Subunit Proximity in the H^+-Translocating NADH-Quinone Oxidoreductase Probed by Zero-Length Cross-Linking[†,‡]

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ABSTRACT: The proton-translocating NADH-quinone oxidoreductase (NDH-1) of *Paracoccus denitrificans* is composed of 14 different subunits (designated Nqo1−14), seven of which are located in the membrane domain and the other seven in the peripheral domain. It has been previously reported that membrane domain subunit Nqo7 (ND3) directly interacts with peripheral subunit Nqo6 (PSST) by using a cross-linker, *m*-maleimidobenzoyl-*N*-hydrosuccinimide ester, and heterologous expression [Di Bernardo, S., and Yagi, T. (2001) *FEBS Lett. 508*, 385−388]. To further explore the near-neighbor relationship of the subunits, a zero-length cross-linker, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC), and the *Paracoccus* membranes were used, and the cross-linked products were examined with antibodies specific to subunits Nqo1−11. The Nqo6 subunit was cross-linked to subunit Nqo9 (TYKY). In addition, a ternary product of Nqo3 (75k), Nqo6, and Nqo7 and binary products of Nqo3 and Nqo6 and of Nqo6 and Nqo7 were observed, but a binary product of Nqo3 and Nqo7 was not detected. The Nqo4 (49k) subunit was found to be associated with the Nqo7 subunit. Furthermore, *Paracoccus* subunits Nqo3, Nqo6, and Nqo7 were heterologously coexpressed in *Escherichia coli*, and EDC cross-linking experiments were carried out using the *E. coli* membranes expressing these three subunits. The results were the same as those obtained with *Paracoccus* membranes. On the basis of the data, subunit arrangements of NDH-1 were discussed.

The proton-translocating NADH-quinone oxidoreductases are members of the bacterial and mitochondrial respiratory chains (designated NDH-1[1] and complex I in bacteria and mitochondria, respectively) (*1*, *2*). The bacterial NDH-1 generally contains one FMN and eight iron−sulfur clusters as cofactors (*3*). Of these iron−sulfur clusters, two are binuclear clusters (N1a and N1b) and the remaining six are tetranuclear clusters (N2−N5, N6A, and N6B) (*4*). These cofactors are involved in electron transfer from NADH to quinone, and during this process, protons (or sodium ions) are translocated across the cytoplasmic membrane. The H^+/2e ratio has been reported to be 4 (*5*). Elucidation of the mechanisms of electron transfer and proton translocation is challenging because of the complexity of the enzyme. Complex I is composed of 46 different subunits, and the bacterial NDH-1, albeit smaller, still contains 14 dissimilar subunits (Nqo1−14)[2] (*6*−*8*). The NDH-1 consists of at least two domains (*9*). One is a peripheral domain composed of seven subunits (Nqo1−6 and Nqo9) and extruded into the cytoplasmic phase (*10*, *11*). This segment bears all known cofactors (*4*). The other is a membrane domain segment bearing seven hydrophobic subunits (Nqo7, Nqo8, and Nqo10−14) which are homologues of seven mitochondrially encoded subunits of complex I (ND1−6 and 4L) (*12*, *13*). The Nqo6/PSST subunit is believed to house center N2 (the E_m value of N2 is the highest of all the iron−sulfur clusters and is known to be pH-dependent)[3] (*14*−*16*) and was labeled with a photoaffinity analogue of pyridaben, a potent complex I inhibitor (*18*). In addition, we have recently demonstrated that the Nqo6/PSST subunit directly interacts with the membrane domain subunit Nqo7/ND3 (*19*). It has been suggested that the Nqo6/PSST and Nqo9/TYKY subunits (*Paracoccus*/bovine) are at the interface between the peripheral domain and the membrane segment and thus play a central role in energy transduction (*10*, *11*). Therefore, it is of interest to investigate which subunits are associated with the Nqo6 and Nqo9 subunits in NDH-1.

In this paper, we explore subunit proximity around the central core of *Paracoccus* NDH-1 by using a zero-length cross-linker, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride. The results indicated that subunits Nqo6/

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[1] Abbreviations: NDH-1, bacterial proton (sodium ion)-translocating NADH-quinone oxidoreductase; complex I, mitochondrial proton-translocating NADH-quinone oxidoreductase; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride; Sulfo-NHS, *N*-hydroxysulfosuccinimide; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

[2] Subunit naming is based on the *Paracoccus* nomenclature. For clarification, bovine naming was also used when appropriate.

[3] Nqo9/TYKY cannot be totally ruled out as the binding subunit of center N2 (*17*).

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PSST, Nqo9/TYKY, Nqo3/75k, Nqo4/49k, and Nqo7/ND3 are close to each other and some are in direct contact.

EXPERIMENTAL PROCEDURES

Antibody Production. Antibodies specific to the individual subunits of *Paracoccus* NDH-1 were raised as reported previously (*11*, *20*−*22*) and used after affinity purification. The Nqo1−6 and Nqo9 antibodies were raised in rabbits against the whole subunit, while the Nqo7, Nqo8, Nqo10, and Nqo11 antibodies were produced in rabbits using C-terminal oligopeptides of the individual subunits.

Chemical Cross-Linking Experiments. Cholate-treated *Paracoccus* membranes were prepared as described previously (*10*). The membranes were suspended at a protein concentration of 1 mg/mL in 50 mM potassium phosphate buffer (pH 7.5). The membrane samples were incubated with 5 mM EDC and 5 mM Sulfo-NHS for 1 h at room temperature in the dark on a rotating wheel. The reaction was terminated by the addition of 50 mM Tris-HCl (pH 7.5). After a 30 min incubation, an equal volume of 2× Laemmli sample buffer was added, and the resulting samples were subjected to SDS−PAGE and Western blotting analyses. Cross-linked species were identified using affinity-purified antibodies raised against the individual subunits of *Paracoccus* NDH-1. Extraction of peripheral subunits was carried out as follows. Membrane samples obtained after the cross-linking procedure were further incubated with 1.5 M NaI for 15 min at 30 °C and frozen and thawed three times in a liquid nitrogen/water bath at 30 °C. The suspensions were centrifuged in a Beckman Airfuge at 30 psi for 15 min. The supernatant and resuspended pellets [in 50 mM potassium phosphate buffer (pH 7.5)] were subjected to immunoblotting analysis as described above.

Construction of Coexpression Vectors. For coexpression of the *Paracoccus* Nqo3, Nqo7, and Nqo6 subunits in *Escherichia coli*, a two-plasmid coexpression system was employed in this study. The first expression vector for the Nqo3 subunit, named pET11a(Nqo3), was constructed as described previously (*23*). The second vector used for coexpression was generated as follows. A DNA fragment containing the *nqo7* and *nqo6* genes was constructed from plasmid pXT-3 (*24*) using PCR. A sense primer (5′-AGGGAGCCCAACCATATGGAATACCTGCTG-3′) and an antisense primer (5′-GAGAGGGCTTCGGATCCGGCT-CACCTCACC-3′) were synthesized (underlined bases altered to generate an *Nde*I site at the translation initiation codon of the *nqo7* gene and a *Bam*HI site downstream of the stop codon of the *nqo6* gene, respectively). The amplified DNA was subcloned into pCR-Script Amp SK(+), and its sequence was verified by sequencing. The resulting plasmid was designated pCR(Nqo7-6). The *Nde*I−*Bam*HI DNA fragment digested from pCR(Nqo7-6) was then purified and ligated into the *Nde*I and *Bam*HI sites of the pET11a vector. The plasmid thus obtained was designated pET11a(Nqo7-6). The pET11a(Nqo7-6) plasmid was digested with *Bgl*II and *Eco*RI. The *Bgl*II−*Eco*RI fragment, which contains the *nqo7* and *nqo6* genes within the T7 expression cassette, was then subcloned into the *Bam*HI and *Eco*RI sites of plasmid pKT230 to generate the pKT230(Nqo7-6) expression vector. The pKT230 vector, which is an IncQ broad host-range multicopy plasmid, was used to prevent the instability of two incompatible plasmids introduced into the same cells which may lead to the ultimate loss of one plasmid.

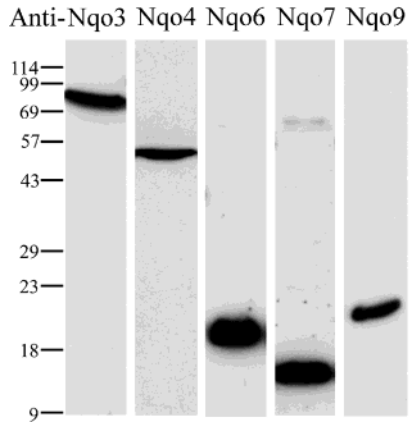

FIGURE 1: Immunoblotting of the *Paracoccus* membranes with affinity-purified antibodies specific to subunits Nqo3, Nqo4, Nqo6, Nqo7, and Nqo9 of *Paracoccus* NDH-1. Membrane samples (10 μg of protein) were applied on each lane of a Laemmli SDS−12.5% polyacrylamide gel. The numbers to the left of the gel denote molecular sizes of marker proteins (kilodaltons).

Coexpression of the Paracoccus Nqo3, Nqo6, and Nqo7 Subunits in E. coli Membranes. The pKT230(Nqo7-6) plasmid was first introduced into *E. coli* strain BL21(DE3) by $CaCl_2$-mediated transformation, and the transformed cells were spread on LB agar plates containing 50 μg/mL kanamycin. A well-isolated colony retrieved from the plate described above was grown in 2× YT medium and then made competent. These cells were then transformed with plasmid pET11a(Nqo3) and screened on LB agar plates for both kanamycin and ampicillin resistance. A single colony was selected and used to inoculate 5 mL of 2× YT medium with both kanamycin (50 μg/mL) and ampicillin (100 μg/mL) added and incubated overnight at 37 °C. Two milliliters of this start culture was used to inoculate 250 mL of TB medium supplemented with 1% glucose, 50 μg/mL kanamycin, and 100 μg/mL ampicillin. Cells were grown at 25 °C with shaking until the A_{600} reached 0.5. Isopropyl β-D-thiogalactopyranoside was then added to a final concentration of 0.2 mM, and the culture was incubated for an additional 20 h at 20 °C with shaking at 250 rpm. Cell membranes were prepared as described previously (*25*).

Other Analytical Procedures. The protein concentration of membrane preparations was determined by the BCA protein assay kit (Pierce) according to the manufacturer's protocol after solubilization of the membrane in 2% SDS. SDS−PAGE was performed using the discontinuous system of Laemmli (*26*). Instead of being boiled, protein samples were incubated at room temperature for 1 h before being loaded onto the gel. Immunoblotting was carried out using the SuperSignal West Pico system (Pierce). Any variations from the procedures and other details are described in the figure legends.

RESULTS

Figure 1 displays the reactivity of the *Paracoccus* membranes with the antibodies specific to subunits Nqo3, Nqo4, Nqo6, Nqo7, and Nqo9. It is clear that each antibody strongly reacted with a single band in the SDS gels of the *Paracoccus* membranes. The molecular sizes of the bands (72, 48, 19,

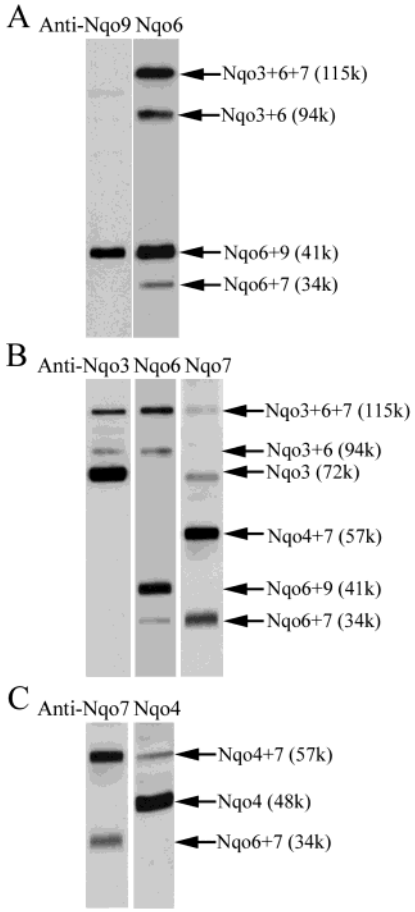

FIGURE 2: Immunoblotting of the *Paracoccus* membranes after treatment with a zero-length cross-linking reagent, EDC. Membranes were incubated in the presence of 5 mM EDC as described in Experimental Procedures. Membrane samples (10 μg of protein) were applied on each lane of Laemmli SDS−10% polyacrylamide gels. Immunoblotting was performed using affinity-purified antibodies specific to the individual subunits of *Paracoccus* NDH-1 [(A) anti-Nqo9 and anti-Nqo6 antibodies, (B) anti-Nqo3, anti-Nqo6, and anti-Nqo7 antibodies, and (C) anti-Nqo7 and anti-Nqo4 antibodies]. The identified cross-linked products are marked on the right with the M_r values. Note that in some cases overexposure of the film was necessary to reveal minor cross-linked bands. For reproducibility, the experiments were carried out at least three times.

14, and 21 kDa for Nqo3, Nqo4, Nqo6, Nqo7, and Nqo9, respectively) are consistent with those of subunits isolated from the *Paracoccus* NDH-1 and expressed in *E. coli* (*10*, *19*, *22*, *23*, *27*−*31*). The antibody to subunit Nqo7 faintly reacted with a band with an M_r of ∼69 kDa. This band could be extracted from the *Paracoccus* membranes by treatment with NaI (see below), suggesting that it was not related to membrane domain subunit Nqo7 (and did not disturb cross-linking experiments). Together with antibodies raised against the Nqo1, Nqo2, Nqo5, Nqo8, Nqo10, and Nqo11 subunits (*10*, *18*−*21*, *31*), a total of 11 subunits can be detected in our cross-linking experiments of NDH-1 in the *Paracoccus* membranes.

It has previously been demonstrated that the Nqo7 subunit directly interacts with the Nqo6 subunit by using a cross-linker, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester, with a 9.9 Å arm (*19*). In contrast, EDC catalyzes formation of a peptide bond by coupling a carboxyl group to an amino group and, therefore, works as a zero-length cross-linking reagent. According to the literature (*32*), Sulfo-NHS can

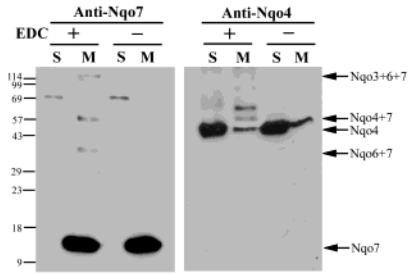

FIGURE 3: Effect of extraction by a chaotropic agent on the EDC-cross-linked products. *Paracoccus* membranes were first treated with (+) or without (−) 5 mM EDC as described in the legend of Figure 2. The membranes were then incubated in the presence of 1.5 M NaI for 15 min at 30 °C. This procedure is known to remove peripheral subunits of NDH-1. The membrane suspensions were centrifuged with a Beckman airfuge, and the resulting supernatant (S) and membrane suspensions (M) were mixed with an equal volume of 2× Laemmli's sample buffer. Thirty microliters of each sample was loaded on Laemmli SDS−12.5% polyacrylamide gels. Immunoblotting was carried out with affinity-purified antibodies specific to the *Paracoccus* NDH-1 subunits indicated in the figure.

enhance the coupling reaction by EDC at physiological pH. Utilizing the EDC/Sulfo-NHS system, we investigated the spatial relationship of the subunits that directly interact with each other. *Paracoccus* membranes were incubated in the presence of EDC and analyzed by immunoblotting using affinity-purified antibodies specific to the 11 subunits of *Paracoccus* NDH-1 described above. The EDC treatment gave rise to several new bands. They were detected by antibodies to subunits Nqo3, Nqo4, Nqo6, Nqo7, and Nqo9, but none of them reacted with antibodies to subunits Nqo1, Nqo2, Nqo5, Nqo8, Nqo10, and Nqo11. Figure 2A shows that a cross-linked product of the 41 kDa band was recognized by antibodies against subunits Nqo6 and Nqo9. Furthermore, as shown in Figure 2B, the 34 kDa band was detected by Nqo6 and Nqo7 antibodies, the 94 kDa band by Nqo6 and Nqo3 antibodies, and the 115 kDa band by the Nqo3, Nqo6, and Nqo7 antibodies. It should be noted that a binary product composed of Nqo3 and Nqo7 was not detected in our experiments. In addition, in Figure 2C, the 57 kDa band reacted with antibodies against Nqo4 and Nqo7. A band with an M_r of ∼70 kDa that appeared in the anti-Nqo7 blot (Figure 2B) is not a cross-linked product but the nonspecific band which was also seen in Figure 1.

We have shown that the peripheral subunits can be extracted with a chaotropic agent, while the membrane domain subunits resist the extraction (*10*, *11*). Therefore, if cross-linking occurs within the peripheral subunits, the product should still be able to be extracted from the membranes by NaI. In contrast, if a peripheral subunit is linked to a membrane domain subunit, then it may no longer be released from the membrane. Of particular interest was the Nqo4−Nqo7 pair which would presumably span the two domains. Therefore, we carried out the extraction experiment, and the result is presented in Figure 3. After incubation of the *Paracoccus* membranes with EDC, the membranes were subjected to treatment with NaI. The suspensions were centrifuged and separated into a soluble fraction and a membrane fraction. Both fractions were examined by immunoblotting using anti-Nqo4 and anti-Nqo7 antibodies. The Nqo7 subunit remained in the membrane, and the Nqo4 was mostly extracted from the membrane, in agreement with our previous observations (*10*). The 57 kDa band that reacts with

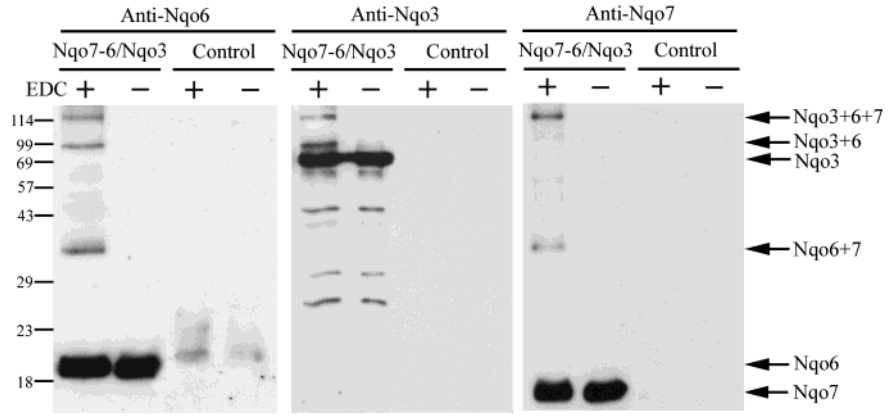

FIGURE 4: Coexpression of the *Paracoccus* Nqo7, Nqo6, and Nqo3 subunits in *E. coli* membranes and effects of EDC treatment on the expressed proteins. *E. coli* was cotransformed with *Paracoccus* genes *nqo7*, *nqo6*, and *nqo3*. Membranes were prepared from the transformed *E. coli* cells and incubated in the presence (+) or absence (−) of 5 mM EDC. Immunoblotting was carried out with affinity-purified antibody against *Paracoccus* subunits Nqo6 (left), Nqo3 (center), and Nqo7 (right). Although the Nqo3 protein tended to be partially degraded, the immunoblotting patterns of the degraded Nqo3 subunit were not changed after treatment with 5 mM EDC. Control membranes were prepared from the nontransformed *E. coli* membranes. Membrane samples (10 μg of protein) were used for immunoblotting analyses. The numbers on the left are the molecular sizes of the marker proteins.

the anti-Nqo4 and anti-Nqo7 antibodies was found predominantly in the membrane fraction, supporting the identification of this cross-linked product. In addition, the anti-Nqo4 antibody reacted with a band with an M_r of ~65 kDa in the EDC-treated *Paracoccus* membranes. The fact that the 65 kDa band was not extracted by NaI suggests that the other subunit cross-linked to Nqo4 in this product may be a membrane domain subunit. Given the M_r of 48 kDa for Nqo4, the partner subunit may have an M_r of 15−25 kDa. Membrane domain subunits that fall in this range are Nqo7, Nqo8, Nqo10, and Nqo11. Antibodies to these subunits were raised against their C-terminal oligopeptides. If the C-terminal parts of these subunits are involved in the cross-linking, antigenic sites may be lost. Therefore, these membrane domain subunits cannot be excluded from the possible candidates.

To further confirm that the Nqo3 and Nqo7 subunits interact with the Nqo6 subunit, we attempted to coexpress *Paracoccus* genes *nqo3*, *nqo6*, and *nqo7* in *E. coli* and investigated whether the expressed subunits are assembled in *E. coli* membranes. It should be noted that when these genes were individually expressed in *E. coli*, the Nqo6 and Nqo7 subunits were found in the membrane (*10*, *19*) and the Nqo3 subunit was in the cytosol (*23*, *33*). For the coexpression experiment, *E. coli* was transformed with two expression plasmids; one bears the *nqo7* and *nqo6* genes, and the other carries the *nqo3* gene. After the transformation, *E. coli* membranes were prepared and subjected to immunoblotting analyses. The *E. coli* membrane preparation contains its own NDH-1. As seen in Figure 4, neither the *E. coli* Nqo3 or Nqo7 subunit reacted with the antibody raised against the respective *Paracoccus* subunit. In the case of Nqo6, the *E. coli* protein exhibited a weak cross reactivity with the anti-*Paracoccus* Nqo6 antibody as reported elsewhere (*19*) but not to an extent that would interfere with the assay. Interestingly, the expressed *Paracoccus* Nqo3 subunit was located in the membrane, indicating that the presence of the other two subunits caused migration of Nqo3 to the membrane fraction. When the *E. coli* membranes containing the three expressed *Paracoccus* subunits were treated with EDC, three new bands appeared at 34, 94, and 115 kDa. The 34 kDa band was recognized by anti-Nqo6 and anti-Nqo7 antibodies, and the 94 kDa band was detected by anti-Nqo6 and anti-Nqo3 antibodies. The 115 kDa band was recognized by all three antibodies. A Nqo3−Nqo7 product was not detected. All the results from the heterologous expression system are in complete agreement with those obtained with the *Paracoccus* membranes. Furthermore, we previously demonstrated that the Nqo7 subunit is attached to the Nqo6 subunit (*19*). Therefore, it is conceivable that the expressed Nqo3 and Nqo7 subunits were cross-linked through the Nqo6 subunit in the *E. coli* membranes. These agree with the concept that the Nqo3 subunit directly interacts with the Nqo6 subunit which then connects to the Nqo7 subunit in the *Paracoccus* membranes and *vice versa*. On the basis of the available data, we have drawn a schematic model of subunit arrangements of Nqo1−7 and Nqo9 as shown in Figure 5.

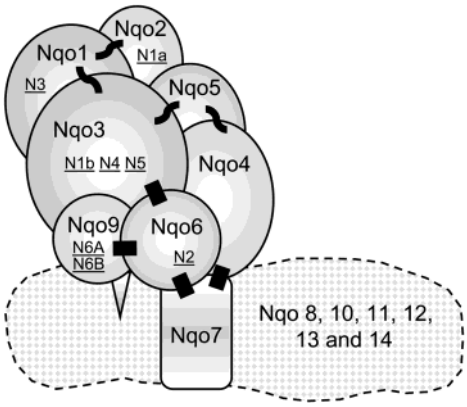

FIGURE 5: Speculative model of subunit interactions among Nqo7, Nqo6, Nqo9, Nqo4, Nqo3, Nqo5, Nqo1, and Nqo2. It should be noted that the sizes of the subunits in the figure are not proportional to their molecular mass. The iron−sulfur centers are underlined. Subunits connected by a solid block were demonstrated to interact in this study. Interactions represented with wavy lines were reported in earlier publications (*19*, *34*).

DISCUSSION

It is known that cofactors of the NDH-1/complex I, FMN, and iron−sulfur centers are all located in the peripheral domain of the enzyme. However, the electron pathway

through the carriers is still largely speculative. Understanding near-neighbor relationships of the subunits should provide a clue as to how the redox events take place within the domain. Earlier cross-linking experiments using isolated bovine complex I and a cross-linker ethylene glycol bis-(succinimidylsuccinate) showed that three subunits, 51k/Nqo1, 24k/Nqo2, and 9k, are associated with each other and that the 51k/Nqo1 subunit is in the proximity of the 75k/Nqo3 subunit (*34*). As illustrated in Figure 5, the Nqo1−3 subunits of the *Paracoccus* enzyme house five iron−sulfur centers, namely, N1a, N1b, and N3−N5 (*31*, *33*, *35*). Given the fact that the Nqo1 subunit binds NADH and presumably FMN, it is likely that the three subunits are involved in earlier steps of the electron transfer (*31*). As for the downstream events, many lines of evidence lead us to believe that center N2 is near or at the end of the chain and plays a key role in the reaction with quinones and that N2 is located in the Nqo6/PSST subunit (*15*, *16*). We have demonstrated in this paper that the Nqo3/75k subunit is in direct contact with the Nqo6/PSST subunit, which now connects the two segments of the electron transfer pathway. In addition, zero-length cross-linking experiments showed that the Nqo9/TYKY subunit harboring centers N6A and N6B is also close to the Nqo6/PSST subunit. The proximity of the Nqo6 and Nqo9 subunits agrees with our previous observations that both subunits are located near the membranes (*19*, *25*). With all things combined, it seems likely that a conduit of the electron pathway in the NDH-1/complex I from NADH to center N2 has been compactly constructed.

Another interesting subunit association revealed by zero-length cross-linking is a Nqo4/49k−Nqo7/ND3 pair. This is the first experimental result that demonstrates a possible direct interaction of the Nqo4/49 kDa subunit with a membrane domain subunit. It is also in agreement with our observation that the *Paracoccus* Nqo4 subunit, when expressed in *E. coli*, is found in both the cytosol and membranes (*10*). The proximity of this subunit to the membrane has been suggested in the case of *Neurospora crassa* complex I by immunolabeling and electron microscopy image analysis (*36*). It should be noted, however, that a similar analysis of *Yarrowia lipolytica* complex I placed the Nqo4/49k subunit near the top of the peripheral domain (*37*). Obviously, more structural information about the Nqo4 subunit is required to clarify this apparent discrepancy.

The Nqo7/ND3 subunit also cross-links to the Nqo6/PSST subunit as we previously reported using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester which has a 9.9 Å arm and contains two distinct reactive groups toward SH and NH_2 residues (*19*). According to our topological study, the *Paracoccus* Nqo7 subunit has three transmembrane segments, and not much is exposed on the cytosolic side (one short loop and a short N-terminus, each consisting of ≤10 amino acid residues) (*22*). The data clearly showed that both the Nqo4 and Nqo6 subunits are cross-linked to rather limited stretches of membrane subunit Nqo7, suggesting that the two subunits are close to each other. This would position the Nqo4/49k subunit close to center N2 in the Nqo6/PSST subunit. The Nqo4 subunit does not carry iron−sulfur clusters but may play a key role in the mechanism of coupling. Dupuis' group (*38*, *39*) hypothesized that the Nqo4 subunit of *Rhodobacter capsulatus* NDH-1 bears the ubiquinone-binding site based on the inhibitor sensitivity of Nqo4 site-specific mutants together with sequence motif analyses of the ubiquinone-binding site (*40*). Interestingly, Ohnishi's group observed strong magnetic interaction between center N2 and semiquinone radical in the oligomycin-treated bovine heart submitochondrial particles, which indicated the proximity (8−11 Å) between the semiquinone radicals and center N2 (*41*). Therefore, this semiquinone radical might represent the quinone-binding site proposed to be on the Nqo4/49k subunit. We cannot, however, eliminate the possibility of the presence of another quinone-binding site as recently reported by using photoaffinity labeling with isolated *E. coli* NDH-1 (*42*).

We are beginning to see the links, albeit slowly, around the central core subunits that connect the peripheral part and the membrane domain of the NDH-1/complex I. A more detailed picture should help elucidate the entire electron pathways and energy coupling of this enzyme.

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