and Von Jagow (*44*). Preparation of cytoplasmic, membrane, and inclusion body fractions was performed as described previously (*21*). The GST-fused NQO7 subunit in the inclusion body fraction was purified as described previously (*14*, *18*). Any variations from the procedures and other detail are described in the figure legends.

RESULTS

Expression of the NQO7 Subunit. It is generally recognized that expression of hydrophobic proteins is difficult (*45*). We have previously attempted to express the full-length *Paracoccus* NQO7 subunit, as well as the N-terminal $(His)_6$ tag fused *Paracoccus* NQO7 subunits, in *E. coli*. These attempts proved unsuccessful. However, Yu's group (*46*) demonstrated that the hydrophobic QPc 9.5 kDa subunit (subunit VII) of bovine heart complex III could be successfully expressed in *E. coli* by use of the GST-fused expression

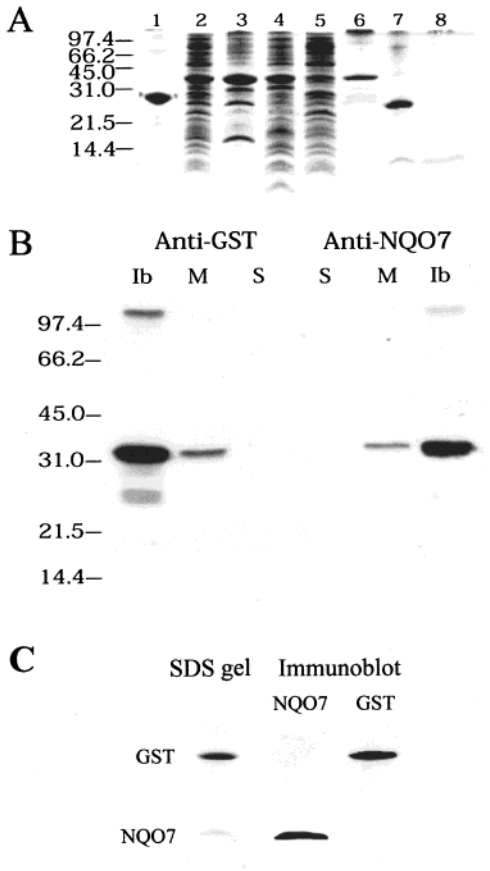

FIGURE 2: (A) SDS−Polyacrylamide gel pattern of the GST-fused *Paracoccus* NQO7 subunit expressed in *E. coli*. Lane 1, GST; lane 2, cell lysate of *E. coli* expressing GST-fused *Paracoccus* NQO7 subunit; lane 3, inclusion body fraction; lane 4, membrane fraction, lane 5, supernatant fraction; lane 6, GST-fused NQO7 subunit isolated by glutathione affinity column chromatography; lane 7, thrombin-treated GST-fused NQO7 subunit; lane 8, NQO7 subunit isolated from the thrombin-treated GST-fused NQO7 subunit. The numbers on the left side indicate the molecular mass (kilodaltons) of the marker proteins. Ten micrograms of protein were loaded per lane on a Schägger SDS−12% polyacrylamide gel. After electrophoresis the gel was stained with Coomassie Brilliant Blue. (B) Immunoblotting of the membrane (M), soluble (S), and inclusion body (Ib) fractions of *E. coli* bearing the pGEX2TK$_{CS}$-(NQO7) expression vector with anti-GST antibody (left panel) and anti-*Paracoccus* NQO7c antibody (right panel). Five micrograms of protein were applied to each lane of a Laemmli SDS−13% polyacrylamide gel. The electrophoresis and electronic transfer were performed according to refs *32*−*34*. Immunoblotting was performed with the ECL system. (C) Identification of the two fragments derived from the thrombin-treated GST-fused NQO7 subunit by use of the antibodies against the *Paracoccus* NQO7c and GST. Left panel, SDS−polyacrylamide gel pattern; center panel, immunoblotting with the affinity-purified anti-*Paracoccus* NQO7c antibody; right panel, immunoblotting with anti-GST antibody. Aliquots (30 μg, left panel; 20 μg, center and right panels) of the proteins were loaded on Schägger SDS−16.5% polyacrylamide gel. The electrophoresis and immunoblotting were carried out as in panel B.

system. Therefore, we have attempted to express the *Paracoccus* NQO7 subunit in *E. coli* by utilizing this same GST fused expression system. The procedure employed is outlined under Experimental Procedures. The data revealed that when the BLR(DE3)pLysS or BL21(DE3)pLysS were used as competent cells, the GST-fused *Paracoccus* NQO7 subunit was, in fact, overexpressed (Figure 2A) in these cells. Studies conducted to localize the subunit indicated that the GST-fused NQO7 subunit was expressed both in the membrane fraction and in the inclusion body fraction (Figure 2B). Despite various attempts, the GST-fused NQO7 subunit in the inclusion bodies could not be recovered as a soluble, renatured protein. Similar results have been reported for the GST-fused QPc 9.5 kDa subunit expressed in the inclusion bodies (*46*). In contrast, the GST-fused NQO7 subunit expressed in the membrane fraction was extracted with nonionic detergents such as Triton X-100 and dodecyl maltoside (see Experimental Procedures). After extraction the GST-fused NQO7 subunit was purified by glutathione affinity chromatography (Figure 2A). The purity and identity of the isolated material was assessed by SDS−PAGE. The data indicated that the purified GST-fused NQO7 subunit migrated by SDS−PAGE with M_r = 38 000, which reasonably corresponds to the molecular size of this fusion product (MW = 41 000) as deduced from the DNA sequence. The purified GST-fused NQO7 subunit was then treated with thrombin to separate the fused protein into its component parts, GST and NQO7. As shown in Figure 2A,C, when the cleaved product was subjected to SDS−PAGE followed by staining, two bands were observed. When these SDS−PAGE fractionated proteins were transferred to nitrocellulose membranes and subjected to immunoblotting, the upper band (M_r = 28 000) reacted only with antibodies specific to the GST while the lower band (M_r = 13 000) reacted only with antibodies specific to *Paracoccus* NQO7c (Figure 2C). In addition, the mass spectroscopic data of the fragments derived from trypsin treatment of NQO7 subunit part are consistent with the deduced primary structure of the NQO7 subunit (data not shown). These results indicate that the GST-fused *Paracoccus* NQO7 subunit is correctly expressed in *E. coli*. To assess the effect that the GST domain and the NQO7 domain had on each other, the fluorescence anisotropy of the GST-fused NQO7 product was compared with the anisotropy induced by the GST protein alone. This study was based on the knowledge that NQO7 is purported to be hydrophobic, membrane-embedded protein while the GST protein is known to be water-soluble. The data indicated that the NQO7 domain of the GST-fused NQO7 subunit interacted with the phospholipid vesicles and suppressed their membrane fluidity, while the GST alone did not have this effect (see Figure 3). Taken together with the fact that thrombin could interact with the GST-fused NQO7 subunit and cleave it into the individual GST and NQO7 parts, it seems likely that the GST and NQO7 domains of the GST-fused NQO7 subunit exhibit their own properties. In other words, the two domains appear to behave independently.

Identification of the Paracoccus NQO7 and Its Homologues. Although the presence of the human complex I homologue of the NQO7 subunit (ND3) has been immunochemically demonstrated in situ (*47*), similar information is not available for the NQO7 subunit of the bacterial NDH-1. Therefore, it was of interest to identify the NQO7 subunit in situ. To pursue this objective, immunoblotting experiments were conducted on *Paracoccus* membranes and antibodies directed against the N-terminal (anti-NQO7n) and C-terminal (anti-NQO7c) of the NQO7 subunit. The results (Figure 4A) demonstrated that both antibodies clearly recognized the same 15 kDa band in Laemmli SDS gels. In addition, subsequent immunoprecipitation experiments of the *Paracoccus* membranes with the anti-NQO7c antibody confirmed

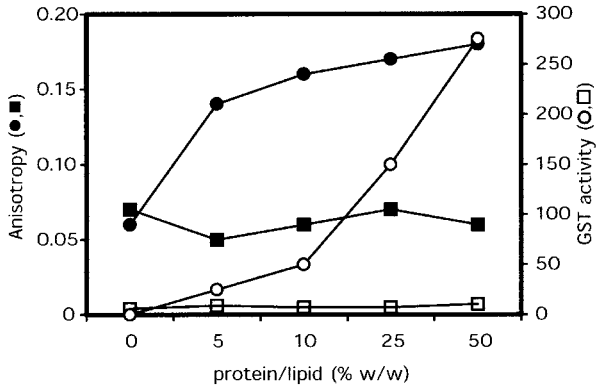

FIGURE 3: Effect of GST-fused *Paracoccus* NQO7 subunit on the membrane fluidity of phospholipid vesicles. The open and solid symbols indicate, respectively, GST enzymatic activity and fluorescence anisotropy. The reconstituted phospholipid vesicles contained either GST-fused *Paracoccus* NQO7 subunit (●, ○) or GST polypeptide (■, □). The GST enzymatic activity is given in ΔA_{340} min^{-1} mL^{-1}. Fluorescence anisotropy of 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene intercalated into the phospholipid vesicles was determined by an SLM spectrofluorometer according to refs *57* and *58*. Excitation wavelength was 363 nm and emission was measured at 429 nm. It should be noted that GST was dissociated with phospholipid vesicles during the step of Sephadex G-150 column chromatography (see Experimental Procedures).

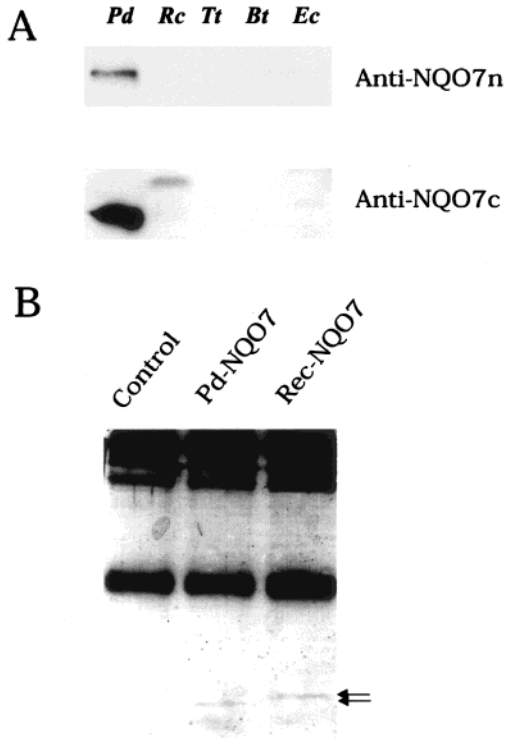

FIGURE 4: (A) Cross-immunoreactivity of the *Paracoccus* membranes (*Pd*), *R. capsulatus* chromatophores (*Rc*), *T. thermophilus* HB-8 membranes (*Tt*), bovine heart SMP (*Bt*), and *E. coli* membranes (*Ec*) with affinity-purified antibodies directed against the *Paracoccus* NQO7c and NQO7n. Ten micrograms of the *Paracoccus* membranes, *R. capsulatus* chromatophores, *T. thermophilus* HB-8 membranes, bovine heart SMP, and *E. coli* membranes were loaded into the wells of and SDS−13% polyacrylamide gel and subjected to electrophoresis as described under Experimental Procedures. The immunoblotting procedures were performed as described previously (*14*, *18*). (B) Immunoprecipitation of the NQO7 subunit from the *Paracoccus* membranes (Pd-NQO7) and from *E. coli* membranes containing the GST-fused NQO7 (Rec-NQO7) by the antiserum to the *Paracoccus* NQO7 C-terminus. The upper and lower arrows indicate, respectively, the GSRRASVES-tagged NQO7 subunit part and NQO7 subunit. Immunoprecipitation experiments with anti-NQO7c antiserum were carried out according to refs *25* and *59*.

these results (see Figure 4B). Finally, the 15 kDa polypeptide was identified as a membrane-embedded protein by demonstrating that it could not be extracted from the membranes by treatment of the *Paracoccus* membranes with NaI and alkaline pH (*11*, *12*). Under the same conditions, the peripheral subunits (NQO1−6 and NQO9) were completely extracted from the membranes (*14*, *18*).

Figure 4A also demonstrates the results of cross-immunoreactivity studies of the antibodies directed against the *Paracoccus* NQO7 N- and C-termini with *R. capsulatus* chromatophores, *T. thermophilus* HB-8 membranes, bovine heart SMP, and *E. coli* membranes. The data reveal that the anti-*Paracoccus* NQO7n antibody did not cross-react with any membranes of these organisms. On the other hand, the anti-*Paracoccus* NQO7c antibody cross-reacted with an 18 kDa polypeptide of *R. capsulatus* chromatophores but did not react with the bovine heart SMP or *T. thermophilus* or *E. coli* membranes. As seen in Figure 1, these results are consistent with the sequence homology and identity between the *Paracoccus* NQO7 subunit and its homologues.

Topographical Studies of the NQO7 Subunit. To carry out topological studies we prepared the inside-out and right-side-out membrane vesicles as described under Experimental Procedures. The NQO1 (NADH-binding) subunit of the *Paracoccus* NDH-1 (*13*) and Rieske iron−sulfur protein (ISP) of complex III (*48*) were selected as marker proteins of cytoplasmic and periplasmic sides of the membrane, respectively, on the basis of work previously reported (*49*, *50*). As shown in Figure 5, the data clearly indicate that inside-out vesicles reacted with the antibody to the *Paracoccus* NQO1 subunit but did not react with the antibody to the Rieske iron−sulfur protein (ISP). In contrast, the right-side-out vesicles were recognized by the latter antibody but not by the former antibody. These results demonstrate that the sidedness of the constructed *Paracoccus* membrane vesicles is correct. The data further indicate that the anti-NQO7n antibody only reacted with the inside-out vesicles (but not with the right-side-out vesicles), while the anti-NQO7c antibody reacted only with the right-side-out vesicles (but not with the inside-out vesicles). These results indicate that the N- and C-termini of the NQO7 subunit face the cytoplasmic and periplasmic compartments, respectively. In addition, the data suggest that since the antibodies were able to interact with N- and C-terminal regions of the NQO7 subunit in the *Paracoccus* membranes, it is likely that both of these terminal regions are freely accessible and neither is covered by other polypeptides.

The hydropathy plots of the deduced primary structure of the *Paracoccus* NQO7 subunit suggest that this subunit houses three transmembrane helices as shown in Figure 1. These three helices, from the N-terminus to C-terminus, are tentatively designated TM1, TM2, and TM3. As illustrated in Figure 1, the *Paracoccus* NQO7 subunit contains a unique cysteine at the 47th position. This Cys47 is conserved in the *Paracoccus* NQO7 homologues of *R. capsulatus* and bovine heart but not in those of *T. thermophilus* HB-8 or *E.*

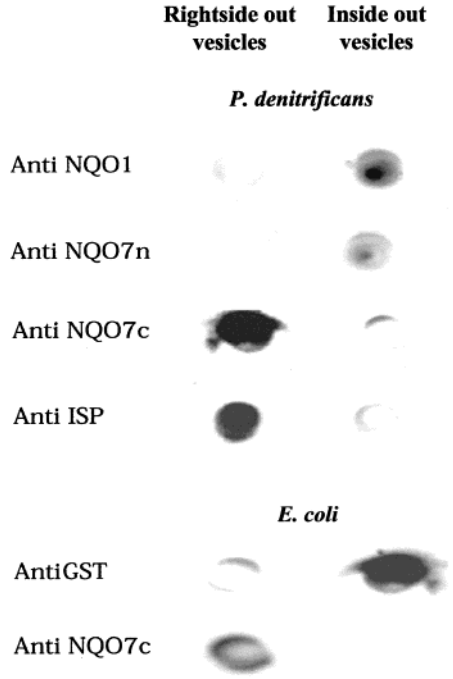

FIGURE 5: Localization of the N- and C-terminal regions of the NQO7 subunit in the membranes of *P. denitrificans* and *E. coli* harboring the pGEX2TK$_{CS}$(NQO7) vector. Aliquots (50 μL) of right-side-out and inside-out membrane vesicles from *Paracoccus* and *E. coli* were bound directly to nitrocellulose membranes by incubation of the membranes with the vesicles in a Dot Blot apparatus for 2 h at room temperature. The wells were then washed 3 times with TES buffer and incubated with 2% skim milk in PBS 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 detecting antibodies as described previously (*14*, *18*). The primary antibodies employed included antibodies directed against NQO1 (positive control for inside-out vesicles), against the N-terminus of NQO7 (anti-NQO7n), against the C-terminal region of NQO7 (anti-NQO7c), against the Rieske iron−sulfur protein (anti-ISP; positive control for right-side-out vesicles), and against GST.

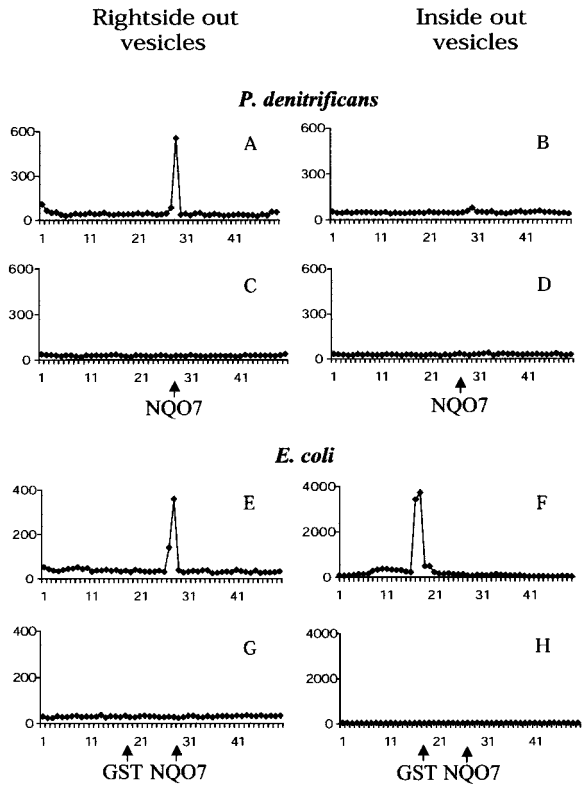

FIGURE 6: Localization of the NQO7 subunit Cys47 in the membranes of *P. denitrificans* and in *E. coli* carrying the pGEX2TK$_{CS}$(NQO7) vector. In panels A, B, E, and F, 600 μg of right-side-out and inside-out vesicles of *Paracoccus* and *E. coli* were treated with 2 μL of 1 mCi/mL [^{3}H]NEM for 2 h at room temperature. In panels C, D, G, and H, the membrane vesicles were preincubated with 2 μL of 40 mM AIAS before [^{3}H]NEM treatment. After treatment with [^{3}H]NEM, membrane vesicles were treated with 4.5% SDS and then immunoprecipitation was performed as described previously (*22*, *25*) with the anti-*Paracoccus* NQO7c antiserum. The immunoprecipitate recovered from *E. coli* was subjected to thrombin treatment to separate the GST and NQO7 domains. The immunoprecipitated samples were then subjected to SDS−PAGE and the gels were cut in 2-mm-thick slices. Each slice was digested overnight with 0.5 mL of 30% H_2O_2 at 65 °C and then mixed with 5 mL of scintillation mixture and counted for ^{3}H radioactivity in a Beckman LS-1801 scintillation counter.

coli. The Cys47 is surrounded by hydrophilic amino acid residues, suggesting that Cys47 may be exposed to either the cytoplasmic or the periplasmic phase but is most likely not embedded in the membranes. On the basis of this information it is expected that localization of the Cys47 would provide useful information on topology of the *Paracoccus* NQO7 subunit. Therefore, we attempted to identify the location of this Cys47 by reacting both right-side-out and inside-out membrane vesicles with [^{3}H]NEM as well as with the anti-NQO7c antibody. The results of this study, exhibited in Figure 6, reveal that only the NQO7 subunit in the right-side-out vesicles (but not in the inside-out vesicles) could be labeled by [^{3}H]NEM, suggesting that the Cys47 is exposed on the periplasmic side of the membrane (but not on the cytoplasmic side of the membrane). However, these data are not entirely conclusive because it is still possible that [^{3}H]NEM labeled the Cys in the hydrophobic area instead of in the hydrophilic area. For example, the [^{3}H]NEM may have labeled a Cys located in a transmembrane helix as reported for the transposon 10-encoded metal-tetracycline/proton antiporter in *E. coli* (*51*). To eliminate this possibility we performed a displacement experiment using the membrane-impermeable cysteine modifier 4-acetamido-4′-[(iodoacetyl)amino]stilbene-2,2′-disulfonic acid (AIAS). This membrane-impermeable SH modifier completely protected against labeling of the NQO7 subunit in the right-side-out membrane vesicles with [^{3}H]NEM (see Figure 6). Together these data provide strong support for the concept that Cys47 is exposed to the periplasmic compartment of the *Paracoccus* membranes. With all these results taken into consideration, the speculated topology of the *Paracoccus* NQO7 subunit has been displayed in Figure 7.

A similar strategy was taken to determine the topology of the GST-fused *Paracoccus* NQO7 subunit expressed in *E. coli* membranes. The antibody against GST reacted with the inside-out vesicles but did not react with right-side-out vesicles, confirming the presence of GST on the cytoplasmic side of the *E. coli* membranes (see Figure 5). In contrast, the anti-NQO7c antibody reacted only with the right-side-out vesicles, not with the inside-out vesicles (see Figure 5). In addition, [^{3}H]NEM labeled the NQO7 subunit part of the GST-fused *Paracoccus* NQO7 subunit in the right-side-out vesicles (but not in the inside-out vesicles) and the GST part in the inside-out vesicles (but not in the right-side-out

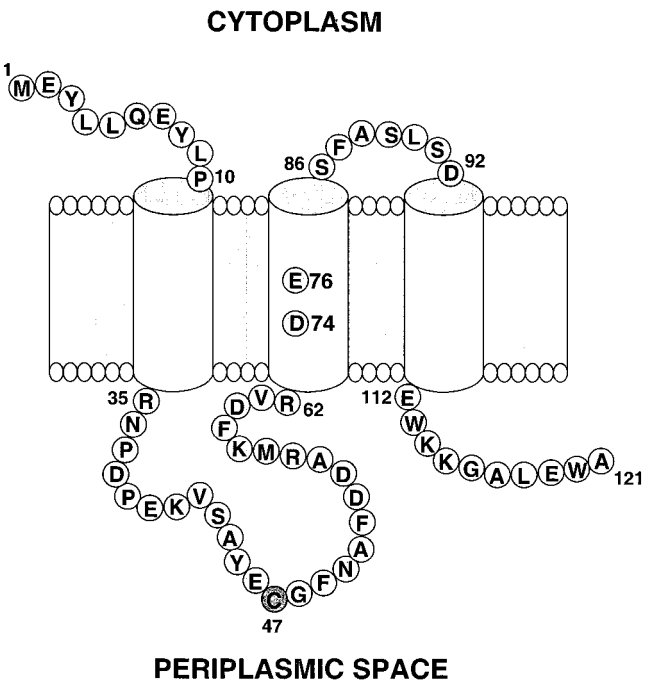

FIGURE 7: Proposed topology of the *Paracoccus* NQO7 subunit. As described in the text, the three transmembrane segments of the *Paracoccus* NQO7 subunit from the N-terminus to the C-terminus are tentatively designated TM1, TM2, and TM3, respectively. The N-terminus of the subunit is exposed to the cytoplasmic side of the membrane. The C-terminus and the Cys47 residue are exposed to the periplasmic space.

vesicles) (Figure 6). In addition, the AIAS protected against labeling of the NQO7 and GST parts with [^{3}H]NEM. These data suggest that the GST-fused *Paracoccus* NQO7 subunit in *E. coli* is similar to the NQO7 subunit in the *Paracoccus* membranes in terms of topology.

DISCUSSION

The *Paracoccus* NQO7 subunit has been successfully overexpressed in the membrane fraction of *E. coli* as a GST-fused protein. The expressed GST-fused *Paracoccus* NQO7 subunit appears to have retained the same native topology as in the *Paracoccus* membranes. Thus, the native expression of the *Paracoccus* NQO7 subunit in *E. coli* membranes suggests that it may be possible to functionally express any or all of the individual hydrophobic subunits of the *Paracoccus* NDH-1, although a number of problems are, of course, anticipated. In addition to demonstrating the native topology of the expressed subunit, the successful overexpression of the *Paracoccus* NQO7 subunit produced sufficient material to allow us to easily conduct research on the biochemical characteristics of this subunit. As demonstrated concerning the DCCD-binding subunit of the ATP synthase (*52*, *53*) and subunit VII (Q-binding subunit) of complex III (*46*), studies of the properties of the individual hydrophobic subunits will provide useful information regarding the structure and function of the *Paracoccus* NDH-1 enzyme complex as a whole.

As described above, the deduced primary structures of the *Paracoccus* NQO7 and its homologues are not well conserved. In fact, only 14 amino acid residues (K41, E46, G48, F63, V66, F70, F73, D74, E76, L80, F101, L105, Y111, and W120) of the *Paracoccus* NQO7 subunit are conserved among its bovine, *R. capsulatus*, *T. thermophilus* HB-8, and *E. coli* homologues (see Figure 1). Of these, only five amino acid residues, K41, E46, D74, E76, and Y111, are related to protonation and, as such, are anticipated to be involved in proton translocation. The K41 and E46 appear to be located in the periplasmically exposed loop between transmembrane segments TM1 and TM2 (Figure 7). It is likely that Y111 is present at the interface between the TM3 segment and the C-terminal region of the protein, which is also exposed to the periplasmic phase. In contrast, D74 and E76 seem to be located in the middle (12th and 14th amino acid) of the 23 amino acid TM2 segment. Interestingly, these two carboxyl residues are conserved in at least 250 counterparts of the *Paracoccus* NQO7 subunit according to the nonredundant database at NCBI (March 2000). The only exception noted is the ND3 subunit of *Cyanidium caldarium* mitochondria (D74 → C) (accession number P48911). It is well-known that the DCCD-binding protein of the ATP synthase houses one highly conserved carboxyl residue in the center of its transmembrane helix (*52*). This carboxyl residue is clearly involved in the proton translocation of the membrane sector of the ATP synthase (*52*). At the present time, little is known about the mechanism of proton translocation of the energy coupling site 1. Comparison to the ATP synthase system, however, suggests a possibility that conserved amino acid residues, which are not only involved in protonation but also located in the center of the membranes, may play important roles of the proton translocation of the energy coupling site 1 as well. If so, D74 and E76 (*Paracoccus* numbering) appear to be candidates for involvement in proton translocation of NDH-1. Clarifying the structural and functional roles of these residues is expected to provide useful information about the mechanism of proton translocation of coupling site 1. Therefore, it will be of interest to conduct site-directed mutation of these two residues in future.

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