Direct interaction between a membrane domain subunit and a connector subunit in the H^+-translocating NADH-quinone oxidoreductase

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Abstract When *Paracoccus denitrificans* membranes were treated with a crosslinker, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS), a cross-linked product of M_r ~31 kDa was found which reacted with antibodies against the hydrophobic subunit Nqo7 and the connector subunit Nqo6. NaI treatment of the *Paracoccus* membranes before, but not after, the crosslinking step prevented the formation of the 31 kDa band. When Nqo7 and Nqo6 were coexpressed in *Escherichia coli*, both subunits were located in the membrane fraction. MBS treatment of the *E. coli* membranes generated the 31 kDa band as in the *Paracoccus* membranes. These results indicate that Nqo7 interacts with probable N2-binding Nqo6. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: NADH dehydrogenase; Subunit crosslink; Proton pump; Iron–sulfur clusters; Bacterial proton-translocating NADH-quinone oxidoreductase; *Paracoccus denitrificans*

1. Introduction

The proton-translocating NADH-quinone oxidoreductase (NDH-1) in *Paracoccus denitrificans* (a Gram-negative soil bacterium which has been called 'a free-living mitochondrion' [1,2]) is a multi-subunit enzyme complex composed of at least 14 unlike subunits (designated Nqo1–14) [3,4]. The enzyme is located in the cytoplasmic membrane and contains one non-covalently bound flavin mononucleotide and eight iron–sulfur clusters as cofactors [5]. Of these iron–sulfur clusters, six are electron paramagnetic resonance (EPR)-detectable (designated N1a and N1b for binuclear iron–sulfur ([2Fe–2S]) clusters and N2, N3, N4, and N5 for tetranuclear iron–sulfur ([4Fe–4S]) clusters). These prosthetic groups are utilized to transfer electrons from NADH to quinone (Q) and during this process protons are pumped across the cytoplasmic membrane. It is generally accepted that NDH-1 and complex I (mitochondrial proton-translocating NADH-quinone oxidoreductase) can be divided into two segments [6–8]. One is a peripheral segment made up of seven subunits (Nqo1–6 and 9) and extruded into the cytoplasmic side (or mitochondrial matrix) and bears all prosthetic groups. The other is a membrane domain segment bearing seven hydrophobic subunits (Nqo7, 8 and 10–14) [8]. The Nqo6 and 9 subunits are believed to function as connectors between the peripheral and the membrane segments [8]. It is a pre-requisite for structural studies of NDH-1/complex I to clarify subunit interaction of these enzyme complexes. Although to date there is some information about subunit interaction of the peripheral segment [9–17], little is known about subunit interaction of the membrane segment and about subunit interaction between the peripheral and membrane segments [18].

Recently we have determined the topology of the *P. denitrificans* Nqo7 subunit [19]. This subunit contains three transmembrane segments (tentatively designated TM1, TM2 and TM3 from the N-terminus to C-terminus) and its N- and C-terminal regions are directed toward the cytoplasmic and periplasmic phases of the membrane, respectively [20]. In the present paper, utilizing crosslinking experiments and heterologous expression, we have shown that the membrane domain subunit Nqo7 directly interacts with the connector subunit Nqo6 which is believed to house center N2.

2. Materials and methods

Cholate-treated *P. denitrificans* membranes were prepared as described previously [7]. To 150 μl of the cholate-treated membrane suspension (1 mg/ml) in 50 mM K-phosphate buffer (pH 7.5) were added 2 μl of 10 mM *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS, from Pierce) in dimethyl sulfoxide and the suspension was incubated for 45 min at room temperature on a rotating wheel. Then the suspension was immediately diluted two-fold with 2× Laemmli sample buffer and applied on sodium dodecyl sulfate (SDS) gels.

For removal of the peripheral subunit from the membranes, the cholate-treated membranes were incubated with 2 M NaI for 10 min at 30°C, and freeze–thawed three times in liquid nitrogen/water bath at 30°C. The suspension was airfuged at 30 psi for 5 min. The pellet was resuspended in 50 mM K-phosphate buffer (pH 7.5). This NaI treatment was performed twice on each sample to remove the peripheral subunits from the *P. denitrificans* membranes. The membranes thus prepared were used immediately for crosslinking experiments. The NaI treatment removed the Nqo1 (NADH-binding) subunit completely and 70–80% of the Nqo6 subunit from the *P. denitrificans* membranes. However, the Nqo7 subunit was not extracted by this treatment.

A DNA fragment bearing *nqo7* and *nqo6* genes was prepared by polymerase chain reaction with pXT-3 as the template [21]. A sense primer (5′-CCAAC*CATA*TGGAATACCTGCTGCAAG-3′) was employed to generate a *Nde*I site at the translation initiation codon of *nqo7* gene and an antisense primer (5′-CTTCGGA*CTCGAG*TCAC-

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Abbreviations: Q, quinone; NDH-1, bacterial proton-translocating NADH-quinone oxidoreductase; complex I, mitochondrial proton-translocating NADH-quinone oxidoreductase; EPR, electron paramagnetic resonance; [2Fe–2S], binuclear iron–sulfur cluster; [4Fe–4S], tetranuclear iron–sulfur cluster; MBS, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester

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CTCACCAGCG-3′) was used to produce a *Xho*I site in the downstream of the termination codon of the *nqo6* gene. The underlined bases were altered from *Paracoccus* DNA for the mutations and the italicized bases exhibit the *Nde*I and *Xho*I sites. The DNA was cloned into expression plasmid pET24a(+). The resulting expression plasmid is designated pET24a(NQO7,6). Competent *Escherichia coli* strain BLR(DE3)pLysS was transformed with pET24a(NQO7,6). The transformed cells were spread on 2×YT plates containing 100 μg/ml kanamycin and cultivated at 30°C overnight. A well-isolated colony was inoculated into 2 ml of 2×YT medium containing 100 μg/ml kanamycin and cultivated to stationary phase at 37°C. The 2 ml seed culture was transferred into 1 l TB culture medium containing 100 μg/ml kanamycin. Cells were grown at 24°C until $A_{600} = 0.2$, at which time 0.05 mM isopropyl-β-D-thiogalactopyranoside was added, and cultured overnight in an orbit shaker at 150 rpm. Cell membranes were prepared as described in [22]. It should be noted that the inclusion bodies were completely removed from the cell membrane preparation by repeated centrifugations.

Protein concentration was estimated by the method of Lowry et al. [23]. Any variations from the procedures and details are described in the figure legends.

3. Results

As described previously [19], antibody against the C-terminal region of the deduced primary structure of the Nqo7 subunit (Nqo7c) mainly reacted with a 14 kDa-band of the *P. denitrificans* membranes (see Fig. 1). Although a weak band at 18 kDa was occasionally recognized by the Nqo7c antibody, this band did not react with the antibody directed to the N-terminal oligopeptide of the Nqo7 (Nqo7n). It is previously reported that the antibody against Nqo7n did not recognize the Nqo7 subunit when the α-amino group was modified [19]. Therefore, it seems likely that the weak 18 kDa band may be due to the Nqo7 subunit whose α-amino group was modified. After the *P. denitrificans* membranes were treated with a crosslinker MBS which has a 9.9 Å arm and contains two distinct reactive groups toward SH and NH_2 residues, two new bands were recognized by the anti-Nqo7c antibody. As shown in Figs. 1 and 2, the M_rs of the strong and faint bands were 31 and 38 kDa, respectively. On the basis of the molecular size of the *Paracoccus* NDH-1 subunits in Table 1, we can exclude the possibility that subunit Nqo1, 3, 4, 12, 13, or 14 is involved in the 31 or 38 kDa band. Therefore, possible candidates for the 31 and 38 kDa bands are the Nqo2, 5, 6, 8, 9, 10, and 11 subunits. Antibodies directed against the individual candidate subunits (except for Nqo10) are available in this laboratory. Of these, only the antibody against the Nqo6 subunit recognized the 31 kDa band (Fig. 1). None of the seven antibodies reacted with the 38 kDa band (data not shown). The molecular size of the 31 kDa appears to reasonably fit the sum (33 kDa) of Nqo7 (14 kDa) and Nqo6 (19 kDa). As described in Section 1, the Nqo6 subunit belongs to the peripheral segment, which can be detached from the membranes by a chaotropic agent such as NaI [8]. On the other hand, the Nqo7 subunit is located in the membrane domain segment and thus cannot be extracted by chaotrope treatment. In order to confirm that the 31 kDa band is composed of the Nqo7 and Nqo6 subunits, we investigated whether the 31 kDa band was recognized by the antibodies specific to the Nqo7c and the Nqo6 subunit using *P. denitrificans* membranes treated two times with 2 M NaI prior to the MBS incubation. As seen in Fig. 2, pre-treatment with NaI prevented the formation of the cross-linked product (31 kDa band) by MBS. On the other hand, post-treatment with NaI did not affect the amount of MBS-cross-linked 31 kDa band in the membranes. Therefore, it is conceivable that, in the *P. denitrificans* NDH-1, the Nqo7 subunit is located in the proximity of the Nqo6 subunit in the range less than 9.9 Å. These data also exclude the possibilities that the Nqo10 subunit is responsible for the 31 kDa band and that the 31 kDa band is made up of an oligomer of Nqo7 subunit.

Table 1
Comparison of molecular size of NDH-1 subunits in *P. denitrificans*

Subunit (Nqo-)	MW deduced from DNA sequence	M_r determined by Laemmli SDS gel
1	47k	50k
2	26k	25k
3	73k	66k
4	47k	48k
5	24k	28k
6	19k	20k
7	14k	14k
8	39k	30k
9	19k	22k
10	22k	–
11	11k	10k
12	78k	–
13	56k	–
14	53k	–

–, not determined.

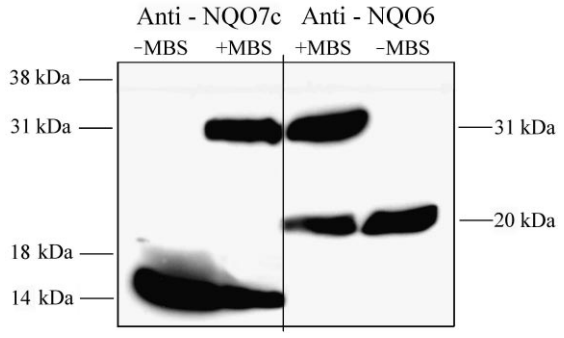

Fig. 1. Immunoblotting of the cholate-treated *P. denitrificans* membranes with and without MBS treatment using Nqo7c antibody (left) and Nqo6 antibody (right). The membranes (10 μg of protein) were applied on each lane of a Laemmli SDS–13% polyacrylamide gel. Immunoblotting was performed with the Pierce Western Pico system.

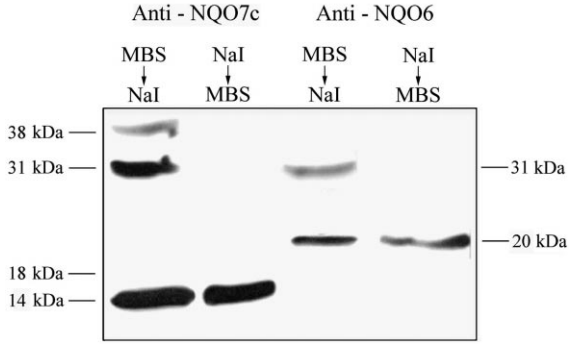

Fig. 2. Effect of NaI extraction on the cross-linked 31 and 38 kDa products. Membranes were treated with 2 M NaI treatment either before crosslinking (NaI → MBS) or after crosslinking (MBS → NaI). Anti-NQO7c and Anti-NQO6 show the immunoblotting with the antibodies specific to Nqo7c and Nqo6, respectively.

The next question is whether these two subunits directly interact with each other. For this purpose, we attempted to coexpress *P. denitrificans nqo6* and *nqo7* genes in *E. coli* and to examine whether the two subunits are assembled in *E. coli* membranes. If assembled, it is expected that MBS treatment could generate the same 31 kDa cross-linked product as seen in the *P. denitrificans* membranes. It has been reported that the expression of non-fused full-length Nqo7 in *E. coli* was unsuccessful [19]. In contrast, the Nqo7 subunit was stably expressed when a DNA fragment bearing the *nqo7* and *nqo6* genes was used (Fig. 3). The Nqo7- and Nqo6-coexpressed *E. coli* membranes exhibit a greenish brown color, whereas control *E. coli* membranes looked whitish. The observed color of the coexpressed membranes might come from an iron–sulfur cluster in the Nqo6 subunit. Fig. 3 shows the effect of MBS on coexpressed *P. denitrificans* Nqo7 and Nqo6 in *E. coli* membranes. The antibody directed against the *P. denitrificans* Nqo7c did not react with its *E. coli* homolog as shown in the previous paper [19], whereas the antibody against *P. denitrificans* Nqo6 subunit weakly cross-reacted with its *E. coli* counterpart (Fig. 3). In contrast, the Nqo7- and Nqo6-coexpressed *E. coli* membranes were strongly recognized by both antibodies at the predicted molecular sizes. After MBS treatment of the coexpressed *E. coli* membranes, both antibodies reacted with a 31 kDa band just as observed with the *P. denitrificans* membranes (Fig. 3). No other cross-linked product was detected by the antibody to Nqo7c or Nqo6. Taking these results together, we conclude that the Nqo7 subunit directly interacts with the Nqo6 subunit in the *P. denitrificans* NDH-1.

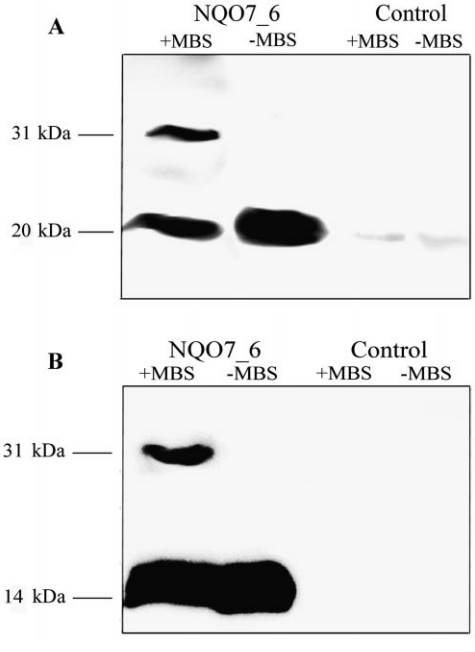

Fig. 3. Effect of MBS treatment on the Nqo7- and 6-coexpressed *E. coli* membranes. Immunoblotting was performed with the Nqo6 antibody (A) and the Nqo7c antibody (B). Control, membranes prepared from *E. coli* transformed by pET24a(+); NQO7_6, the membranes isolated from Nqo7- and 6-coexpressed *E. coli*. (+MBS) and (−MBS) reveal with and without MBS treatment, respectively. The membranes (10 μg) were loaded on each lane.

4. Discussion

This paper demonstrates that the Nqo7 subunit and the Nqo6 subunit are tightly associated physically, and most likely functionally as well. It may be worthwhile to note that gene arrangements of the NDH-1 operons (gene clusters) are highly conserved [5] and that the *nqo7* and *nqo6* genes are adjacent to each other, being the first and the second. As described above, only coexpression of the *nqo7* and *nqo6* genes stably produced the Nqo7 subunit. Similar results were observed for the *nqo2* (fifth) and *nqo1* (sixth) genes. Expression of the Nqo1 (NADH-binding) subunit in the cytoplasmic phase instead of inclusion bodies required coexpression of the Nqo2 subunit [9]. Furthermore, the *nqo5* (third) and *nqo4* (fourth) genes are fused in certain bacteria (including *E. coli*). Thus, it may be speculated that the gene order in the NDH-1 operons is related to the spatial arrangement of the subunits.

A question arises as to which parts of the subunits are involved in the interaction between the Nqo7 and the Nqo6 subunit. We recently reported that antibodies directed against N- and C-terminal oligopeptides of the Nqo7 subunit react with the *P. denitrificans* membranes [19]. This fact indicates that the N- and C-terminal stretches of the Nqo7 subunit do not participate in assembly between the Nqo7 and Nqo6 subunit. Hydropathy plots of the Nqo6 subunit may suggest the absence of a transmembrane segment in this subunit. Since the Nqo6 subunit is known to be located in the cytoplasmic side, it seems unlikely that the Nqo6 subunit interacts with a long loop between the transmembrane segment 1 (TM1) and TM2 of the Nqo7 subunit. That is because this loop is exposed to the periplasmic side [19]. (Recently it has been reported that mutation of non-conserved Ser45 to Pro in the human ND3 subunit (a Nqo7 homolog) in this loop caused complex I-deficient mitochondrial disease [24], so that this loop may contribute to the activity of complex I.) Therefore, it is likely that some parts of the three transmembrane segments and a short loop between TM2 and TM3 of the Nqo7 subunit are involved in the assembly between the Nqo7 and Nqo6 subunits. The refinement of the interaction part(s) remains to be seen.

The Nqo6/PSST/NuoB (*Paracoccus*/mitochondria/*E. coli* terminology) subunit is considered to be a probable candidate subunit bearing center N2 [6]. Center N2 has the highest and pH-dependent redox potential. EPR studies exhibited that rotenone and piericidin A dissect electron transfer from N2 to Q [25]. In addition, it has been reported that the Nqo6/PSST subunit is a target of photoaffinity labeling of pyridaben, a specific inhibitor of NDH-1/complex I [26]. This labeling was displaced by rotenone, piericidin A, and rolliniastatin. Therefore, it is conceivable that the Nqo6/PSST subunit is involved in the reaction mechanism of the coupling site 1. As described above, the Nqo6/PSST subunit appears to lack a transmembrane segment which is indispensable for proton translocation. Our results indicate that the Nqo7/ND3 subunit having three transmembrane segments is associated with the Nqo6/PSST subunit and thus provides a membrane foundation for the Nqo6/PSST subunit. It may be hypothesized that the Nqo7/ND3 subunit is a constituent of a proton channel (or proton wire) because it bears two highly conserved carboxyl residues (D74 and E76, *Paracoccus* numbering) in the middle of TM2. It will be of interest to investigate whether these

conserved residues of the Nqo7/ND3 subunit are involved in the proton translocation utilizing site-specific mutation procedures.

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