Functional Roles of Four Conserved Charged Residues in the Membrane Domain Subunit NuoA of the Proton-translocating NADH-Quinone Oxidoreductase from Escherichia coli*

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The H⁺(Na⁺)-translocating NADH-quinone (Q) oxidoreductase (NDH-1) of Escherichia coli is composed of 13 different subunits (NuoA–N). Subunit NuoA (ND3, Nqo7) is one of the seven membrane domain subunits that are considered to be involved in H⁺(Na⁺) translocation. We demonstrated that in the Paracoccus denitrificans NDH-1 subunit, Nqo7 (ND3) directly interacts with peripheral subunits Nqo6 (PSST) and Nqo4 (49 kDa) by using cross-linkers (Di Bernardo, S., and Yagi, T. (2001) FEBS Lett. 508, 385–388 and Kao, M.-C., Matsuno-Yagi, A., and Yagi, T. (2004) Biochemistry 43, 3750–3755). To investigate the structural and functional roles of conserved charged amino acid residues, a nuoA knock-out mutant and site-specific mutants K46A, E51A, D79N, D79A, E81Q, E81A, and D79N/E81Q were constructed by utilizing chromosomal DNA manipulation. In terms of immunochemical and NADH dehydrogenase activity-staining analyses, all site-specific mutants are similar to the wild type, suggesting that those NuoA site-specific mutations do not significantly affect the assembly of peripheral subunits in situ. In addition, site-specific mutants showed similar deamino-NADH-K₃Fe(CN)₆ reductase activity to the wild type. The K46A mutation scarcely inhibited deamino-NADH-Q reductase activity. In contrast, E51A, D79A, D79N, E81A, and E81Q mutation partially suppressed deamino-NADH-Q reductase activity to 30, 90, 40, 40, and 50%, respectively. The double mutant D79N/E81Q almost completely lost the energy-transducing NDH-1 activities but did not display any loss of deamino-NADH-K₃Fe(CN)₆ reductase activity. The possible functional roles of residues Asp-79 and Glu-81 were discussed.

The bacterial H⁺(Na⁺)-translocating NADH-quinone oxidoreductase (NDH-1),† also known as complex I in mitochon-
Roles of Glu-81 and Asp-79 of NuoA Subunit in E. coli NDH-1

Cloning and Mutagenesis of the E. coli nuoA Gene—

The gene encoding the NuoA subunit together with a 1-kb DNA segment upstream and downstream of the translation site sequence. The spectinomycin-encoding gene from transposon Tn554 of Staphylococcus aureus (28) was cloned by the PCR methodology using the sense primer 5'-CCAGGAAAATTCC-3', which contains a BglII restriction site (italicized), was used together with the NotI-generating antisense primer to produce a DNA fragment that was cloned in pCRScript. Then the sense primer 5'-CCACGCGAGTTGCGAACGTGCTTTGTCG-3', which contains a ClaI restriction site (italicized) was used together with the SalI-generating antisense primer to produce a DNA fragment used for the generation of nuoA point mutants. The DNA insert in the pCRScript cloning plasmid was mutagenized with the mutagenesis primers shown in Table I. These fragments were also assembled in pCRScript and then cloned into pKO3.

The first step of site-specific mutation of the nuoA gene of the E. coli NDH-1 operon was to construct a nuoA gene knock-out mutant. For this purpose, we employed the pKO3 system developed by Church and co-workers (29). In brief, the pKO3 vector contains a repA(TEs) (temperature-sensitive replication origin), a chloramphenicol-resistant gene (cat), and a Bacillus subtilis sacB gene encoding levansucrase. The pKO3 carrying nuoA-knock-out DNA was prepared as follows (see Fig. 1, a and b). DNA fragments, Smal/NotI (1467 bp) and NotI/Sall (1269 bp), were amplified from E. coli chromosomal DNA by PCR and individually cloned in vector pCRScript at the SrfI site as a blunt end fragment. A PCR-amplified spc cassette carrying NotI site (1200 bp) was also inserted at the SrfI site of pCRScript. The two DNA fragments and the spc cassette were assembled in pKO3. The resulting plasmid, pKO3(nuoA::spc), lacks 90 bp of the nuoA gene, which has been replaced by the spc cassette.

Electrophoresis of Knock-out and Mutant Cells—E. coli strain MC4100 (F^-, araD139, Δarg F-lac U169, ptsF25, relA1, ftsK301, rpsL 150.9^+) was transformed with pKO3(nuoA::Spc) plasmid, and recombination was carried out as described in Link et al. (29). In brief, several isolates of LB agar plates containing 20 μg/ml chloramphenicol and 100 μg/ml Spc grew overnight at 30 °C, were transferred into 100 μl of LB and serially diluted. The dilutions corresponding to 10^-1 to 10^-9 were then plated on LB agar plates containing 20 μg/ml chloramphenicol and 100 μg/ml Spc. The plates were incubated at 43 °C overnight. The next day several colonies (typically 5) were transferred from the 43 °C plates into 100 μl of LB, serially diluted, and plated on LB agar plates containing 5% sucrose at 30 °C overnight. The surviving colonies were then replica-plated on LB agar plates containing 20 μg/ml chloramphenicol and on LB plates containing 100 μg/ml Spc and grown at 30 °C overnight. Colonies sensitive to chloramphenicol but resistant to Spc were used for the PCR amplification of the nuoA region using the 5'-oligonucleotide CTGAAACATGGGACTTCC (chof) and the 3'-oligonucleotide AAGGAGCGCCGCTTTCTATTTTCAATGTTAC (spc3). The chof oligonucleotide was designed to amplify DNA from within

| Primer | Mutation | Mutagenic primer sequence^a |
|--------|----------|-----------------------------|
| NuoA(K46A) | 5'-CACGGCGGAGCTGGCAGACGTGGCTTTGTCG-3' |
| NuoA(E51A) | 5'-GACCCCCGCTCGAATTCCATGCGCGCT-3' |
| NuoA(D79A) | 5'-CGTATCTCGAGCATGTCGAAGC-3' |
| NuoA(E81A) | 5'-GATCTTGCACTGCGCTGCTTGTCG-3' |
| NuoA(E81Q) | 5'-CTTGAGGTCTAAGGCGCTGTGA-3' |
| NuoA(D79N) | 5'-GGTATCTGCGACATGTCGAAGC-3' |
| NuoA(D79N) | 5'-GGTATCTGCGACATGTCGAAGC-3' |

^a Underline indicates mutation.
the E. coli chromosomal and the spc' oligonucleotide from within the Spc cassette. In this way the presence of the Spc cassette and its location in the genomic DNA was confirmed. The knocked-out MC4100 cells where then stored as glycerol stocks at −80 °C. Knocked-out MC4100 competent cells were then employed to introduce nuoA mutated DNA in the E. coli genome using a similar procedure except that the identification of recombinants was carried out by screening for spectinomycin sensitivity in addition to chloramphenicol sensitivity.

To confirm the presence of the mutations, the sense oligonucleotide chro5' and the antisense oligonucleotide CATACGTCGCGGCCTG (nuoA3), which is located inside the nuoA gene, were used as primers for PCR amplification of the nuoA DNA fragments. The nuoA DNA fragments produced were subjected to direct sequencing.

Antibody Production—Antibodies directed against the 12-amino-acid oligopeptide corresponding to the C-terminus region of the E. coli NuoA subunit were produced as follows. An oligopeptide H-CNPFTNSIAN-RQR-OH was synthesized (designated NuoAc) and conjugated to maleimide-activated bovine serum albumin (Pierce) according to the manufacturer's protocol. It should be noted that, for the purpose of conjugation with bovine serum albumin, a cysteine residue was added to the N terminus. For raising antibodies specific to subunits NuoB, NuoE, NuoF, NuoG, and NuoI inclusion bodies of the overexpressed subunits were used as described previously (7). The antibodies were affinity-purified according to Han et al. (30).

Cell Growth and Membrane Preparation—For the preparation of membranes suitable for enzymatic assays wild type, knock-out, and point mutants were grown in 250 ml of Terrific Broth medium until A600 was 2–2.2. The cells were then harvested in a GSA rotor at 4000 rpm for 10 min. The cell pellet was resuspended at 10% (w/v) in a buffer containing 10 mM Tris-HCl (pH 7.0), 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 15% (v/v) glycerol. The cell suspension was then passed once in a French press at 25,000 p.s.i. and centrifuged again in the GSA rotor at 12,000 rpm for 10 min. Cell debris was discarded, and the supernatant was then ultracentrifuged in a 70Ti rotor at 50,000 rpm for 30 min. The pellet was resuspended in the same buffer and was used immediately for enzymatic activity measurements.

**Enzymatic Assay**—It is recognized that deamino-NADH can be catalyzed by only 5-kb long (35, 36). Because this length does not allow incorporation of the whole operon into expression vectors, a site-specific mutation is traditionally carried out by complementation of a cassette-inserted gene with a mutated gene in the expression plasmid (designated in trans complementation) (37–49). However, in trans complementation procedure presents some problems when applied to a gene cluster. For example, the cassette inserted in chromosomal DNA might interrupt the expression of the downstream genes (44). The only case that does not suffer this polar effect is when the mutated gene is at the last position in the operon (38). Another problem is that the mutated gene is under the control of a promoter in the expression plasmid, which often leads to overexpression of the mutated subunit. In mutation studies of the NDH-1 using the in trans complementation, it has been reported that the enzyme activities were significantly low (~20% of the wild type cells) even when the unmutated gene was used (42, 43, 45). An alternative method of site-directed mutation is to introduce mutations directly in chromosomal DNA as detailed in this work (designated chromosomal DNA mutation) (Fig. 2A) (39, 42). In this procedure expression of all genes of the operon are regulated by the authentic promoter. Although chromosomal DNA mutation is laborious and time-consuming, we adopted this technique to produce NuoA mutants to minimize any complications derived from disruption of the operon. As anticipated, the NDH1 was apparently expressed at the same level in all mutants as in the wild type (see below).

**Sequence Analysis of the NuoA Subunit**—Fig. 2A is an amino acid sequence comparison between the E. coli NuoA subunit and its counterparts of various organisms. In terms of hydropathy plots, the E. coli NuoA subunit is akin to its counterpart of P. denitrificans. Fig. 2B is a hypothetical topology of the E. coli NuoA subunit deduced from topological studies of the P. denitrificans NuoA subunit (11). The E. coli NuoA subunit is predicted to contain the three transmembrane segments (designated TM1–3 from N to C terminus). The N- and C-terminal regions are also predicted to be directed toward the cytoplasmic and periplasmic phases of the membrane, respectively. In addition, a long loop (L1) between TM1 and TM2 is exposed to the periplasmic side. As far as our data base search is concerned (more than 250 organisms), Asp-79 (E. coli numbering) is conserved except for its homologues of Cyanidium caldarium mitochondria (Asp-79 → C, CA88774) and Pseudomonas aeruginosa (Asp-79 → G, D83410). On the other hand, Glu-81 is perfectly conserved. Asp-79 and Glu-81 (E. coli numbering) seem to be located in the middle of the TM2. Carboxyl residues are rarely located in the middle of TM of the hydrophobic polypeptides. Therefore, it has been generally recognized that...
carboxyl residues present in the TM may play important roles in cation translocation of the membrane-associated enzyme complexes (46, 47). One well known example is a perfectly conserved carboxyl residue in the center of a transmembrane helix of the N, N-dicyclohexylcarbodiimide (DCCD)-binding protein (also called subunit c or proteolipid subunit) of the ATP synthase (48). This carboxyl residue is clearly involved in the mechanism of proton translocation catalyzed by the membrane sector of the ATP synthase (49). It has been demonstrated that DCCD also inhibits energy-transducing electron transfer of the NDH-1/complex I (50, 51). It is therefore possible that the Asp-79 and/or Glu-81 may be involved in the proton translocation of the energy coupling site 1. To clarify the structural and functional roles of these carboxyl residues, we have constructed site-directed mutants of these two residues (D79A, D79N, E81A, E81Q, D79N/E81Q) by using chromosomal DNA mutation procedures instead of the in trans complementation method. In addition, it has been reported that a heteroplasmic mutation of S34P and S45P (human numbering) in the human ND3 subunit (a homologue of the NuoA) drastically reduced the complex I activity and caused encephalopathy (25, 26). These Ser residues appear to be located in the Loop 1 directed against the cytoplasmic phase and periplasmic phase in eukaryotes and bacteria, respectively (see Fig. 2A). Unfortunately, neither Ser-34 nor Ser-45 is conserved in the bacterial NDH-1. Therefore, we constructed mutations in charged residues that are well conserved (K46A, E51A) to assess whether this loop is involved in function of the NDH-1/complex I. Seven site-specific mutants (nuoA-K46A, -E51A, -D79A, -D79N, -E81A, -E81Q, -D79N/E81Q) were generated. The mutations were confirmed by direct DNA sequencing analyses.

**Subunit Assembly of NDH-1 in NuoA Mutants**—Fig. 3 illustrates Western blot analyses of the membranes isolated from wild type and the NuoA null mutant with affinity-purified antibodies to the NuoAc and peripheral subunits NuoE, NuoF, NuoG, and NuoI of the E. coli NDH-1. The antibody to the NuoAc reacted with the wild type but did not react with the nuoA::spc mutant (knock-out mutant). In addition, mutants...
Roles of Glu-81 and Asp-79 of NuoA Subunit in E. coli NDH-1

K46A, E51A, D79A, D79N, E81A, E81Q, and D79N/E81Q were recognized by the NuoAc antibody. In contrast, membranes isolated from the wild type and all available site-specific NuoA mutants seem to bear similar amounts of peripheral subunits NuoE, NuoF, NuoG, and NuoI. Subunits NuoE and NuoF (the NADH-binding subunit) are known to be essential for deamino-NADH oxidase activity and deamino-NADH-DB reductase activity (52). These results suggest that site-specific NuoA mutants apparently remain intact in subunit assembly in all NuoA mutants examined. To further confirm this point, isolated membranes were treated with dodecylmaltoside and subjected to blue native polyacrylamide gel electrophoresis. Then, the gels were stained for NADH dehydrogenase activity using NADH and p-nitroblue tetrazolium (Fig. 4). Two bands appeared in the wild type membranes. The upper band, but not the lower band, was recognized by the antibody to the peripheral subunit NuoE. In addition, the antibody specific to NuoAc reacted with the upper band (data not shown). Furthermore, the membranes isolated from the knock-out mutant lacked the NADH dehydrogenase activity and reactivity with the NuoE antibody in the upper band position. The data indicate that the NADH dehydrogenase activity of upper band is because of NDH-1. As shown in Fig. 4, all of the site-specific nuaA mutants showed a comparable NADH dehydrogenase activity band because of the NDH-1. In addition, the site-specific NuoA mutants are similar to wild type in terms of relative molecular size of the NDH-1 bands. It seems likely that constructed site-specific mutants are similar to wild type in terms of subunit assembly.

Effects of NuoA Mutation on the NDH-1 Activity—We measured activities of NDH-1 using membranes prepared from wild type and NuoA mutants (Table II). E. coli membranes contain a second type of NADH dehydrogenases (NDH-2). To eliminate contribution from the NDH-2, deamino-NDH was used as the substrate in all assays, because NDH-2 cannot utilize this compound (32). First, it should be noted that deamino-NDH-K3Fe(CN)6 reductase activity of all site-specific mutants was comparable with that of the wild type, whereas the activity of the knock-out mutant was almost null. These results are consistent with the data from the NADH dehydrogenase activity staining of the native gels, suggesting that none of the mutations affected the assembly of the NDH-1 subunits. Second, deamino-NDH oxidase activity and deamino-NDH-DB reductase activity behaved in a similar fashion among the mutants tested, indicating that the inhibitory effect observed was solely because of the NDH-1 mutation.

Single-residue mutations introduced into the Loop 1 (Lys-46 and Glu-51) or the middle of the TM2 (Asp-79 and Glu-81) resulted in either no inhibition or partial (up to 70%) inactivation. However, when the two carboxyl residues in the TM2 were mutated simultaneously (D79N/E81Q), NDH-1 activities were almost completely abolished (see also Fig. 5). It was, therefore, of particular interest to examine the sensitivity of the NDH-1 activity of these mutants to DCCD. As anticipated, mutations in the Loop 1 region (Lys-46 and Glu-51) showed the same degree of DCCD inhibition as the wild type. Of the two mutants in the TM2, Asp-79 had the same DCCD sensitivity as the wild type. In contrast, the Glu-81 mutants were less sensitive to DCCD treatment than other mutants and the wild type. It remains to be seen whether Glu-81 is one of the target sites of DCCD binding.

Miyoshi and co-workers (34) reported that cap-40 acts as a competitive inhibitor for quinone in the NDH-1/complex I and suppresses only the energy-coupled activity. We found that all NuoA mutants described above were almost completely inhibited by cap-40 (data not shown). Furthermore, the I50 values of cap-40 for the mutants were about the same as that of the wild type (Table II), suggesting that the quinone-binding site is not modified by these mutations.

**DISCUSSION**

The membrane domain of the NDH-1 is composed of seven subunits, which are homologues of mitochondrially encoded ND subunits. This domain is involved in H+ (or Na+) translocation in the coupling site 1 (18) but lacks any cofactors. In addition, DCCD, known to specifically modify carboxyl residues located in the hydrophobic environment, inhibits the energy-coupled activities of the NDH-1/complex I. It is therefore speculated that conserved carboxyl residues located in the middle of membranes might participate in cation translocation in the coupling site 1. On the basis of the deduced primary sequence analysis, we predicted that there are eight highly conserved
roles of Glu-81 and Asp-79 of NuoA subunit in E. coli NDH-1

The mutants had moderately reduced amounts (44–65% of wild type) of dNADH-O₂ reductase activity and removal of one of them leads to a partial or substantial loss of coupled activity. Although we do not know the relative positioning of the two subunits, NuoA and NuoK, it might be possible that together they provide negatively charged groups that constitute the H⁺ or Na⁺ binding site as reported for certain cation transporters (47, 53, 54).

Recently, it has been reported that two mutations of the ND3 gene (S34P, T10158C; S45P, T10191C) induced infantile mitochondrial encephalopathy (26) and a progressive mitochondrial disease (25). The two Ser residues are not conserved and located in Loop 1 segment of the NuoA/ND3 subunit (see Fig. 2). The mutants had moderately reduced amounts (44–65% of complex I but drastically reduced amounts of complex I activity (1–11% remaining) (26). According to the predicted topology of the NuoA subunit, the Loop 1 segment is localized in the periplasmic phase and, therefore, may not interact with the peripheral segment of NDH-1/complex I. We have constructed and characterized mutants of highly conserved charged residues, Asp-79 and Glu-81, of the E. coli NuoA subunit. As it turned out, mutating only one of them caused either little or partial inhibition of energy-coupled activities (deamino-NADH-O₂ and deamino-NADH-DB). However, these activities were almost completely lost when both carboxyl groups were removed. There are at least two explanations for the results obtained with the NuoA mutants. One is that the residues Asp-79 and Glu-81 synergistically contribute to the maintenance of an intact architecture of the NDH-1, and thus disruption of both but not either one of them results in drastic change in the structure. However, this seems unlikely because in all mutants examined the assembly of the whole enzyme seems to be normal, and the deamino-NADH-Fe(CN)₆ activity remained unchanged. Another possibility is that Asp-79 and Glu-81 are both involved in the mechanism of ion translocation, but they may work in a compensatory manner. In other words, subunit NuoA needs to have at least one carboxyl group in the area where the two residues are located for the NDH-1 to function as a pump. It can be further postulated that Glu-81 may be in a more favorable position than Asp-79, because omission of the latter has much less impact on the coupled activities. In fact, Glu-81 is perfectly conserved in all sequences available in data bases, whereas Asp-79 is replaced with C in C. caldarium mitochondria (CAA88774) and with G in P. aeruginosa (D83410). The significance of the presence of two carboxyl groups in NuoA is somewhat similar to that in NuoK. As described above, the NuoK subunit has two highly conserved glutamic acids presumably in the middle of transmembrane segments. Both residues seem to be required for the optimal activity and removal of one of them leads to a partial or substantial loss of coupled activity. Although we do not know the relative positioning of the two subunits, NuoA and NuoK, it might be possible that together they provide negatively charged groups that constitute the H⁺ or Na⁺ binding site as reported for certain cation transporters (47, 53, 54).

Roles of Glu-81 and Asp-79 of NuoA Subunit in E. coli NDH-1

Table II: Enzyme activities of the membrane-bound NDH-1 of E. coli wild type and various NuoA mutants

| NuoA mutant   | dNADH-O₂ | dNADH-DB | DCCD inhibition | dNADH-Fe(CN)₆ | dNADH-DB DCCD inhibition |
|---------------|----------|----------|----------------|--------------|--------------------------|
| Wild          | 461 (100%) | 0.13     | 588 (100%)     | 79%          | 1264 (100%)              |
| KO            | 5 (1%)   | 0.10     | 47 (8%)        | 1210 (97%)   | 1210 (97%)               |
| K46A          | 460 (100%) | 0.10     | 534 (94%)      | 1248 (100%)  | 1248 (100%)              |
| E51A          | 143 (31%) | 0.10     | 171 (30%)      | 1275 (102%)  | 1275 (102%)              |
| E81A          | 205 (44%) | 0.11     | 208 (37%)      | 1255 (99%)   | 1255 (99%)               |
| E81Q          | 194 (42%) | 0.11     | 207 (36%)      | 1200 (96%)   | 1200 (96%)               |
| D79N/E81Q     | 357 (77%) | 0.12     | 285 (50%)      | 1170 (94%)   | 1170 (94%)               |
dues Lys-46 and Glu-51, which are present in the Loop 1 segment. Although the K46A mutation did not affect any NDH-1 activities, E51A mutation showed a significant inhibition (−70% inhibition) of the energy-transducing NDH-1 activities. On the other hand, neither mutation induced any drastic modification of subunit assembly or capsaicin sensitivity. The data suggest that the Loop 1 segment may be involved in the NDH-I/complex I activity, although this loop faces the periplasmic phase (the cytoplasmic phase in mitochondria). In eu-

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