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Transmembrane topology of subunit N of complex I (NADH:ubiquinone oxidoreductase) from *Escherichia coli*

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Abstract The transmembrane topology of subunit N from *E. coli* Complex I has been investigated. Chemical labeling of mono-substituted cysteine mutants was carried out in inverted membrane vesicles, and in whole cells, using 3-N-maleimidyl-propionyl biocytin (MPB). The results support a model of 14 transmembrane spans with both termini in the periplasm, and are consistent with the models of subunits L, M and N from the crystal structure of the membrane arm of the *E. coli* Complex I (Efremov et al. (2010) Nature 465, 441–445). In particular, the results do not support an unusual cytoplasmic localization of two likely transmembrane regions, as proposed in previous studies (Mathiesen and Hägerhäll (2002) Biochim Biophys Acta 1556, 121–132; Torres-Bacete, et al. (2009) J Biol Chem 284, 33062–33069).

Keywords Complex I · NADH:ubiquinone oxidoreductase · Transmembrane topology · Cysteine mutagenesis · Chemical labeling · 3-N-maleimidyl-propionyl biocytin

Abbreviations

BA101	strain deficient in subunit N of the *E. coli* Complex I
HA	hemagglutinin
MPB	3-N-maleimidyl-propionyl biocytin
phoA	gene for alkaline phosphatase
PMBN	polymyxin B nonapeptide
TM	transmembrane

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Introduction

Complex I, or NADH:ubiquinone oxidoreductase, is a multi-subunit enzyme that serves as an entry point for reducing equivalents in the electron transport chains found in both mitochondria and in many bacteria. In addition to its function as a redox-coupled ion translocator, it has an important role in the regeneration of NAD for oxidative processes such as found in the citric acid cycle. A prominent feature of this enzyme is the large number of Fe-S centers that are involved in the transport of electrons from NADH to quinones (For a review of Complex I see (Brandt 2006)).

Bovine Complex I contains 45 different protein subunits (Carroll et al. 2006), which are arranged in an L-shape, with a peripheral arm and a membrane arm (Grigorieff 1998). Similar shapes have been observed in fungal and bacterial enzymes (Guénebaut et al. 1997; Guénebaut et al. 1998; Radermacher et al. 2006; Clason et al. 2010), including a series of recent crystal structures (Hinchliffe and Sazanov 2005; Sazanov and Hinchliffe 2006; Efremov et al. 2010; Hunte et al. 2010). In the mammalian enzyme there are seven integral membrane proteins that are encoded by mitochondrial DNA (Chomyn et al. 1985; Chomyn et al. 1986). These seven are all present in the bacterial enzymes, which contain only 13–14 subunits overall. Among the seven are three related subunits (Kikuno and Miyata 1985) called L, M and N in *E. coli*. It had also been noticed that these proteins appear to be related to subunits of several

cation antiporters (Friedrich and Weiss 1997; Mathiesen and Hägerhäll 2003). Several proposals have implicated these subunits in proton (Zickermann et al. 2009; Efremov et al. 2010; Hunte et al. 2010; Ohnishi et al. 2010) or sodium ion (Steuber 2003) translocation activities.

Recently, the crystal structure of the *E. coli* membrane arm of Complex I was reported at 3.9 Å resolution (Efremov et al. 2010). This allowed a clear view of the transmembrane helices, but lacking connectivity and side chains. Based on prior findings, N, M and L subunits could be identified, with 14, 14 and 16 transmembrane helices, respectively. The structures of the 3 related subunits were all very similar, and each had the same unusual features: 2 of the likely transmembrane spans were composed of 2 discontinuous, or broken helices, each of which traversed about half of the membrane bilayer.

Independent experimental analyses of the transmembrane topology of subunit L from *R. capsulatus* and subunit M from *E. coli* have been reported. One approach, using the alkaline phosphatase gene fusion methodology (Mathiesen and Hägerhäll 2002), yielded a model for L with 16 transmembrane spans, although two were placed out of the membrane and near the cytoplasmic surface. A similar result was obtained for subunit M from *E. coli* using a series of insertions of histidine tags (Torres-Bacete et al. 2009). In each case, the predicted transmembrane helices 10 and 11 were concluded to be in the cytoplasm, since the predicted periplasmic loop appeared to have cytoplasmic localization. In this report we have focused on the transmembrane topology of subunit N from *E. coli*, using a labeling procedure that has been applied previously in this lab to subunit *a* of the ATP synthase (Wada et al. 1999; Zhang and Vik 2003). We find that two residues in the corresponding region of N, between predicted transmembrane helices 10 and 11, can be labeled from the periplasmic space. This results in a transmembrane model for N of 14 transmembrane spans, consistent with a straightforward interpretation of the recent crystal structure (Efremov et al. 2010).

Materials and methods

Materials

Restriction enzymes were obtained from New England BioLabs. MPB was obtained from Molecular Probes. Other chemicals were from Sigma-Aldrich. DNA miniprep columns and Ni-NTA agarose were obtained from Qiagen. Nitrocellulose membranes, 12% acrylamide gels, 5-bromo-4-chloro-3-indoylphosphate *p*-toluidine salt (BCIP), *p*-nitro blue tetrazolium chloride (NBT) and the DC protein assay were from Bio-Rad. Rat anti-HA monoclonal antibody was from Roche. Synthetic oligonucleotides were obtained from Operon Technologies (Huntsville, AL). DNA sequencing was done by Lone Star Labs (Houston, TX).

Strains, plasmids and construction of mutants

Strain BA101 is deficient in subunit N of Complex I from *E. coli*, and lacks deamino-NADH driven proton translocation (Amarneh and Vik 2003). Plasmid pBA200 carries *nuoN*, the gene for subunit N, and additionally encodes an HA epitope and an octahistidine sequence at the carboxyl terminus. Mutations were constructed in pBA200 as described previously (Amarneh and Vik 2003).

Growth of cultures and preparation of membrane vesicles

Cells were grown in rich media containing 3% tryptone, 1.5% yeast extract, 0.15% NaCl and 1% (v/v) glycerol at 37 °C with ampicillin added at 0.1 g/L to cells, as described previously. Cells grown overnight at 30 °C were used to inoculate a 100 ml of culture. Induction with IPTG was done at A_{600}=0.6 and the cultures were harvested at A_{600}= 1.85. The cells were resuspended in 50 mM MES, 25% glycerol, 10 mM $MgSO_4$ pH 6.0, and passed through the French press at 8,000 psi. The supernatant fraction after a low speed centrifugation (2 min at 15,000×g) was centrifuged at 250,000×g for 20 min. The pellet was resuspended in the same buffer, and enzyme assays and labeling experiments were carried out the same day.

Labeling of membrane vesicles and cells

Membrane vesicles were prepared as described above from 200 mL cultures. Labeling occurred in 100 mM K-phosphate buffer (pH 7.4), 1 mM $MgSO_4$ with 100 μM MPB for 60 min at room temperature. The reaction was quenched with 20 mM 2-mercaptoethanol. Membranes were solubilized with 1.5% Triton X-100 and 0.05% SDS in 100 mM K-phosphate buffer (pH 7.2) with 500 mM NaCl. Following a 10 min centrifugation at 1500×g, 30 μL Ni-NTA-agarose and imidazole (30 mM) were added. The resin was allowed to bind overnight at 4 °C. The resin was washed with the same detergent solution containing 40 mM imidazole, and eluted with 30 μL of 1 M imidazole containing 1.5% Triton X-100 and 0.2% SDS at pH 8.0.

Cells were grown as described above. Thirty mL of culture were washed with 20 mM K-MOPS, 250 mM KCl, 1 mM $MgSO_4$ (pH 7.0) and resuspended to 1.5 mL. Cells were labeled with 100 μM MPB for 2 h at room temperature in the presence of 50 μM polymyxin B nonapeptide (PMBN). The reaction was quenched with 20 mM 2-mercaptoethanol. Cells were treated with lysozyme (3 mg) for 10 min at room temperature, and lysed by

freeze-thaw. Cells were solubilized and subunit N purified as described above.

Assays and immunnoblotting

Deamino-NADH assays were performed in 50 mM MOPS, 10 mM $MgCl_2$ pH 7.3 at 21 °C with 0.25 mM deamino-NADH, as described previously (Amarneh and Vik 2003). Immunoblotting of subunit N was carried out as described previously (Amarneh and Vik 2003). Analysis of MPB labeling was carried out as described previously (Wada et al. 1999; Zhang and Vik 2003).

Results

Fifteen mono-substituted cysteine mutants were analyzed in a cysteine-free subunit N background. Subunit N contains one naturally-occurring cysteine, residue 88, and this was changed to serine for the labeling studies. This mutation was shown previously to have no effect on function (Amarneh and Vik 2003). Twelve of the mutants have been described previously (88, 104, 133, 151, 154, 226, 229, 247, 295, 300, 395, and 482) and were shown to have normal levels of subunit N expression (Amarneh and Vik 2003). Four new mutations were constructed for this study (2, 320, 324, C-terminus). The four new mutants had normal levels (80–100%) of membrane-bound deamino-NADH oxidase activity (results not shown).

Periplasmic labeling

Whole cells were treated with MPB, in the presence of polymyxin B nonapeptide (PMBN), an outer membrane permeabilizing agent (Dixon and Chopra 1986), to test the exposure of cysteine residues in the periplasm. Following the labeling step, the cells were lysed and subunit N was extracted and purified by Ni-NTA chromatography. As shown in Fig. 1a, no labeling was seen with the cysteine-free protein (C88S), with W226C, or with K395C. A cysteine at the amino-terminus, T2C, was strongly labeled. Two positions at the carboxy-terminus were tested, M482C and at the extreme carboxyl terminus following the epitope tags, called 486. Both showed low levels of label. Finally, two residues that would be predicted to be periplasmic, between transmembrane helices 10 and 11, E320C and E324C, were both labeled from the periplasm. As shown in Fig. 1b, five additional mutants tested, including E154C, K158C, D229C, K295C, and Y300C were not labeled from the periplasm. In each panel, the samples were also blotted in a second gel for the level of subunit N as detected by the HA antibody, shown just below the labeling results.

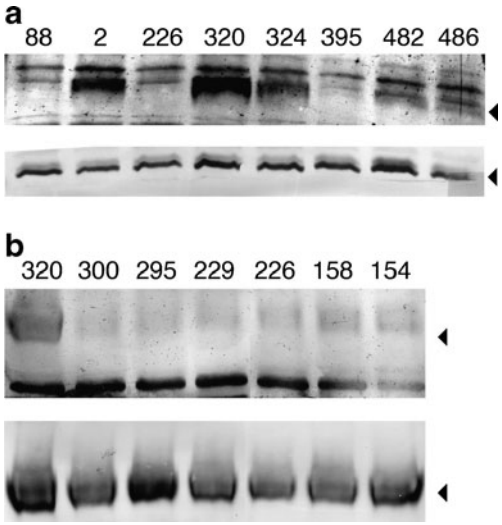

Fig. 1 Labeling of mono-cysteine mutants of subunit N in whole cells, partially-permeabilized with PMBN. **a** 88 is the cysteine-free nuoN mutant, C88S. 2 is T2C, 226 is W226C, 320 is E320C, 324 is E324C, 395 is K395C, 482 is M482C, and 486 contains a cysteine residue following the carboxy-terminal HA and octa-His tags. **b** 320 is E320C, 300 is Y300C, 295 is K295C, 229 is D229C, 226 is W226C, 158 is K158C, and 154 is E154C. In each panel the upper blot is MPB labeling, and the lower one is an immunoblot of an equivalent amount of material. Migration of subunit N is indicated by the arrowheads at the far right

Cytoplasmic labeling

Cytoplasmic labeling was carried out in preparations of French press-inverted membrane vesicles. As shown in Fig. 2a, neither the wild-type subunit N (with native Cys88) nor the cysteine-free protein (C88S) are labeled in inverted membrane vesicles after treatment with MPB, and purification of subunit N by Ni-NTA chromatography. Residue E154C is found in a conserved hydrophilic region of subunit N, and is labeled by MPB in inverted membrane

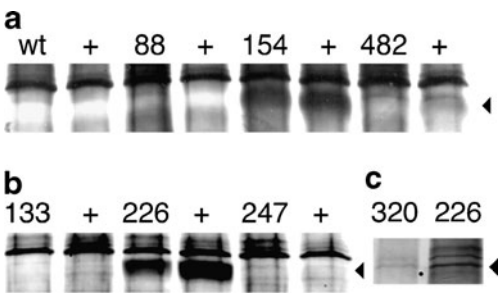

Fig. 2 Labeling of mono-cysteine mutants of subunit N in inverted membrane vesicles. The (+) sign at the right of the residue number indicates that the labeling occurred after the addition of detergent, 1.5% Triton X-100 and 0.05% SDS. All lanes were loaded with equal amounts of membrane protein. **a** wt indicates the true wild-type, with Cys-88. 88 is the cysteine-free subunit N, C88S. 154 is E154C and 482 is M482C. **b** 133 is E133C, 226 is W226C, and 247 is K247C. **c** 320 is E320C and 226 is W226C. The arrowheads at the far right indicate the position of subunit N

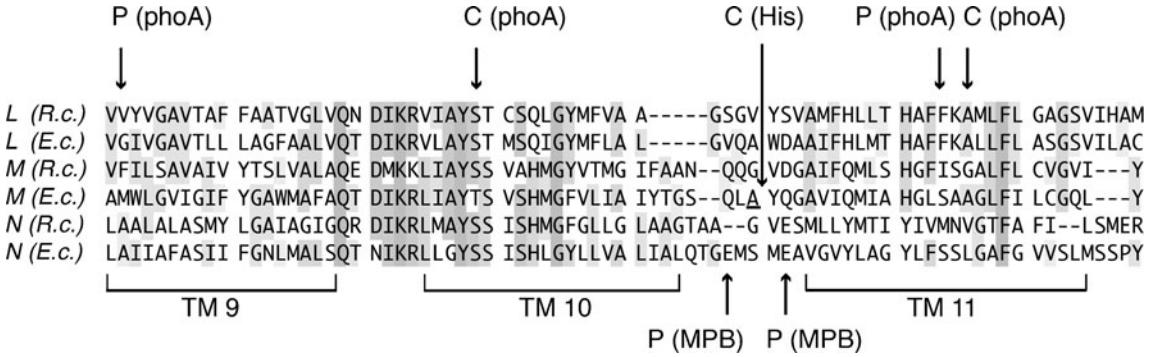

Fig. 3 Sequence alignment of subunits L, M, and N in the region of predicted transmembrane helices 10 and 11, from both *R. capsulatus* (*R.c.*) and *E. coli* (*E.c.*). Conservation of amino acid sequence is indicated by shading. Approximate locations of the predicted transmembrane spans are indicated below as TM9, TM10, and TM11. Results from alkaline phosphatase (*phoA*) gene fusion experiments (Mathiesen and Hägerhäll 2002) are indicated above, where P indicates high activity (likely periplasmic location), and C indicates low activity (likely cytoplasmic location). The site of a His-tag insertion in the *E. coli* M subunit is indicated by a long vertical arrow and C (His). The actual insertion site is to the left of the underlined A residue. The results indicated a cytoplasmic location of this insertion (Torres-Bacete et al. 2009). The locations of E320 and E324 in the *E. coli* N subunit, which were labeled from the periplasm when mutated to cysteine, in the current study, are indicated below by P (MPB)

vesicles. The addition of detergent (1.2% Triton X-100) increased the level of labeling, suggesting that the residue is partially shielded. M482C, previously found to be accessible to MPB in the periplasm, showed no labeling in inverted membrane vesicles. Upon addition of detergent, a low level of labeling was seen, suggesting that the membrane might have been partially permeabilized. In Fig. 2b, W226C, found in a conserved hydrophilic region of subunit N, is shown to be labeled by MPB in inverted membrane vesicles. The presence of detergent also slightly increased this level of labeling. Other residues shown in Fig. 2b, E133C and K247C, showed no labeling, even in the presence of detergent, suggesting that they are membrane-buried. In panel C, the labeling of E320C and W226C in inverted membrane vesicles is compared, in the absence of added detergent. The E320C shows little or no

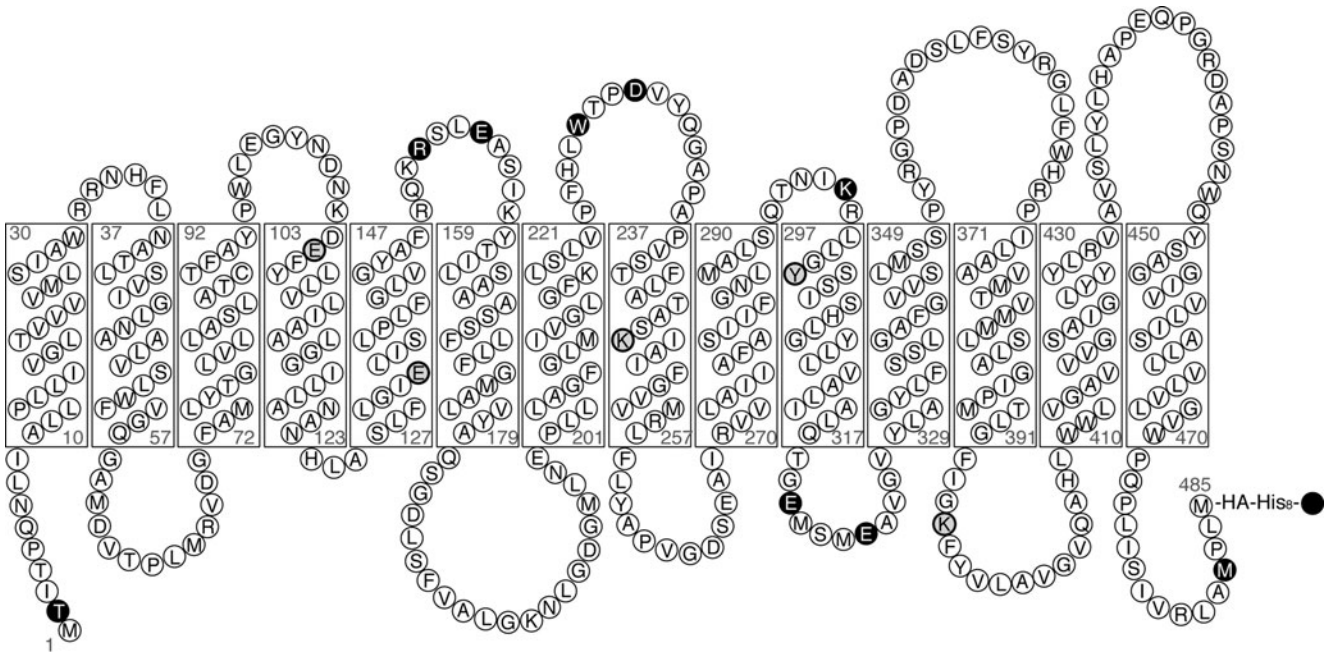

Fig. 4 Transmembrane model of the N subunit of Complex I from *E. coli*. The residues tested in this study are indicated by either lightly shaded circles, or by black circles. The lightly shaded circles, E104, E133, K247, Y300, and K395 showed little or no labeling in both whole cells and membrane vesicles. The residues indicated by black circles, T2, R151, E154, W226, D229, K295, E320, E324, M482, and C-terminal, were labeled significantly on either the cytoplasmic surface or from the periplasm, as indicated. The cytoplasm is above and the periplasm is below

labeling relative to W226C. Of the other mutants tested, only R151C, D229C, K295C, and to a small extent, Y300C, were labeled in inverted membrane vesicles (results not shown).

Discussion

The recently published crystal structure of the membrane arm of the *E. coli* Complex I indicated that there are 14 transmembrane helices in subunits N and M, and 16 in subunit L. This is consistent with many previous analyses of these membrane proteins (Mathiesen and Hägerhäll 2002; Amarneh and Vik 2003; Torres-Bacete et al. 2007; Vik 2007; Torres-Bacete et al. 2009). However, in the crystal structure, each subunit contains two transmembrane helices that seem to be composed of two discontinuous segments. Since the resolution was not high enough to determine the presence of side chains, the direction of the polypeptide chains, or the connectivity of the transmembrane helices, some uncertainties still remain about the identities of the visible helices.

The membrane topology of subunit L of the *R. capulatus* Complex I was analyzed previously by a gene fusion approach (Mathiesen and Hägerhäll 2002). In this method, fusion of alkaline phosphatase to periplasmic regions of a membrane protein should yield high activity, while fusions to a cytoplasmic region should yield low activity (Boyd et al. 1987; Traxler et al. 1993). In the study of subunit L (Mathiesen and Hägerhäll 2002), the authors concluded that the periplasmic loop between predicted transmembrane helices 10 and 11 was in the cytoplasm. A closer look at those results suggests that such a conclusion might not be warranted. Two fusions were made only 2 residues apart and gave different results (Fig. 3). But since it is likely that these fusions occurred within a transmembrane helix, the localization of alkaline phosphatase is expected to be very sensitive to the precise position of fusion. In this case, fusion after a lysine led to cytoplasmic localization, whereas a fusion before the lysine led to periplasmic localization. Therefore, those results seem consistent with a standard model of 16 transmembrane helices in subunit L.

A recent analysis of transmembrane topology of subunit M from *E. coli* used a different approach (Torres-Bacete et al. 2009). A histidine tag was inserted into subunit M at each predicted loop, assuming a model of 14 transmembrane helices with both termini in the periplasm. Both inside-out and right side-out membrane vesicles were prepared and analyzed in a dot-blot apparatus for reaction with penta-His antibody. All of the results supported the standard model of 14 transmembrane helices, except for one insertion at a predicted periplasmic loop. Once again, that was the loop between transmembrane helices 10 and 11. In this case the authors showed that the extent of antibody reaction was very high, and that the membranes had normal levels of Complex I activity, so it is not easy to discount the results (Torres-Bacete et al. 2009). The location of the His tag insertion is shown in Fig. 3 with the long vertical arrow.

The methods used in this report were developed in this laboratory for analysis of subunit *a* of the ATP synthase, and are well-established (Zhang et al. 2005). Single cysteine substitutions are incorporated into a membrane protein at various positions. Residues in the periplasm can be detected by labeling in whole cells, with the assistance of PMBN, which helps to permeabilize the outer membrane of *E. coli* (Dixon and Chopra 1986). Residues in the cytoplasm are extremely resistant to labeling using this whole cell procedure (Wada et al. 1999; Zhang and Vik 2003). The findings presented in this report indicate that the region including residues 320–324 in subunit N of *E. coli* is accessible in the periplasm, and so it is likely that TM spans 10 and 11 have the predicted arrangement in the membrane. (See Fig. 4). The results presented here also indicate that residues Glu-154 and Arg-151, between TM spans 5 and 6, are exposed at the cytoplasmic surface. This is also true of residues Trp-226 and Asp-229, between TM spans 7 and 8, and residue Lys-295 between TM spans 9 and 10. The placement of these residues is consistent with the results presented for subunit L from *R. capsulatus* (Mathiesen and Hägerhäll 2002) and subunit M from *E. coli* (Torres-Bacete et al. 2009). In addition, the results presented here provide evidence that both termini of subunit N are in the periplasm. Therefore it seems likely that the broken transmembrane helices seen in the crystal structure are composed of continuous polypeptide segments, and that subunits L, M and N are composed of 16, 14, and 14 transmembrane spans.

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