The Location of NuoL and NuoM Subunits in the Membrane Domain of the <i>Escherichia coli</i> Complex I

IMPLICATIONS FOR THE MECHANISM OF PROTON PUMPING*

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The molecular organization of bacterial NADH:ubiquinone oxidoreductase (complex I or NDH-1) is not established, apart from a rough separation into dehydrogenase, connecting and membrane domains. In this work, complex I was purified from <i>Escherichia coli</i> and fragmented by replacing dodecylmaltoside with other detergents. Exchange into decyl maltoside led to further disruption of the membrane domain into fragments containing NuoM/N, NuoA/K/N, and NuoH/J subunits. Among the hydrophilic subunits, NuoCD was most readily dissociated from the complex, whereas NuoB was partially dissociated from the peripheral arm assembly in <i>N</i>,<i>N</i>-dimethyldodecylamine N-oxide or Triton X-100 led to further disruption of the membrane domain into fragments containing NuoLM/N, NuoA/K/N, and NuoH/J subunits. Among the hydrophilic subunits, NuoCD was most readily dissociated from the complex, whereas NuoB was partially dissociated from the peripheral arm assembly in <i>N</i>,<i>N</i>-dimethyldodecylamine N-oxide. A model of subunit arrangement in bacterial complex I based on these data is proposed. Subunits NuoL and NuoM, which are homologous to antiporters and are implicated in proton pumping, are located at the distal end of the membrane arm, spatially separated from the redox centers of the peripheral arm. This is consistent with proposals that the mechanism of proton pumping by complex I is likely to involve long range conformational changes.

NADH:ubiquinone oxidoreductase (complex I, EC 1.6.5.3) is the first enzyme of the respiratory chains of most mitochondria and many bacteria. It catalyzes the transfer of two electrons from NADH to quinone, coupled to the translocation of about 4 protons across the membrane (for reviews, see Refs. 1–4). Complex I is one of the largest known membrane protein complexes.

The bovine enzyme has a mass of ~980 kDa and is composed of about 46 subunits, including the seven hydrophobic ND subunits encoded in the mitochondrial genome (5). The simplest version of the complex in terms of protein content is the procaryotic enzyme that has 13–14 subunits and a combined molecular mass of about 550 kDa (6). All of the subunits of bacterial complex I (also referred to as NDH-1) have analogues in the mitochondrial enzyme (4). Because of its relatively simple subunit composition, NDH-1 represents a useful "minimal" model to study the structure and function of complex I. It is currently the least understood component of the respiratory chain. In contrast to other enzymes of the respiratory chain, the atomic structure of complex I is not known and the mechanisms of proton pumping and electron transfer are not established.

Electron microscopy has shown that both the mitochondrial and the bacterial enzyme have a characteristic L-shaped structure. One arm is embedded in the membrane and the other, the peripheral arm, protrudes into the mitochondrial matrix or bacterial cytoplasm. This was demonstrated at about 20 to 30 Å resolution for the enzyme from fungus <i>Neurospora crassa</i> (7, 8), yeast <i>Yarrowia lipolytica</i> (9), beef heart mitochondria (10), <i>Escherichia coli</i> (11, 12), and the thermophile <i>Aquifex aeolicus</i> (13). An alternative "horseshoe"-like conformation of active complex I from <i>E. coli</i> has been proposed recently (14), but this observation was not confirmed in our laboratory (15). The membrane and peripheral arms contain mainly hydrophobic and hydrophilic subunits, respectively. Because a more precise allocation of subunits is not possible at the current resolution, biochemical studies have been considered to refine the structural model. The disruption of complex I into smaller subcomplexes and the cross-linking of subunits are potentially ways that can provide such information.

Dissociation of complex I by chaotropes and detergents indicates that all the redox centers of the enzyme (FMN and up to 3–9 iron-sulfur clusters) are located in the peripheral arm (2, 16–18). The peripheral arm of the bovine enzyme has been resolved into smaller fragments, namely a flavoprotein fraction and an iron-sulfur protein fraction. The main components of the flavoprotein fraction were the 51-kDa (NuoF in <i>E. coli</i>) and 24-kDa (NuoE) subunits, whereas the iron-sulfur protein fraction comprised mainly the 75- (NuoG), 49-, and 30-kDa (NuoCD, fused in <i>E. coli</i>) and PSST (NuoB) subunits (19). With the <i>E. coli</i> enzyme, resolution of the peripheral arm into a dehydrogenase fragment containing NuoE, NuoF, and NuoG subunits, and a connecting fragment, containing NuoCD, NuoB, and NuoI (bovine TYKY) subunits, has been described (20).

The membrane spanning part of the enzyme, which seemingly lacks prosthetic groups, has been studied far less than the peripheral part. However, it is clear that essential components of the proton translocating machinery must reside in the transmembrane subunits. Sequence comparisons have suggested that subunits ND2 (NuoN in <i>E. coli</i>), ND4 (NuoM), and ND5 (NuoL) evolved from a common ancestor related to K<sup>+</sup> or Na<sup>+</sup>/H<sup>+</sup> antiporters and thus are likely to be involved in the proton translocation (1, 21, 22). The predicted molecular mass of <i>E. coli</i> subunit NuoL is 66 kDa, NuoM is 57 kDa, and NuoN is 52 kDa (23). These are the largest hydrophobic subunits of

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complex I, adding up to about 2/3 of the molecular mass of the membrane arm of the bacterial enzyme. They are likely to have between 12 and 16 transmembrane helices each (24). Clearly, the position of these large subunits within the membrane arm has important implications for our understanding of the organization of the complex.

To date, evidence available regarding the location of antiporter-like subunits in complex I has been contradictory, with some reports placing these subunits in proximity to the peripheral arm and some suggesting a distal location. We have demonstrated by disruption of the bovine enzyme that subunits ND4 and ND5 are likely situated in a different part of the membrane arm to subunits ND1 (NuoH in E. coli), ND2, ND3 (NuoA), and ND4L (NuoK) (18). This is in agreement with our electron crystallography studies, which located subunit ND5 to the distal end of the membrane arm (25). Analyses of ND subunits mutants in human and mouse cell lines (26, 27) and in Chlamydomonas reinhardtii (28) are consistent with this proposal.

In contrast, comparison of sequences of complex I with membrane-bound [NiFe] hydrogenases have suggested that subunits NuoL and NuoH, along with subunits of the connecting fragment, form part of a core “hydrogenase” module, whereas NuoM and NuoN are a part of a “transporter” module spatially separated from the peripheral arm (29, 30). Several transmembraneic subunits have been implicated in quinone binding, with initial studies concentrating on ND1 (NuoI) (31). More recently, on the basis of specific labeling, it was suggested that ND5 (32) and/or NuoM (33) are likely to form a part of the inhibitor/Q-binding pocket located probably at the junction of the peripheral and membrane arms.

Two main models for the mechanism of proton pumping by complex I are being discussed in the literature: direct (redox-driven) and indirect (conformation-driven) (1, 4). The first model, employing modifications of the Q cycle, assumes that the electron carriers are located in the membrane and are directly involved in proton translocation (34, 35). In this case quinone binding sites and proton translocation machinery would be located close to the peripheral arm with its redox centers to allow direct interaction. This would be consistent with a location of subunits NuoLM/N proximal to the peripheral arm and associated with the Q-binding pocket (29, 32, 33). In the second model, the catalytic module with electron carriers is an entity distinct from the proton pumping module and energy transduction takes place through long range conformational changes (4, 25, 36, 37). In this second case, the two modules can be spatially separated, which would be consistent with a location for NuoLM/N distal from the peripheral arm. A combination of the two mechanisms to explain a high H+/e− ratio in complex I has also been discussed (1, 25). It has been suggested recently that complex I from a bacterium closely related to E. coli, namely Klebsiella pneumoniae, might pump two sodium ions instead of four protons per two electrons (38, 39). If this observation is confirmed, it would support an indirect mechanism for complex I, as the Q cycle with Na+ is not possible.

Here, by disruption of complex I from E. coli into subcomplexes, we show that antiporter-like subunits NuoL and NuoM are located in a distal part of the membrane arm of the enzyme, spatially separated from the redox centers of the peripheral arm. This provides experimental evidence in support of an indirect, conformation-driven, coupling between the redox reactions and proton translocation as at least a part of the complex I proton pumping machinery. Analysis of the observed subcomplexes allows us to propose the most detailed model to date of subunit arrangement in bacterial complex I.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—Dodecyl-, undecyl-, and decylmaltoside were purchased from Avanti Polar Lipids (Alabaster, AL), other detergents were from Calbiochem (Nottingham, UK). Complete protease inhibitors tablets were obtained from Roche Applied Science, Bio-scale DEAE column was from Bio-Rad, and other chromatography columns were from Amersham Biosciences. All other chemicals were purchased from Sigma.

**Purification of Complex I, Detergent Exchange, and Resolution into Subcomplexes**—The enzyme was purified from E. coli BL21 cells grown under limited oxygen as described previously (15). Purified complex I was stored in small aliquots under liquid nitrogen and used for detergent exchange experiments as required.

The procedure for detergent exchange was as follows: a sample in dodecylmaltoside was diluted 10–20-fold in buffer A, containing 20 mM MES, pH 6.0, 10% glycerol, 0.002% phenylmethanesulfonyl fluoride, and the new detergent at the described concentrations. The sample was then loaded onto an anion exchange column (DEAE HiTrap 5 ml or Mono-Q 5/5) equilibrated in buffer A. After elution with a gradient of NaCl in buffer A, fractions containing either intact complex or putative subcomplexes were pooled, concentrated by ultrafiltration, and applied to a gel filtration column for further analysis. Gel filtration chromatography was performed on either a Superose 6 or a Superose 12 column calibrated using a high molecular weight gel filtration calibration kit (Amersham Biosciences). Buffers for gel filtration comprised 20 mM MES, pH 6.0, 200 mM NaCl, 10% glycerol, 0.005% phenylmethanesulfonyl fluoride, and the relevant detergent at the described concentrations. A flow rate of 0.3 ml/min was used and fractions of 0.5 ml were collected and analyzed by SDS-PAGE. In cases where Triton X-100 was used, all buffers contained 20 mM Tris-HCl at pH 7.5 instead of MES.

The positions of all individual subunits of complex I on our SDSPAGE system were positively determined by mass spectrometry (15), allowing for unambiguous assignment of subunits constituting observed subcomplexes. Additionally, positions of all hydrophilic subunits were confirmed by N-terminal sequencing (data not shown). In the case of subunits NuoN and NuoJ overlapping on the gel, additional identification of subunits in subcomplexes was performed as described previously (15).

**Analytical Methods**—Protein concentrations were determined by the Bradford (Bio-Rad) method with bovine serum albumin as standard. Pre-prepared Novex Tris glycine polyacrylamide gels containing a 10–20% acrylamide gradient (Invitrogen) were used according to the manufacturer's instructions. Enzyme activity assays were performed at 30 °C under constant stirring in a Shimadzu UV-1601 spectrophotometer with a magnetic stirrer attachment. Ferricyanide (FeCy) reduction was followed at 420 nm in an assay buffer containing 10 mM MES, pH 6.0, 50 mM NaCl, and 0.03% n-dodecyl-β-D-maltoside. Reactions were started by the addition of 1–2 μl of protein solution (2–4 μg of protein) to 2.0 ml of assay mixture containing 0.1 mM NADH and 1 mM FeCy. The oxidation of NADH in the presence of decyl-ubiquinone (DQ) or ubiquinone-1 (Q1) was followed at 340 nm. Assay buffer contained 10 mM MES, pH 6.0, 50 mM NaCl, 0.25 mg/ml E. coli total lipids, and 0.1% CHAPS carried over with the lipids. Reactions were initiated by the addition of 0.1 mM NADH after preincubation of 2.0 ml of otherwise complete assay mixture (containing 4–10 μg of protein and 0.1 mM of DQ or Q1) at 30 °C with stirring for 2 min.

**RESULTS**

**Subunit NuoL Is Dissociated from Complex I in Decylmaltoside**—DDM is one of the mildest detergents and it can support the integrity of complex I from E. coli during purification and several days incubation at 4 °C (15). As part of a strategy employed in the search for crystallization conditions and for characterization of putative subcomplexes, we explored the stability of the enzyme in detergents other than DDM. Subcomplexes were studied by performing two different chromatography steps in the presence of the new detergent, anion

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1. The abbreviations used are: MES, 2-(N-morpholino)ethanesulfonic acid; DDM, dodecylmaltoside, DM, decylmaltoside; DHPC, 1,2-dihexanoyl-sn-glycero-3-phosphocholine; DQ, 2,3-dimethoxy-5-methyl-6-decyl-ubiquinone; FeCy, ferricyanide; LDAO, N-N-dimethyldicyanovinyl-N oxide; Q1, Q2, Q3; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.
exchange followed by gel filtration.

Among detergents with shorter hydrophobic carbon chains and higher critical micellar concentrations than DDM, n-decyl-β-D-maltoside preserved complex I intact (data not shown). However, exchange into n-decyl-β-D-maltoside (DM) at 0.5% (w/v) led to partial dissociation of the enzyme. During the first chromatography step (anion exchange) the complex remained intact, apart from a minor dissociation of the hydrophilic subunit NuoCD (probably less than 10% of the protein), which eluted partially in a first peak (Fig. 1, lane A1 and C). The second and major peak (Fig. 1, lane B1) contained apparently intact complex (Fig. 1C, lane A2). The fractions from this peak were concentrated and applied to a gel filtration column. SDS-PAGE analysis of the eluate indicated that along with intact complex (Fig. 1B, fraction 1 and C, lane A1) the preparation contained in later fractions of complex I a diminished amount of subunit NuoL (Fig. 1B, fraction 2, and C, lane B2). Additionally, a clear peak of subunit NuoL was observed late in the elution profile (Fig. 1B, fraction 3, and C, lane B3). Other subunits visible in lane B3 were because of the tail of the main protein peak. The main peak itself was shifted slightly (−13 ml) compared with the elution of intact complex I on a Superose 6 column (−12.5 ml; −550 kDa plus detergent), consistent with it being a mixture of intact enzyme and complex I without NuoL. The position of the NuoL peak in the elution profile (−15.5 ml) corresponds to about 150–200 kDa. Although close to the resolution limit of the column, this value is consistent with the elution of NuoL (−66 kDa) surrounded by a detergent micelle.

Thus, the chromatography in the presence of DM shows that the large hydrophobic subunit NuoL of the membrane part of complex I can be removed, whereas the integrity of the rest of the complex is preserved. This indicates a distal location of this subunit in the L-shaped assembly.

Subunits NuoL and NuoM Are Removed from Complex I in Diheptanoyl Phosphocholine—1,2-Diheptanoyl-sn-glycero-3-phosphocholine (DHPC) is a short chain lipid that can be used as a relatively mild detergent for the solubilization of membrane proteins (40). During exchange of complex I into DHPC (0.15% w/v) by anion exchange chromatography, we observed a significant protein peak (Fig. 2A, fraction 2) before the elution of the bulk of the protein (Fig. 2A, fraction 2). SDS-PAGE indicated that this first peak contained subunits NuoL and NuoM (Fig. 2C, lane A1), which were completely removed from the rest of the complex (Fig. 2C, lane A2). When the fractions containing the bulk of the protein were concentrated and subjected to gel filtration chromatography, a symmetrical peak eluting at −13.3 ml (corresponding to about 450 kDa plus detergent) was observed (Fig. 2B). All subunits except NuoL and NuoM were present and eluted in concert throughout the fractions, with the peak fraction shown in Fig. 2C, lane B1.

Thus, after removal of two large subunits of the membrane arm, NuoL and NuoM, the other subunits of complex I remained in a stable assembly. This demonstrates that neither subunit is involved in contacts between the membrane and peripheral arms of the enzyme.

Peak fractions from gel filtration chromatography in DHPC, containing complex I lacking subunits NuoL and NuoM, were pooled, concentrated, and assayed for NADH dehydrogenase activity. As can be seen from Table I, the activity with artificial electron acceptor FeCy was still observed, although at somewhat lower levels than with intact enzyme. However, with water-soluble quinone Q1 the rate was severely reduced, about 20-fold lower as compared with the intact enzyme. There was no measurable activity when hydrophobic ubiquinone analogue DQ was used as an acceptor.

Further Fragmentation of Complex I—The observations on the relative lability of subunits NuoL and NuoM were confirmed by the dissociation of the complex after detergent exchange into Triton X-100. In the experiments described so far, we used pH 6.0 to keep the enzyme as stable as possible (15, 20). However, Triton X-100 (0.15% v/v) was used at pH 7.5 with a preincubation of the enzyme with 0.3 M NaCl to promote dissociation of the complex (20). Anion exchange chromatography indicated the presence of three major fractions: a membrane domain without subunits NuoL and NuoM (Fig. 3, A, fraction 1, and B, lane A1), an apparently intact membrane domain complete with subunits NuoL, NuoM, and a minor proportion of NuoCD (Fig. 3, A, fraction 2, and B, lane A2), and a peripheral domain (Fig. 3, A, fraction 3, and B, lane A3). The peripheral domain appeared to be a stable assembly, such that during subsequent gel filtration chromatography on a Superose 6 column it eluted as a single species of about 300 kDa, containing all six hydrophilic subunits (data not shown). As with the 1α subcomplex of bovine complex I (18), the presence of detergent was necessary to keep the peripheral domain in solution.

When the membrane domain without NuoL and NuoM subunits (Fig. 3A, fraction 1) was concentrated and subjected to gel filtration chromatography in Triton X-100, two apparent subcomplexes were observed. First, a larger assembly of NuoN, NuoA, and NuoK subunits, eluting with a peak at fractions 3–4
and a second smaller assembly, peaking at fraction 5 and containing subunits NuoH and NuoJ (Fig. 3).

Thus, chromatography in Triton X-100 at elevated pH shows that not only can the complex be separated into peripheral and membrane domains, but also that subunits NuoL and NuoM can be removed from the rest of the membrane domain, which is consistent with our findings in other detergents. The fragmentation patterns that we observed with Triton X-100 differ from those previously reported, where under similar conditions the dehydrogenase domain (subunits NuoE/F/G), the connecting domain (NuoCD/B/I), and the membrane domain (all other subunits) were obtained (20). This is likely because of differences in the chromatographic steps employed, rather than in the initial treatment of complex I. We have, however, observed a similar fragmentation into dehydrogenase, connecting, and membrane domains after incubation of the enzyme at pH 9.0 with 2.0 M NaCl in DDM (not shown).

The “harshest” detergent used in our study was LDAO. Initial anion exchange chromatography at pH 6.0 indicated that subunits NuoL, NuoM, and NuoN co-elute in one peak (Fig. 4, A, fraction 1, and B, lane A1), followed by subunits of the peripheral domain (Fig. 4, A, fraction 2, and B, lane A2). Other hydrophobic subunits eluted earlier in the gradient. When fractions containing hydrophobic subunits were concentrated and subjected to gel filtration chromatography, all subunits eluted individually with no evidence of subcomplexes, indicating that prolonged exposure to LDAO disrupts the membrane domain completely. As would be expected with individual subunits, the smaller subunits NuoA, NuoK, and NuoJ eluted from the gel filtration column later than the larger subunits NuoN or NuoH.

**Fig. 2. Chromatography of E. coli complex I in DHPC.** A, chromatography on a HiTrap DEAE column. B, chromatography on a Superose 6 HR 10/30 column. C, Coomassie Blue-stained SDS-PAGE gels of fractions as indicated, with subunit names assigned to each visible band. **Solid line**, 280 nm absorbance. **Dotted line**, 420 nm absorbance. **Dashed line**, NaCl concentration. **Black bars** indicate fractions from A and B shown on SDS-PAGE in C. **Black arrow** indicates fractions pooled and concentrated for Superose 6 chromatography.

**Graphs**

![Graph A](image1.png)

**Graph B**

![Graph B](image2.png)

**Graph C**

![Graph C](image3.png)

**Table 1**

Activities of intact E. coli complex I and of the subcomplex obtained in DHPC (with subunits NuoL and NuoM removed)

| Preparation                  | NADH:PeCy activity | NADH:Q₆ activity | NADH:DQ activity |
|------------------------------|--------------------|------------------|------------------|
| Intact complex I             | 54.2 ± 3.3         | 17.7 ± 2.6       | 21.3 ± 2.1       |
| Complex I without NuoL and NuoM | 28.4 ± 4.3        | 0.80 ± 0.15      | 0.0              |

**Fig. 3. Chromatography of E. coli complex I in Triton X-100.** A, chromatography on a Mono-Q HR 5/5 column. B, Coomassie Blue-stained SDS-PAGE gel showing fractions from chromatography on a Superose 12 HR 10/30 column. **Solid line**, 280 nm absorbance. **Dotted line**, 420 nm absorbance. **Dashed line**, NaCl concentration. **Black bars** indicate fractions from A shown on SDS-PAGE in B. **Black arrow** indicates fractions pooled and concentrated for Superose 12 chromatography.

![Graph A](image4.png)

![Graph B](image5.png)

![Graph C](image6.png)
for Superose 12 chromatography.

ND1 (NuoH) or ND6 do not assemble complex I, whereas the 43118 line, NaCl concentration. eluted with a peak at fractions 3
analysis of the eluate. Most of the hydrophilic subunits co-
some dissociation of subunit NuoB, evident from SDS-PAGE
subcomplexes rather than single dissociated subunits.

eluted earlier, and NuoH/J, which eluted later, were genuine
experiments described above the associated NuoA/K/N, which
in E. coli, mutants lacking subunit ND5 still assembled nearly
mice cell lines, where mutants lacking subunits ND4 or ND6
subunit NuoB peaked at fractions 4–5 (Fig. 4C).

Thus, the chromatography in LDAO shows that subunits
NuoL, NuoM, and NuoN are likely to form a large hydrophobic
subcomplex, which was observed transiently during our first
chromatography step. Subunit NuoB is partially dissociated
from the otherwise intact hydrophilic subcomplex, indicating
that the location of this subunit is likely at the edge of the
peripheral arm assembly.

DISCUSSION

We have shown here that subunit NuoL can be removed on
its own from otherwise intact E. coli complex I (Fig. 1), which
indicates that it is located at the extremity of the membrane
domain. If this subunit were located close to the peripheral
arm, its large size (about a quarter of the entire membrane
arm) would mean that removing it should have resulted in the
dissociation of the peripheral arm from the membrane arm. A
distal location for NuoL is supported also by our observation
that both subunits NuoL and NuoM, which together make up
about a half of the membrane domain, can be removed fully
while the rest of the complex remains assembled (Fig. 2). These
two subunits are not, therefore, involved in contact between the
hydrophilic and hydrophobic domains of the enzyme and sub-
unit NuoM must be situated next to NuoL at the distal end of
the membrane arm.

For bovine complex I, we have shown previously that subunit
ND5 (NuoL) is located at the distal end of the membrane arm,
most likely next to subunit ND4 (NuoM) (18, 25). This is consis-
tent with observations on complex I mutants in human and
mouse cell lines, where mutants lacking subunits ND4 or ND6
(NuoJ) failed to form the membrane arm of the complex (41,
42), but mutants lacking subunit ND5 still assembled nearly
intact complex I (26, 27). In C. reinhardtii, mutants lacking
ND1 (NuoH) or ND6 do not assemble complex I, whereas the
loss of ND4 or ND4 and ND5 leads to the formation of a large
650-kDa subcomplex (28).

Sequence analysis work on a modular evolution of complex I
placed subunit NuoL in the core hydrogenase module next to
the peripheral arm. This alternative proposal for the location of
NuoL was based on its similarity to a single copy subunit found
in membrane-bound hydrogenases (29, 30). However, more rec-
cent phylogenetic analysis with a larger number of sequences
did not show such a preference, with hydrogenase subunits
scattered randomly in the evolutionary tree. It was concluded
that a single antiporter-like subunit in membrane-bound
[NiFe] hydrogenases should not be labeled specifically “NuoL”
in preference to NuoL/M/N, nor should it be inferred that NuoL
is part of a hydrogenase module in complex I (24).

Sequence comparisons have suggested also that not only
subunit ND1, but also ND4 and ND5 might contain quinone-
binding sites (43). Specific labeling of bovine subunit ND5 by
an analogue of the inhibitor fenpyroximate was stimulated by
NADH/NADPH and was prevented by other complex I inhibi-
tors (32). It was suggested, therefore, that subunit ND5 is a
part of the inhibitor/Q-binding pocket together with subunits
PSST (NuoB), 49 kDa (NuoD), and ND1, at the junction of the
membrane and peripheral arms (4). Labeling of E. coli subunit

(data not shown). This confirms that in the Triton X-100 ex-
periments described above the associated NuoA/K/N, which
eluted earlier, and NuoH/J, which eluted later, were genuine
subcomplexes rather than single dissociated subunits.

The peripheral domain remained mostly intact during sub-
sequent gel filtration chromatography in LDAO, except for
some dissociation of subunit NuoB, evident from SDS-PAGE
analysis of the eluate. Most of the hydrophilic subunits co-
eluted with a peak at fractions 3–4 (~300 kDa), whereas sub-
unit NuoB peaked at fractions 4–5 (Fig. 4C).

FIG. 4. Chromatography of E. coli complex I in LDAO. A, chromatography on a HiTrap DEAE column. B, Coomassie Blue-stained SDS-PAGE gels of fractions from A as indicated, with subunit names assigned to each visible band. C, Coomassie Blue-stained SDS-PAGE gel showing fractions from chromatography on a Superose 12 HR 10/30 column. Solid line, 280 nm absorbance. Dotted line, 420 nm absorbance. Dashed line, NaCl concentration. Black bars indicate fractions from A shown on SDS-PAGE in B. Black arrow indicates fractions pooled and concentrated for Superose 12 chromatography.

FIG. 5. A model of subunit arrangement in bacterial complex I. The dehydrogenase domain (subunits NuoE, NuoF, and NuoG) and the connecting domain (subunits NuoB, NuoCD, and NuoL) comprise the peripheral arm, the rest of subunits are in the membrane arm. This model is based on the molecular outline obtained from single particle analysis, fragmentation patterns reported here, and other available data as described in the text. Borders between subunits or subcomplexes in the model are delineated approximately in accordance with their molecular mass.
NuoM by an azidoubiquinone derivative was not affected by NADH and inhibitors had a minor effect only, but this subunit was also proposed to be a part of a Q-binding pocket (33).

We have observed that complex I lacking NuoL and NuoM subunits still possesses NADH:FeCy oxidoreductase activity, but no NADH:DQ activity and very low NADH:Q1 activity (Table I). This does not seem to be consistent with the location of the ubiquinone-binding site in subunit NuoH, which is present in this subcomplex. However, our observations are in agreement with previous findings on complex I mutants lacking subunits ND4 and/or ND5, where the activity with artificial substrates was also observed, but rates with ubiquinone analogues were negligible (26–28, 41). Our results are also consistent with proposals that subunits NuoL and/or NuoM contain at least one ubiquinone-binding site. Alternatively, it is possible that the removal of subunits Nuo/NuoM leads to a conformational change in the complex, so that the Q-binding pocket formed by subunits NuoH/NuoB/NuoD (4) is no longer accessible to hydrophobic DQ. The hydrophilic subunits of the peripheral arm on its own are capable of catalyzing the residual activity that we observe with water-soluble Q1 (18).

Thus, the number of quinone-binding sites and which subunits are involved in forming them in complex I are still unsolved questions, with several possible candidates. The results of labeling with inhibitor/quinone analogues in the presence or absence of substrates have been interpreted so far mostly as implying that all labeled subunits are involved in the single Q-binding pocket proximal to the peripheral arm of the enzyme. However, long range conformational changes induced by NADH/NADPH binding have been observed with the bovine complex (37) and may be part of a mechanism of the enzyme. If this is taken into account, the labeling results are also compatible with a distal position for subunits NuoL and NuoM. If there are Q-binding sites in these subunits, it is possible that these additional quinones do not participate directly in the electron transfer pathway but may have a different role as cofactors.

The fragmentation patterns of E. coli complex I reported here can be assessed in conjunction with structures available from EM studies. Our reconstructions of dimers and monomers of the enzyme show an L-shaped molecule similar to that described in earlier work (11). Slightly more detail is visible from the dimer, possibly because the larger size of the dimer enables particles to be aligned better (Fig. 3 in Ref. 15). An outline of the molecule as determined by such EM work, with the arrangement of subunits within the complex proposed on the basis of our data and other available evidence, is given in Fig. 5. We have demonstrated here that subunit NuoL is located at the extremity of the membrane arm of E. coli complex I and that subunit NuoM is located next to it, as shown.

As well as a clear demonstration of the distal position of NuoL and NuoM, it is possible to propose an arrangement of the other subunits in the complex. Within the membrane arm of the enzyme, subunit NuoN is likely situated close to NuoM, because a transient subcomplex comprising NuoL, NuoM, and NuoO was observed in LDAO (Fig. 4). Subunit NuoO also occurred in a more stable subcomplex with NuoA and NuoK in Triton X-100, whereas NuoJ co-eluted with NuoH (Fig. 3C). This suggests a position of NuoN together with the small subunits NuoA and NuoK, between subunits NuoJ/NuoH and NuoM, as indicated (Fig. 5). Such an arrangement is consistent with observations on mutants lacking analogues of subunits NuoH, NuoD, or NuoA, in which complex I is not assembled, indicating that these subunits are located at the interface between the membrane and peripheral arms (4, 28, 42). As discussed previously (1, 18), because of a high $H^+/e^-$ ratio, it is possible that there is a dual-mode combination of direct and indirect coupling in complex I. The proposed location for the antiporter-like subunit NuoN in the vicinity of the putative Q-binding pocket formed by NuoH/NuoB/NuoD (Fig. 5) does not exclude its participation in a direct coupling of proton translocation to electron transfer as one possible part of the mechanism of complex I.

Subunits in the peripheral arm are shown (Fig. 5) divided into the dehydrogenase and connecting domains identified previously (20), which we observed also. Subunits NuoL and NuoB are amphipathic and numerous cross-linking and mutagenesis experiments indicate that they are likely to form an interface between the peripheral and membrane arms (4, 44–46). This is confirmed by our observation that subunit NuoB can be partially dissociated from the peripheral arm (Fig. 4C). The location shown for NuoB takes into account the direct interaction demonstrated between subunits NuoB and NuoA (47). The location of NuoCD is according to recent antibody labeling studies (48). The fact that NuoCD was readily dissociated from the otherwise intact complex under some conditions (Figs. 1C and 3B) suggests that this subunit does not span completely the peripheral arm, but rather that the dehydrogenase domain and connecting domain are linked also via other subunits.

The model of bacterial complex I subunit arrangement proposed (Fig. 5) is the most detailed, experiment-based model to date and is in agreement with most available data. An important conclusion is that in bacterial complex I the antiporter-like subunits NuoL and NuoM are situated at the extremity of the membrane arm. These subunits are probably involved in proton pumping, but the distance between them and the redox centers in the peripheral arm makes direct coupling between electron transfer and proton translocation through these subunits unlikely. This provides experimental support for conformational change coupling as a part of the complex I mechanism.

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