The C-terminally Truncated NuoL Subunit (ND5 Homologue) of the Na\(^+\)-dependent Complex I from Escherichia coli Transports Na\(^+\)

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The NADH:quinone oxidoreductase (complex I) from Escherichia coli acts as a primary Na\(^+\) pump. Expression of a C-terminally truncated version of the hydrophobic NuoL subunit (ND5 homologue) from E. coli complex I resulted in Na\(^+\)-dependent growth inhibition of the E. coli host cells. Membrane vesicles containing the truncated NuoL subunit (NuoLN) exhibited 2–4-fold higher Na\(^+\) uptake activity than control vesicles without NuoLN. Respiratory proton transport into inverted vesicles containing NuoLN decreased upon addition of Na\(^+\), but was not affected by K\(^+\), indicating a Na\(^+\)-dependent increase of proton permeability of membranes in the presence of NuoLN. The His-tagged NuoL protein was solubilized, enriched by affinity chromatography, and reconstituted into proteoliposomes. Reconstituted His\(^\text{6}\)-NuoLN facilitated the uptake of Na\(^+\) into the proteoliposomes along a concentration gradient. This Na\(^+\) uptake was prevented by EIPA (5-(N-ethyl-N-isopropyl)amiloride), which acts as inhibitor against Na\(^+\)/H\(^+\) antiporters.

Mitochondrial complex I (NADH:quinone oxidoreductase) is the largest multiprotein complex of the oxidative phosphorylation (OXPHOS) system. Diminished complex I activity is associated with Parkinson’s disease (1) and aging (2) and represents the most frequently encountered inherited defect of the OXPHOS system (3). Despite the considerable knowledge on primary sequences, cofactors and assembly (4–8), the mechanism of redox-driven proton transport by complex I and the subunit(s) that guide the proton through its membranous part are unknown. A promising approach is to study bacterial counterparts of complex I that are smaller but possess all subunits required for redox-driven proton transport by complex I and the subunit(s) that guide the proton through its membranous part are unknown. A promising approach is to study bacterial counterparts of complex I that are smaller but possess all subunits required for redox-driven H\(^+\) (or Na\(^+\)) transport (9). In particular, a Na\(^+\)-translocating complex I found in enterobacteria like Escherichia coli (10) or Klebsiella pneumoniae (11, 12) is a useful model to trace the pathway of the coupling cation, as exemplified by the Na\(^+\)-translocating FIP10 ATP synthase (13).

The L-shaped complex I is composed of a peripheral arm extending into the bacterial cytoplasm (or the mitochondrial matrix) and a hydrophobic arm that is embedded in the membrane (14). In addition to the L-shaped conformation, a “horse-shoe-conformation” was observed for E. coli complex I, with two arms arranged side by side (15). Upon purification, the peripheral and the membrane arm tend to dissociate (16–18).

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Solubilization and Reconstitution—Washed membrane vesicles (40 mg protein, 1 ml) from \textit{E. coli} DH5\textalpha{} transformed with pECL5 (NuoL\textsubscript{p}) or pISC3 (control) grown on Luria Bertani medium in the presence of 10 mM glucose were mixed with 9 ml buffer (20 mM Tris-HCl, pH 8.0, 300 mM KCl). Na\textsuperscript{+}—valinomycin. The internal Na\textsuperscript{+} content of the vesicles was determined by atomic absorption spectroscopy. In controls performed with the liposomes (0.5 mM glucose), Na\textsuperscript{+}—valinomycin (Sigma) in 10 mM Tris-HCl, pH 8.0, 300 mM KCl. If indicated, 0.1 mM EIPA\textsuperscript{1} (5-N-ethyl-N-isopropyl)-amiloride (Sigma) was added to the assay. The reaction was started by the addition of 5 mM NaCl (final concentration). At different times, samples of 70 \textmu{}l were analyzed by atomic absorption spectroscopy.

**Fig. 1.** Putative topology of the Nuol subunit (ND5 homologue) of complex I from \textit{E. coli} and relationship with subunit A of multicomponent Na\textsuperscript{+}/H\textsuperscript{+} antiporters. The topology of the \textit{E. coli} Nuol subunit is based on data obtained for Nuol from \textit{R. capsulatus} complex I (32). A total of sixteen hydrophobic helices are predicted for Nuol, and its N and C termini are located in the periplasm. The beginning of a conserved part of the cytoplasmic loop between helices (XI) and XII.

| Region | Description |
|--------|-------------|
| N      | Putative N-terminus |
| 1-369  | Conserved region |
| 370-613| Conserved region |

The abbreviations used are: EIPA, 5-N-ethyl-N-isopropyl)-amiloride; ACMA, 9-amino-6-chloro-2-methoxyacridin.

**RESULTS**

The C-terminally Truncated Nuol Subunit Increases the Na\textsuperscript{+} Permeability of \textit{E. coli} Membranes—Complex I (NDH I) from \textit{E. coli} couples the oxidation of NADH with quinone to the translocation of Na\textsuperscript{+} (10). An important goal is to identify the membrane complex I subunit(s) that participate in cation transport. A prime candidate is the hydrophobic Nuol subunit of complex I from \textit{E. coli} (ND5 in \textit{Bos taurus}, or Nqo12 in \textit{Paracoccus denitrificans}) that exhibits striking sequence similarity to multicomponent Na\textsuperscript{+}/H\textsuperscript{+} antiporters. A sequence alignment of the Nuol subunit from \textit{E. coli} complex I with the MnhA subunit of the Na\textsuperscript{+}/H\textsuperscript{+} antiporter from \textit{Staphylococcus aureus} reveals 133 conserved amino acids in Nuol encompassing a total of 613 amino acid residues. Fig. 1 shows the putative topology of the Nuol subunit from \textit{E. coli} complex I that is based on phylogenetic analyses and experimental data obtained for Nuol of \textit{Rhodobacter capsulatus} complex I (32). The central region of the Nuol subunit encompassing transmembrane helices III to XIII exhibits the highest sequence similarity to Na\textsuperscript{+}/H\textsuperscript{+} antiporters. The region from amino acid 1 to 369 encompasses 107 residues of the 133 amino acids that are fully conserved in the complete \textit{E. coli} Nuol subunit and in the different times, aliquots of 70 \textmu{}l were applied to the Dowex columns as described above.

**Other Methods—Oxidation of NADH or deaminoNADH (nicotinamide hypoxanthine dinucleotide) by membrane vesicles with O\textsubscript{2} as electron acceptor was followed at 340 nm (ε\textsubscript{340} = 6.22 mM\textsuperscript{–1} cm\textsuperscript{–1}).** NADH-induced proton uptake into native membrane vesicles was measured by the quenching of ACMA (9-amino-6-chloro-2-methoxyacridin) in 1.5 mM of 10 mM Tris-HCl, pH 8.0, 200 mM KCl at 25 °C (30). The residual Na\textsuperscript{+} concentration in the buffer was 0.13 mM. DeaminoNADH, NADH (potassium salt), and ACMA were obtained from Sigma. Protein was determined by the bicinchoninic acid method using the reagent obtained from Pierce.

SDS-PAGE was performed with 10% polyacrylamide according to (31). The polypeptides were blotted onto a nitrocellulose membrane (Hybond-C Extra, Amersham Biosciences), and His-tagged proteins were identified by immunostaining using anti-His (4) antibodies (Qiagen).

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MnHA antiporter subunit from *S. aureus*. This portion of the NuoL subunit contains nine putative transmembrane helices (I-IX) and the amphipathic helices (X) and (XI) that are not assumed to traverse the membrane (32). Truncation of NuoL at R369 (Fig. 1) results in a shortened NuoL subunit that includes the conserved parts of the cytoplasmic loop between helices (XI) and (XII). The gene fragment encoding for amino acid residues 1–369 was amplified by PCR and cloned into a low-copy expression vector that is repressed by glucose and derepressed by L-arabinose, resulting in vector pECL3. The truncated NuoL subunit (NuoLN) has a calculated molecular mass of 40,054 Da, compared with 66,440 Da of the NuoL subunit. In addition, a vector encoding for NuoLN encompassing an N-terminal His6-tag (His6-NuoLN) was constructed (pECL5). Detection of His6-NuoLN, but NaCl showed a more drastic effect in cell-free extracts from *E. coli* in the absence of plasmid (not shown). Due to its hydrophobic nature, the truncated NuoL subunit (NuoLN) has a calculated molecular mass of 40,054 Da, compared with 66,440 Da of the NuoL subunit. In six independent experiments, the uptake of Na\(^{+}\) by control vesicles, compared with 9 nmol Na\(^{+}\) mg\(^{-1}\) min\(^{-1}\) observed with the control NuoL subunit, was 2–4-fold higher in membrane vesicles containing NuoLN compared with the control (50–240 nmol Na\(^{+}\) min\(^{-1}\) mg\(^{-1}\), corrected for initial Na\(^{+}\) uptake by control vesicles), demonstrating that NuoLN enhances Na\(^{+}\) transport through native membranes with initial rates that are comparable with secondary Na\(^{+}\) transporters. For example, the purified, reconstituted Na\(^{+}/H\(^{+}\) antiporter NhaA from *E. coli* has a specific activity of 600 nmol Na\(^{+}\) min\(^{-1}\) mg\(^{-1}\) (34).

An important question is whether the presence of the truncated NuoLN subunit in the cytoplasmic membrane of *E. coli* affects the assembly of complex I. The observed Na\(^{+}\) transport might be catalyzed by misassembled complex I containing the truncated NuoL subunit. The specific complex I activity of membrane vesicles was estimated from the oxidation of deaminonADH with O\(_2\) as terminal electron acceptor. In contrast to NADH, which is oxidized both by complex I and the alternative, non-electrogenic NADH dehydrogenase (NDH II), deaminonADH is preferentially oxidized by complex I and is used to distinguish between complex I and NDH II in *E. coli* membranes (35). The rates of deaminonADH oxidation by control membrane vesicles from *E. coli* EP432 (0.06 \(\mu\)mol min\(^{-1}\) mg\(^{-1}\)) were comparable with the rates observed with membrane vesicles containing the truncated NuoL subunit (0.08 \(\mu\)mol min\(^{-1}\) mg\(^{-1}\)) and with the control (0.38 \(\mu\)mol min\(^{-1}\) mg\(^{-1}\)). The NuoLN protein did not diminish the activity of complex I in *E. coli* membranes, suggesting functional assembly of complex I despite the presence of its hydrophobic, truncated NuoL subunit.

### Table I

Expression of the C-terminally truncated NuoL subunit of complex I decreases the salt tolerance of *E. coli*

| Vector | E. coli strain | NaCl or KCl |
|--------|---------------|-------------|
| pISC3  | DH5\(\alpha\)   | NaCl 7.7    |
|        | KCl 7.7       | 9.0         |
| EP432  | NaCl 5.2      | 1.2         |
|        | KCl 5.2       | 5.4         |
| pECL5  | DH5\(\alpha\)  | NaCl 3.3    |
|        | KCl 3.0       | 2.8         |

| Vector | E. coli strain | NaCl or KCl |
|--------|---------------|-------------|
| pISC3  | DH5\(\alpha\)   | NaCl 3.0    |
|        | KCl 3.0       | 2.8         |
| EP432  | NaCl 3.0      | 0.6*        |
|        | KCl 3.0       | 3.4         |
The C-terminally truncated NuoL subunit of complex I increases the Na\(^{+}\) permeability of membrane vesicles from *E. coli*. Membrane vesicles were prepared from the *Na\(^{+}\)/H\(^{+}\)* antiporter-deficient *E. coli* strain EP432 transformed with plasmid pECL5 (His\(_{6}\)-NuoLN\(_{N}\)) or pISC3 (control). Cells were grown on mineral medium in the presence of 45 mM glucose and 5 mM \(\beta\)-arabinose at pH 8.0. Na\(^{+}\) transport was followed in the presence of 20 mM valinomycin and 140 mM KCl. The reaction was started by adding 5 mM NaCl. Open circles, membrane vesicles containing His\(_{6}\)-NuoLN\(_{N}\); closed circles, membrane vesicles prepared from *E. coli* EP432 transformed with the control vector. The graph shows representative data from a total of six experiments.

**Na\(^{+}\)**-dependent Increase of Proton Permeability in Membranes Containing the C-terminally Truncated NuoL Subunit—Respiratory proton transport activity was studied in membrane vesicles from *E. coli* CP875 containing the His\(_{6}\)-NuoLN protein that were energized by the addition of NADH as outlined above. *E. coli* contains two respiratory NADH:quinone oxidoreductases, the Na\(^{+}\)-translocating NDH I or complex I (10), and the non-electrogenic NDH II. During aerobic growth, NDH is mainly oxidized by the NDH II (36), and the quinol formed is oxidized by terminal oxidases under formation of an electrochemical proton gradient. *E. coli* membrane vesicles obtained by French press cell rupture are predominantly oriented inside-out (26), and the addition of NADH in the presence of KCl (20 mM), whereas the addition of NaCl results in a transient accumulation of Na\(^{+}\) (Fig. 4, lower panel). The slight increase in proton permeability of the control liposomes in the Na\(^{+}\)/H\(^{+}\) antiporter from *E. coli* (24). Similar results were obtained with *E. coli* DH5\(\alpha\) transformed with pECL3 encoding for the NuoLN protein (not shown). Note that the rates of NADH oxidation by membrane vesicles with O\(_{2}\) as terminal electron acceptor were not affected by NuoLN (0.34 \(\mu\)mol min\(^{-1}\) mg\(^{-1}\)) in vesicles containing NuoLN, compared with 0.36 \(\mu\)mol min\(^{-1}\) mg\(^{-1}\) in control vesicles). The diminished proton gradient established during NADH oxidation by vesicles in the presence of NuoLN and Na\(^{+}\) ions provides a rationale for the Na\(^{+}\)-dependent decrease in growth yield of *E. coli* cells producing the truncated NuoLN subunit.

**Na\(^{+}\)** Transport by Reconstituted NuoLN and Inhibition by EIPA—The truncated, His-tagged NuoLN subunit from *E. coli* complex I was solubilized, enriched by affinity chromatography (Fig. 2), and reconstituted into proteoliposomes. Addition of proteoliposomes containing His\(_{6}\)-NuoLN\(_{N}\) to buffer in the presence of 5 mM NaCl resulted in a transient accumulation of Na\(^{+}\) inside the liposomes that was 6-fold higher compared with the control liposomes (Fig. 5). Internal Na\(^{+}\) concentrations were calculated assuming a liposome volume of 3–10 \(\mu\)l per mg lipid (37). At the start of the reaction, the Na\(^{+}\) concentration in the lumen of the liposomes was 100–200 \(\mu\)M, corresponding to an initial chemical Na\(^{+}\) concentration gradient (\(\Delta pNa^{+}\)) of 80–100 mV (inside negative). The experiments were performed without respiratory substrates added, and the build-up of a transmembrane voltage, \(\Delta \Psi\), was prevented by valinomycin/K\(^{+}\). The uptake of Na\(^{+}\) by reconstituted His\(_{6}\)-NuoLN\(_{N}\) was driven by the chemical Na\(^{+}\) concentration gradient (\(\Delta pNa^{+}\)). After 30 s, the maximum Na\(^{+}\) concentration in the interior was 6–20 mM. This is in the range of the external Na\(^{+}\) concentration in the buffer and thus in accord with passive downhill transport of Na\(^{+}\) into the proteoliposomes mediated by His\(_{6}\)-NuoLN\(_{N}\). The initial uptake during the first 30 s was followed by an efflux of Na\(^{+}\) to the values observed with the control proteoliposomes (Fig. 5). Similar results were obtained with different preparations of NuoLN and different concentrations of proteoliposomes in the Na\(^{+}\) transport assay (not shown).

The inhibition of the truncated NuoLN subunit by EIPA was investigated. EIPA is an amiloride analogue that inhibits eukaryotic Na\(^{+}/H\(^{+}\) antiporters but not NhaA, the main Na\(^{+}/H\(^{+}\) antiporter in *E. coli* (38). Na\(^{+}\) uptake by His\(_{6}\)-NuoLN\(_{N}\) was drastically diminished in the presence of 100 \(\mu\)M EIPA, whereas the residual uptake of Na\(^{+}\) by the control proteoliposomes was not affected by EIPA (Fig. 5). This result suggests that the truncated NuoLN subunit of complex I from *E. coli* shares features with Na\(^{+}/H\(^{+}\) antiporters.
with respect to function and catalytic activity. 

ponent cation/H\textsuperscript{+}H11001

subunit was produced in belonging to the CPA3 family (22). Because the truncated NuoL and the A subunit of the multicomponent antiporters NuoL subunit comprises most of the regions conserved between NuoL subunit of complex I is presented here. The truncated

I with subunit A from multicomponent cation/H\textsuperscript{+}\textsubscript{11001} transport was due to other complex I subunits copurified with His\textsubscript{6}-NuoLN on the affinity column. On the other hand, the solubilization of E. coli membranes with dodecylmaltoside under the applied conditions of pH and salt concentration cleaves and inactivates complex I (17). It should also be noted that the specific complex I activity in native membranes was not affected by the presence of the truncated NuoL subunit. It is therefore considered very unlikely that the Na\textsuperscript{+} transport observed with reconstituted His\textsubscript{6}-NuoLN\textsubscript{2} is catalyzed by misassembled complex I. The translocation of Na\textsuperscript{+} by the C-terminally truncated NuoL subunit of complex I from E. coli provides strong evidence for the participation of this subunit in cation transport through the membranous fragment of complex I. The Na\textsuperscript{+}-translocating complex I from K. pneumoniae that exhibits high sequence similarity to E. coli complex I exclusively pumps Na\textsuperscript{+} at a ratio of 2 Na\textsuperscript{+}/2 electrons (11). Na\textsuperscript{+} transport by native complex I generates a transmembrane voltage that is not compensated by the counter-flow of protons (12). Therefore, one would expect unidirectional transport of Na\textsuperscript{+} by NuoLN. On the other hand, the results presented herein suggest a Na\textsuperscript{+}-dependent increase of proton permeability of membrane vesicles in the presence of NuoLN, which can be interpreted in terms of Na\textsuperscript{+}/H\textsuperscript{+} antiporter. Na\textsuperscript{+} uptake by reconstituted His\textsubscript{6}-NuoLN\textsubscript{2} was followed by the extrusion of Na\textsuperscript{+}, similar to results obtained with the reconstituted NhaA antiporter from E. coli (34). Na\textsuperscript{+} transport by His-NuoLN\textsubscript{2} was drastically diminished in the presence of EIPA, an amiloride derivative that acts as inhibitor against Na\textsuperscript{+}/H\textsuperscript{+} antiporters. Note that EIPA also inhibits NADH oxidation by bovine complex I (41). It should be considered that the presence of NuoLN in E. coli host cells could result in an increase of the intracellular Na\textsuperscript{+} concentration, which in turn leads to the up-regulation of endogenous Na\textsuperscript{+}/H\textsuperscript{+} antiporters (42). As a consequence, the Na\textsuperscript{+}/H\textsuperscript{+} antiporter activity in membrane vesicles or enriched protein fractions could be higher in E. coli cells producing NuoLN\textsubscript{2} compared with the control cells. Studies aimed at the determination of Na\textsuperscript{+} (or H\textsuperscript{+}) transport stoichiometries using purified, reconstituted NuoLN\textsubscript{2} are currently underway in this laboratory.

Biochemical (18) and structural (43) studies with bovine complex I suggest that the large ND5 (NuoL) subunit might be situated at the distal end of the membrane-embedded arm of the L-shaped molecule together with the ND4 (NuoM) subunit. In complex I from E. coli, the N-terminal part of the NuoL subunit (helices I-XI) may be part of the Na\textsuperscript{+}-translocating machinery, but electrogenic Na\textsuperscript{+} transport by the holo-complex probably requires the C-terminal NuoL domain (Fig. 6). Studies on site-directed mutants support a functional role of the C-terminal part of NuoL (helices XII-XVI) (Fig. 1). In human complex I, a substitution of glycine 465 (Gly-468 in E. coli complex I) in helix XIV with glutamate represents a primary mutation that causes Leber’s hereditary optic neuropathy (44). In the related Na\textsuperscript{+}/H\textsuperscript{+} antiporter from Bacillus C-125, mutation of glycine 382 in helix XII to arginine results in an alkali-sensitive phenotype, indicating diminished activity of the Na\textsuperscript{+}/H\textsuperscript{+} antiporter that confers alkali resistance in the wild type (19).

A central question is how the endergonic transport of Na\textsuperscript{+} (or H\textsuperscript{+}) by complex I is driven by the exergonic oxidation of NADH with quinone (Fig. 6). Two quinones (Q6 and Q8) were found in complex I from K. pneumoniae (11). A quinone-binding site was identified in the vicinity of the NuoH (ND1), NuoB (PSST), and NuoD (49 kDa) subunits (45) (bovine nomenclature in brackets). Based on comparisons of primary sequences and known high-resolution structures of Q-binding proteins, the binding of Q to the NuoL (ND5) subunit was postulated from a putative Q binding motif located in helix (XI) and the loop between helices

![Na\textsuperscript{+} transport by the reconstituted, truncated NuoL subunit of complex I and inhibition by EIPA](http://www.jbc.org/)


**Na⁺ Transport by a Complex I Subunit**

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**Fig. 6. Proposed coupling of electron and Na⁺ transport in complex I.** Only five of thirteen Nuo subunits (A–N) of the *E. coli* complex I are shown. The bovine nomenclature is indicated in parentheses. B, NuoB (PSST); F, NuoF (51 kDa); C, NuoCD (30 kDa and 49 kDa; these subunits are fused in *E. coli* complex I); H, NuoH (ND1); L, helices XI–XII of NuoL (ND5); L, helices XII–XVI of NuoL (ND5). Electron transport starts with the oxidation of NADH by FMN located on the NuoB subunit in the peripheral arm located in the cytoplasm (or in the mitochondrial matrix). Electrons are transferred via several FeS clusters to the high-potential [4Fe–4S] cluster N2 that is located on NuoB (PSST) (5) or on Nuo (TYKY) (47). The reduced cluster N2 is oxidized by quinone (Q) bound in close vicinity of the B and CD subunits and the membranous H subunit (45) under formation of quinol. This exergonic reaction provides the driving force for the endergonic transport of Na⁺ by the N-terminal part of NuoL (L) submitted via the C-terminal NuoL domain (L) (shaded arrow) (5). Per NADH oxidized, two electrons are transferred to quinone, and two sodium ions are transported from the water-soluble ubiquinone-1 (oxidized form). It will now be im-
The C-terminally Truncated NuoL Subunit (ND5 Homologue) of the Na\textsuperscript{+}-dependent Complex I from *Escherichia coli* Transports Na\textsuperscript{+}

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