Structural Contribution of C-terminal Segments of NuoL (ND5) and NuoM (ND4) Subunits of Complex I from Escherichia coli*5

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The proton-translocating NADH-quinone oxidoreductase (complex I, EC 1.6.5.3) is the first enzyme of the respiratory chain in most eukaryotic cells. Complex I catalyzes the electron transfer from NADH to quinone, which is linked to the translocation of four protons through the inner mitochondrial membrane. This enzymatic complex, made up of 45 different polypeptides, is one of the largest enzymes of the respiratory chain, with a molecular mass of ~1000 kDa (1, 2). The physiological importance of complex I is highlighted by the fact that this enzyme is the principal source of reactive oxygen species in mitochondria and that its deficiencies have been shown to be the origin of many human neurodegenerative diseases (3–5).

The bacterial enzyme (NDH-1) is composed only of 14 subunits with a molecular mass of 500 kDa (6, 7). All subunits of NDH-1 are homologous to the 14 subunits that constitute the core of the mitochondrial complex I (8, 9). Both eukaryotic complex I and prokaryotic NDH-1 have a characteristic L-shaped form with two domains, a hydrophilic (complex I/NDH-1) is a multisubunit enzymatic complex. It has a characteristic L-shaped form with two domains, a hydrophilic and a hydrophobic membrane domain. The membrane domain contains three antiporter-like subunits (NuoL, NuoM, and NuoN, Escherichia coli naming) that are considered to be involved in the proton translocation. Deletion of either NuoL or NuoM resulted in an incomplete assembly of NDH-1 and a total loss of the NDH-quinone oxidoreductase activity. We have truncated the C terminus segments of NuoM and NuoL by introducing STOP codons at different locations using site-directed mutagenesis of chromosomal DNA. Our results suggest an important structural role for the C-terminal segments of both subunits. The data further advocate that the elimination of the last transmembrane helix (TM14) of NuoM and the TM16 (at least C-terminal seven residues) or together with the HL helix and the TM15 of the NuoL subunit lead to reduced stability of the membrane arm and therefore of the whole NDH-1 complex. A region of NuoL critical for stability of NDH-1 architecture has been discussed.

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direct coupling) (8, 21–27). Based on the three-dimensional structure, Sazanov and co-workers (7) have hypothesized that the HL helix of NuoL can work in a piston-like mechanism, driving the conformational changes along the antiporter-like subunits.

In the present work, using *E. coli* NDH-1, we attempted to clarify the structural role of the C-terminal segments of NuoL and NuoM that are most distantly located from the peripheral domain. Primary sequence comparison of the NuoL extra stretch or the NuoM C-terminal region among diverse organisms indicated that amino acid residues are barely conserved in these regions. Therefore, conventional mutation approach (mutation of the conserved residues) does not seem to be useful. Therefore, we created truncation mutants by introducing stop codons directly on the *nuoL* and *nuoM* genes in the chromosomal NDH-1 operon by site-directed mutagenesis and investigated their effects on the architecture and enzymatic activities of NDH-1.

Recently, study of the NuoL extra stretch has been published by Steimle et al. (28). The results by Steimle et al. (28) were significantly different from this work. Possible causable factors leading to these differences were also discussed.

**EXPERIMENTAL PROCEDURES**

**Materials**—PCR product, DNA gel extraction, and plasmid purification kits were from Qiagen (Valencia, CA). The pGEM®-T Easy Vector System was from Promega (Madison, WI). The site-directed mutagenesis kit (QuikChange® II XL kit) and the Herculase® Enhanced DNA polymerase were from Stratagene (Cedar Creek, TX). The pKO3 vector was a generous gift from Dr. George M. Church (Harvard Medical School, Boston, MA). The endonucleases were from New England Biosciences (Boston, MA). *p*-Nitroblue tetrazolium was from EMD Biosciences (La Jolla, CA). All other chemicals including dNADH, NADH, and antibiotics were from Sigma-Aldrich.

**Preparation of NuoL and NuoM Knock-out and Mutagenesis of *nuoL* and *nuoM* Gene in *E. coli* Chromosome**—The *E. coli* knock-out (NuoL-KO) and the C terminus-truncated NuoL (ctNuoL) mutants were generated by employing the pKO3 system according to the method described by Link et al. (32) and Nakamaru-Ogiso et al. (25) with minor modifications. In brief, an *E. coli* Nuol-KO was constructed by replacement of the *nuoL* gene in the NDH-1 operon by spectinomycin using the pKO3 system. In parallel, a DNA fragment that includes the *nuoL* gene together with a 1-kb upstream and 1-kb downstream DNA segments from *E. coli* DH5α was cloned in the pGEM®-T Easy Vector system, generating pGEM/*nuol*. pGEM/*nuol* was used as template to obtain the *nuol*-STOP mutants and the *nuol*-ΔV519-K581 mutant. The *nuoL* mutants were subcloned into the pKO3 vector generating the pKO3/*nuoL*(STOP mutants) or pKO3/*nuoL*ΔV519-K581. The pKO3/*nuoL*- (STOP mutants) and the pKO3/*nuoL*(ΔV519-K581) plasmids were used to replace the spectinomycin gene in *E. coli* nuoL-KO by recombination. The strategy used to obtain the NuoM knock-out (NuoM-KO) and the C terminus truncated NuoM (ctNuoM) mutants were the same as we reported previously in Torres-Bacete et al. (31). All mutations were confirmed by DNA sequencing.

**Growth and Membrane Preparation of *E. coli* Mutants**—*E. coli* ctNuoL and ctNuoM mutants were grown and the inverted membrane vesicles were prepared according to the method described previously in our laboratory (31, 33–36). In summary, the *E. coli* cells were grown in Terrific Broth medium at 37 °C until *A*₆₀₀ of 2. Then, the cells were harvested and frozen at −80 °C. The cells were thawed and resuspended at 10% (w/v) in buffer A (10 mM Tris-HCl (pH 7.0), 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 15% glycerol). The cell suspension was sonicated twice (15 s) and passed twice through a French press (15,000 psi). The cell debris was removed by centrifugation (23,400 × g for 10 min). The supernatant was centrifuged again (256,600 × g for 30 min), and the membrane-rich pellet was resuspended in buffer A, frozen in liquid nitrogen, and stored at −80 °C before use.

**Immunoblots and Blue Native Electrophoresis**—The content of the NDH-1 subunits were analyzed by Western blot using antibodies against NuoB, NuoCD, NuoE, NuoF, NuoG, NuoL, NuoK, and NuoM subunits. For the production of the NuoL and NuoM antibodies, we selected the peptides H¹⁵⁷¹HEQNIKFMGGLRKS²⁷¹ (the loop between TM11 and TM12 in NuoL) and F⁵⁰⁲⁵⁵⁰NŸELELLNTPMS²¹⁶ (the loop between TM6 and TM7 in NuoM), respectively (25, 31). Blue native PAGE was performed according to the method described previously (35). The assembly of NDH-1 was examined by immunoblotting using anti-NuoL and NuoCD antibodies and NADH dehydrogenase activity staining (25, 34).

**Activity Analysis**—The measurements of the electron transfer activities by the NDH-1 ctNuoL and ctNuoM mutants were performed using dNADH as substrate. The enzymatic reactions were conducted at 30 °C in a SLM DW-2000 spectrophotometer according to the methods described previously (37). In brief, the dNADH-K₃Fe(CN)₆ reductase activity was performed using 80 μg of protein/ml of membrane samples in 10 mM potassium phosphate (pH 7.0), 1 mM EDTA containing 10 mM KCN, and 1 mM K₃Fe(CN)₆. The reaction was started by the addition of 150 μM dNADH. The absorbance change was monitored at 420 nm. The dNADH-DB reductase activity was conducted in a similar way using 50 μM DB as the electron acceptor instead of K₃Fe(CN)₆. The absorbance change was followed at 340 nm. The dNADH oxidase activity was performed under the same conditions except that neither KCN nor DB were added. Extinction coefficients of ε₄₂₀ = 1.00 mm⁻¹ cm⁻¹ for K₃Fe(CN)₆ and ε₄₉₀ = 6.22 mm⁻¹ cm⁻¹ for dNADH were used for the activity calculations.

We observed that despite the complete absence of dNADH oxidase and dNADH-DB reductase activity the NuoL-KO and NuoM-KO mutants still showed residual (~30%) dNADH-K₃Fe(CN)₆ reductase activity. It is known that dNADH-K₃Fe(CN)₆ reductase activity of NDH-1 needs at least NuoF (the NADH-binding subunit) and NuoE (38). The NuoL-KO...
mutant lacks NuoF and NuoE in the membranes and the NuoM-KO mutant does not house NuoF. In addition, similar activity was observed in a NuoF knock-out mutant. This is most likely a diaphorase activity unrelated to NDH-1. Therefore, the values obtained for the NuoL-KO and NuoM-KO mutants were subtracted as a background activity from the activity values of the wild type and the different mutants analyzed.

The generation of membrane potential for the ctNuoL and ctNuoM mutants was assayed using 0.33 mg of protein/ml of membrane vesicles in 50 mM MOPS (pH 7.3), 10 mM MgCl₂, 50 mM KCl, and 2 μM oxonol VI as described previously (33, 39). The reaction was started by addition of 0.2 mM dNADH, and the absorbance changes at 630–603 nm were recorded. The proton ionophore FCCP (2 μM) was added to uncouple the reaction. The NDH-1 proton pump activity was followed by ACMA fluorescence quenching (40). 50 μg of protein/ml of membrane vesicles was used for the assay, whereas 200 μM dNADH was used as substrate.

**RESULTS**

*Mutant Constructs for Subunits NuoL/ND5 and NuoM/ND4—NuoL and NuoM are two of the most well conserved membrane subunits in NDH-1/complex I from bacteria to mammals (see supplemental Figs. 1 and 2). Both NuoL and NuoM subunits share a similar secondary structure (7), except that the longer NuoL subunit possesses two extra TM helices, TM15 and TM16, connected by a long amphipathic helix (HL). This TM15-HL-TM16 C-terminal region displays low sequence homology among the diverse organisms. As described in the Introduction, we investigated structural role of the extra stretch of ctNuoL and C-terminal region of NuoM by using truncated mutation technique. Fig. 1 shows the locations at which the stop codons were introduced. In the NuoL subunit, these positions are Gly₄₈₈ (NuoL was truncated at the beginning of the HL helix), Thr⁵₁₇ (truncation at the beginning of the HL helix), Trp⁵⁹₃ (truncation toward the end of the HL helix), Ala⁶⁰⁷ (truncation in the middle of the TM16), Val⁶¹₁ (truncated three residues before the C terminus of NuoL) and Leu⁶¹₂ (truncated two residues before the C terminus; last residue of the TM16). We also constructed a NuoL mutant in which the complete HL helix was lacking, whereas the TM helices 15 and 16 were intact.*
The structural role of NuoM and NuoL in complex I was investigated. Antisera against NuoL and NuoM, as well as other NDH-1 subunits, were used to detect their contents. 

*Assembly and Subunit Contents of ctNuoL and ctNuoM Mutants*—We studied the effect of the C terminus truncation mutants, ctNuoL and ctNuoM, on the subunit contents and architecture of NDH-1 by Western blotting and BN-PAGE. Antibodies against the NuoL and NuoM subunits, and another seven NDH-1 subunits: the peripheral subunits NuoB, NuoCD, NuoE, NuoF, NuoG, NuoI, NuoK, and NuoM subunits were used. Immunoblotting in the ctNuoL-G488STOP, -T517STOP, and -A607STOP mutants showed positive activity staining in the BN-PAGE. As shown in Fig. 2B, no NDH-1 assembly was detected in the ctNuoM mutants constructed, only the ctNuoL-V611STOP and -L612STOP mutants showed positive activity staining in the BN-PAGE comparable with the wild type. In agreement with the activity staining results, no NDH-1 assembly was detected by immunoblotting in the ctNuoL-G488STOP, -T517STOP, -W593STOP, -A607STOP, and -ΔV519-K581 mutants. The correct assembly of NDH-1 in the ctNuoM mutants was analyzed by BN-PAGE (Fig. 3B). As expected from the immunoblot experiments, no NDH-1 assembly was detected in the ctNuoM mutants in which the TM14 was truncated (ctNuoM-G459STOP and -M467STOP).

*Effect of NuoL and NuoM Truncations on NDH-1 Electron Transfer Activities*—E. coli contains the alternative NADH-quinone oxidoreductase (or NDH-2) in addition to NDH-1. Because NDH-1 can use dNADH as substrate, whereas NDH-2 cannot, all enzymatic assays were performed using dNADH as the substrate to eliminate contribution from NDH-2 (41). We measured the electron transfer activities of NDH-1 in membrane vesicles obtained from E. coli MC4100 wild type and the ctNuoL and ctNuoM mutants (Table 1). The electron transfer activities assayed were the dNADH-K₃Fe(CN)₆ oxidoreductase, dNADH oxidase, and dNADH-DB oxidoreductase of NDH-1. (ΔV519-K581, Fig. 1). In the NuoM subunit, the positions at which the stop codons were introduced are Asn502 and Asn495 (truncation in the middle of the periplasmic C-terminal segment at two different positions), Ile485 (in which the periplasmic C-terminal segment was eliminated), Met467 (in which TM14 was eliminated), and Gly459 (truncation in the middle of the cytoplasmic loop that connects TM13 and TM14).

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The dNADH-K₃Fe(CN)₆ oxidoreductase activity, derived from the NuoF + NuoE subunits (38), has been used as a measure for the
The analysis of the formation of a proton gradient found to be affected in a similar manner.

The electron transfer activities of these mutants were compromised. As seen in Table 1, the wild type showed a maximum quenching after the addition of dNADH. The signal was completely restored when the ionophore FCCP was added. In contrast, the NuoL-KO mutant did not show any change in the ACMA signal after the addition of dNADH, indicating the absence of proton pump activity (Fig. 4A). A similar result was observed for the NuoM-KO mutant (Fig. 4B).

In the case of the ctNuoM mutant, two different results were observed when the last TM helix was eliminated. The ctNuoM-M467STOP did not show any proton pump activity; however, interestingly the ctNuoM-G459STOP mutant retained ~20% of the proton pump activity as compared with the wild type (Fig. 4B). On the other hand the ctNuoM-I485STOP, -N95STOP, and -N502STOP mutants showed activity similar to the wild type.

In conjunction with the proton pump activity, we analyzed the generation of membrane potential (∆Ψ) in the membrane vesicles of the ctNuoL mutants using oxonol VI as the reporter (Fig. 5). Membrane vesicles of the wild type showed a maximum ∆Ψ after the addition of dNADH, and FCCP totally abolished the ∆Ψ. No ∆Ψ was generated in the case of NuoL-KO and NuoM-KO mutants. Similar results were obtained for the ctNuoL mutants lacking the HL helix and/or the transmembrane α-helix 16. Interestingly, a minor ∆Ψ was observed in the ctNuoL-A607STOP, -W593STOP, -N495STOP, and -N502STOP mutants.

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TABLE 1

| Mutant          | dNADH-O₂* % | dNADH-DB% | dNADH-K₃Fe(CN)₆% |
|-----------------|-------------|-----------|------------------|
| WT              | 100 ± 6.0   | 100 ± 8.0 | 100 ± 4.7        |
| NuoL-KO-rev     | 102 ± 2.7   | 86 ± 5.1  | 100 ± 5.9        |
| NuoM-KO-rev     | 113 ± 4.4   | 95 ± 14.0 | 106 ± 6.1        |
| ctNuoL-G488STOP| 2 ± 0.9     | 2 ± 0.3   | 18 ± 2.3         |
| ctNuoL-T517STOP| 1 ± 1.0     | 1 ± 1.2   | 2 ± 1.5          |
| ctNuoL-W593STOP| 1 ± 0.4     | 3 ± 0.7   | 27 ± 2.9         |
| ctNuoL-A607STOP| 4 ± 1.9     | 2 ± 0.3   | 20 ± 1.9         |
| ctNuoL-V611STOP| 66 ± 6.7    | 67 ± 3.2  | 80 ± 5.8         |
| ctNuoL-L612STOP| 89 ± 11.0   | 73 ± 5.7  | 91 ± 3.5         |
| ctNuoL-ΔV519-K581| 1 ± 0.4   | 1 ± 2.0   | 30 ± 4.7         |
| ctNuoM-G459STOP| 9 ± 1.1     | 9 ± 0.6   | 33 ± 1.2         |
| ctNuoM-M467STOP| 2 ± 0.6     | 1 ± 0.3   | 33 ± 0.6         |
| ctNuoM-I485STOP| 64 ± 2.2    | 60 ± 3.8  | 67 ± 5.6         |
| ctNuoM-N495STOP| 77 ± 0.8    | 67 ± 6.8  | 75 ± 4.5         |
| ctNuoM-N502STOP| 81 ± 9.4    | 79 ± 2.3  | 75 ± 0.6         |

*a Percentage of dNADH-O₂ reductase activity (100% activity of WT was 0.61 μmol dNADH/mg protein/min).

*b Percentage of dNADH-DB reductase activity (100% activity of WT was 0.69 μmol dNADH/mg protein/min).

** Percentage of dNADH-K₃Fe(CN)₆ reductase activity (100% activity of WT was 1.5 μmol K₃Fe(CN)₆/mg protein/min).
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**DISCUSSION**

Our current and earlier results indicated that deletion of a subunit in *E. coli* NDH-1 can lead to incomplete assembly of the complex. When subunit NuoA (35), NuoCD (36), NuoH (42), NuoJ (33), NuoK (34), NuoM (31), or NuoL was individually deleted, the amount of other subunits were reduced to various extents. Mutants lacking NuoCD or NuoH seemed to be most drastically affected with nearly a total loss of NDH-1 assembly, which may agree with their location in the core of the complex (36). The distal location of antiporter-like subunits NuoM and NuoL (7, 43) might suggest that their removal would not affect the assembly of the peripheral domain. However, our results with the deletion mutants demonstrated a complete absence of the NDH-1 reductase activity, whereas other subunits studied are present in the membranes, whereas other subunits studied are present in the membranes to an extent similar to the wild type. The seemingly contradicting data between theirs and ours could be explained by the difference in the experimental approach. We introduce mutations directly in the *E. coli* chromosome assuring a unique copy of the mutated gene and thus avoiding any risk of extra copies of the wild type gene.

Interestingly, the truncation of the NuoM subunit at its C terminus by exclusion of the last TM14 helix resulted in defects of subunit assembly. The ctNuoM mutants in which NuoM was truncated at the last periplasmic segment did not show notable differences in the assembly of NDH-1 when compared with the wild type. Similarly, the electron transfer activities and the proton pump activities were severely affected only in those ctNuoM mutants that were devoid of TM14. In the ctNuoM mutants lacking TM14, neither NuoM nor NuoL was detected in the membranes, whereas other subunits studied are present in the membranes to an extent similar to the wild type. The data suggest that deficiency of NuoM and/or NuoL contributed to incomplete, or lack of, NDH-1 assembly.

Furthermore, none of the ctNuoL mutants lacking the HL helix showed correct NDH-1 assembly when analyzed by BN-PAGE. Similar results were obtained even when the seven C-terminal residues of the NuoL TM16 were eliminated. The assembly of NDH-1 remained unaffected only in two mutants, ctNuoL-V611STOP and -L612STOP, in which the NuoL TM15 was truncated, respectively, at the second and third residues from the C terminus. It is remarkable that, after the elimination of only seven residues of the NuoL subunit, NuoL was detectable in the membrane, thereby causing incomplete assembly or misassembly of the whole complex. In contrast, the ctNuoL truncation mutants scarcely affected the NuoM content in the membranes. Therefore, absence of NuoL seems to trigger NDH-1 disassembly of not only ctNuoL-G488STOP,
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