A Novel Labeling Approach Supports the Five-transmembrane Model of Subunit $a$ of the Escherichia coli ATP Synthase*

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The ATP synthase from Escherichia coli is typical of the ATP synthases found in mitochondria, chloroplasts, and many other bacteria (for reviews, see Refs. 1–3). It contains an F$_1$ sector, with subunits for nucleotide binding and catalysis, and an F$_0$ sector, which conducts protons across the membrane. Five different subunits are found in the E. coli F$_0$: $a$, $b$, $g$, $e$, and $c$, in a stoichiometry of 3:3:1:1:1. Three different subunits named $a$, $b$, and $c$ form the E. coli F$_0$ with a stoichiometry of 1:2:12 (4).

The mechanism by which an electrochemical proton gradient across the membrane drives ATP synthesis is thought to involve a rotary mechanism. The crystallization of F$_1$ from bovine mitochondria (5) led to a high resolution structure of the $a$$\beta$$\gamma$ hexamer, plus parts of $\gamma$, $\delta$, and $\varepsilon$, in a stoichiometry of 3:3:1:1:1:1. The transmembrane topology of subunit $a$ has been analyzed (6), (7). It contains a $\alpha$$\beta$$\gamma$$\delta$$\varepsilon$-helical hairpin structure of the two predicted transmembrane spans, and also provides a simple and reliable technique for detection of periplasmic regions of inner membrane proteins in E. coli.

Cysteine mutagenesis and surface labeling has been used to define more precisely the transmembrane spans of subunit $a$ of the Escherichia coli ATP synthase. Regions of subunit $a$ that are exposed to the periplasmic space have been identified by a new procedure, in which cells are incubated with polymyxin B nonapeptide (PMBN), an antibiotic derivative that partially permeabilizes the outer membrane of E. coli, along with a sulphhydryl reagent, 3-(N-maleimidylpropionyl) biocytin (MPB). This procedure permits reaction of sulphhydryl groups in the periplasmic space with MPB, but residues in the cytoplasm are not labeled. Using this procedure, residues 8, 27, 37, 127, 131, 230, 231, and 232 were labeled and so are thought to be exposed in the periplasm. Using inside-out membrane vesicles, residues near the end of transmembrane spans 1, 64, 67, 68, 69, and 70 and residues near the end of transmembrane spans 5, 260, 263, and 265 were labeled. Residues 62 and 257 were not labeled. None of these residues were labeled in PMBN-permeabilized cells. These results provide a more detailed view of the transmembrane spans of subunit $a$ and also provide a simple and reliable technique for the detection of periplasmic regions of inner membrane proteins.

Information about the tertiary and quaternary structure of F$_0$ subunits will be necessary for an understanding of how F$_0$ translocates protons, and how it might drive rotation of $\gamma$ and $\varepsilon$ subunits in F$_1$. Subunit $b$ seems to be embedded in the membrane via a span of hydrophobic amino acids at its N terminus. A truncated, soluble form of $b$ has been shown to be extended and dimeric (10). Recent NMR studies of $c$ have confirmed the $\alpha$-helical hairpin structure of the two predicted transmembrane spans, and also details of the essential residue Asp$^{13}$ and its local environment (11, 12). Cysteine cross-linking studies have provided information about the oligomeric structure of subunit $c$ (13) and about its interactions with subunit $a$ (14). One face of a transmembrane $\alpha$-helix between residues 207 and 225 of subunit $a$ appears to be in contact with the oligomer of subunit $c$. This region includes Arg$^{210}$, which is thought to be essential for function (15–17).

The transmembrane topology of subunit $a$ has been analyzed by several methods in recent years. In particular, studies using the labeling of cysteine substitutions (18, 19), epitope insertions (20), and peptide-directed antibodies (20, 21), to identify surface accessible regions of the protein have come to significant agreement. However, the location of the N terminus remains controversial, leading to models of five- or six-transmembrane spans. Studies using the labeling of cysteine substitutions (18, 19) concluded five-transmembrane spans, with a periplasmic N terminus. Studies using epitope insertion and peptide-directed antibodies (20, 21) found cytoplasmic localization of the N terminus, leading to six-transmembrane spans. All of the studies required the preparation of oriented membrane vesicles for probing potentially accessible surface residues or regions. A limitation of such experiments is that the membrane vesicles may not be perfectly sealed or oriented. Also, some reagents may be somewhat permeable to membranes because of a limited degree of partitioning into the lipid phase. Such limitations may be responsible for the detection of certain residues or epitopes in membrane vesicles of both orientations.

In this study, we have minimized these problems for the detection of periplasmic regions of inner membrane proteins of E. coli. This procedure requires the use of an antibiotic derivative, PMBN, to partially permeabilize the outer membrane (22) to the labeling reagent, MPB. Essentially zero background labeling is seen with highly accessible cytoplasmic cysteine residues, allowing a more definitive assignment of periplasmic regions.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs. Materials for silver sequencing and

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plasmid mini-preps were obtained from Promega Corp. Synthetic oligonucleotides were obtained from Operon Technologies. Urea was from International Biotechnologies, Inc. MBP was obtained from Molecular Probes. PMBN, mouse and rat anti-HA, anti-His tag antibody, and proteins A and G-agarose were obtained from Roche Molecular Biochemicals. Ni-NTA resin was obtained from Qiagen. Talon resin was obtained from CLONTECH. Octyl glucoside was obtained from Sigma or Anatrace. Anti-α antiserum was provided by Dr. Karlheinz Altendorf (Universität Osnabrück). Immunoblotting reagents were obtained from Bio-Rad.

**Growth and Expression**—For expression, RH305 (uncB205, recA56, srl: Tn10, bgIR, thi-1, rol-1, Hfr PO1) was used as the background strain (23). It produces a defective subunit α that is truncated near Pro240 (24) and is complemented by plasmids containing a wild type uncB gene. Cultures were grown at 37 °C in LB or Minimal A medium supplemented with sucinate (0.2%). Media were also supplemented with chloramphenicol (34 mg/liter) or tetracycline (12.5 mg/liter) as appropriate.

**Plasmids and Mutagenesis**—New mutations were constructed by cassette mutagenesis (25) and are shown in Table I. All mutants, when expressed in RH305, permitted growth on succinate minimal medium, indicating normal ATP synthesis, with the exception of S62C. Plasmids designated “His” code for five additional histidine residues following the natural C-terminal His271 of subunit α, indicating normal ATP synthesis, with the exception of S62C. Plasmids designated “HisHA,” contain, in addition to the histidine segment, the nine-residue HA epitope (underlined) inserted after Ser268. It generates a C-terminal sequence of YPYDDVPDYASSEHHHHHH, with an additional Ser residue following the HA epitope. One new plasmid was constructed for this study, pARP2-HisHA. First, a 600-bp BglI-AflII fragment was isolated from pSBV10 (26), internal to uncB. This fragment was ligated to a 2700-base pair BglII-AflII fragment from pTW1-HisHA (18) to form pARP1-HisHA. A 115-bp SpeI-BglI fragment was excised from pARP1-HisHA and replaced by synthetic DNA to generate pARP2-HisHA. The construction of the other cysteine mutations at residues 8, 27, 69, 128, and 131, was described previously (18). These mutations were all expressed with His-HA tags at the C terminus.

**Preparation and Labeling of Membrane Vesicles**—Inside-out membrane vesicles were prepared by French press as described previously (18). The membrane vesicles were labeled in 120 μM MPB at 15 min at 25 °C, as described previously (18). After labeling, subunit α was detergent extracted and purified by Ni-NTA, as described previously (18).

**Preparation and Labeling of Whole Cells**—A 30-ml culture of cells was grown in LB medium at 37 °C to A600 = 1.0 and harvested. The cells were resuspended in 1 ml of 20 mM K-Mops, 250 mM KCl, 1 mM MgSO4, pH 7.0, and washed twice. For labeling, they were immediately suspended in the same buffer with 50 μM PMBN and 150 μM MPB and incubated at 25 °C for 1 h. Following centrifugation (10 min at 16,000 g), the cells were resuspended in 20 mM Tris-HCl, pH 7.5, 1.5% octyl glucoside, 0.1% deoxycholate, 0.5% cholate, and 1% Tween 20. Following a 2-h incubation at 4 °C, the previous centrifugation step was repeated, and the supernatant fraction was transferred to a new microfuge tube and mixed with 50 μl of protein A-agarose and incubated another 3 h (4 °C). After a short spin to remove agarose, the supernatant fraction was transferred to a new microfuge tube and mixed with 100 μg of anti-HA and incubated another 1.5 h (4 °C). Finally, 50 μl of protein A-agarose was added, incubated for 3 h (4 °C), washed and suspended in 100 μl of 50 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 14.4 mM 2-mercaptoethanol, and 0.1% bromphenol blue.

**MPB Detection and Immunoblotting**—Samples of purified subunit α were subjected to SDS-polyacrylamide gel electrophoresis (13% acrylamide) and transferred to nitrocellulose membrane (0.2 μm) as described previously (18). Subunit α detection, using rabbit anti-α serum, was carried out as described previously (18). Subunit α bearing the HA epitope was detected with rat anti-HA in a similar manner. The use of anti-HA eliminated detection of the mouse anti-HA, present in the immunoprecipitation complex, after reaction with secondary antibodies.

### RESULTS

A model for subunit α of the ATP synthase containing five-transmembrane spans has been presented recently from labeling studies of cysteine mutants (18). In this study, 24 new cysteine substitution mutants have been constructed to define more precisely the ends of the transmembrane spans. Periplasmic exposure of the cysteine residues was detected by labeling with a new procedure in which cells were partially permeabilized by incubating with 50 μM polymyxin B nonapeptide in the presence of the sulfhydryl reagent MPB. During this procedure, only periplasmic residues were labeled. Labeling was also performed with inside-out membrane vesicles prepared by French press. Cytoplasmic residues were identified as those that can be labeled in inside-out membrane vesicles but not in PMBN-permeabilized cells. Both labeling procedures were carried out for each cysteine mutant, and the results are organized into five groups.

In Fig. 1, the results for residues 37, 39, and 44 are shown, along with previously studied cysteine mutants 8 and 27. In panel A, labeling in PMBN-permeabilized cells is shown, and in panel B, labeling in inside-out membrane vesicles is shown. Panel C shows immunoblots of the membrane vesicles using rat anti-HA antibody. Residues 8 and 27 were shown previously to be periplasmic (18), although other methods have indicated that the N terminus is cytoplasmic (20, 21). Here these residues are labeled in PMBN-permeabilized cells (Fig. 1A), and also in inside-out membrane vesicles (Fig. 1B), because of the permeability of MPB under these conditions. Residue 37 is labeled in a similar pattern but not as strongly. Residues 39 and 44 are not labeled by either method.

The C-terminal end of the first transmembrane span was probed by cysteine substitution of residues 62, 64, 67, 68, 69, and 70, and the results are shown in Fig. 2. None of these mutants can be labeled by MPB in PMBN-permeabilized cells (Fig. 2A), whereas all but 62 can be labeled in inside-out membrane vesicles (Fig. 2B). Residues 67, 68, 69, and 70 are labeled strongly, compared with residue 64. These results indicate cytoplasmic exposure for residues 64, 67, 68, 69, and 70. The mutant S62C is impaired in function as indicated by little or no growth in minimal succinate medium, but immunoblots indicate the presence of the protein in membrane preparations at a significant level (Fig. 2C).

The periplasmic loop between transmembrane spans 2 and 3 was probed by cysteine substitution at positions 127, 128, and 131, and the results are shown in Fig. 3. Residue 131, consistent with earlier results (18, 19), can be labeled in PMBN-permeabilized cells, indicating periplasmic exposure.
The residues changed to Cys are indicated by number above each lane. Panel A shows labeling of cysteine residues by MPB in PMBN-permeabilized cells, as detected by avidin-conjugated alkaline phosphatase color assay. Panel B shows labeling of cysteine residues by MPB in inside-out membrane vesicles, as detected by the same color assay. Panel C shows an immunoblot of the samples from panel A, using rat anti-HA for detection of subunit α.

127 is labeled similarly, whereas residue 128 is resistant to labeling. All three residues are labeled somewhat in inside-out membrane vesicles (Fig. 3B).

The periplasmic loop between transmembrane spans 4 and 5 was probed by cysteine substitution at positions 226–228, 230–234, 240, 241, 244, and 246. These results are shown in Fig. 4. Only residues 230, 231, and 232 were labeled by MPB in PMBN-permeabilized cells (Fig. 4A), indicating a very limited periplasmic exposure in this region. None of the residues were labeled in inside-out membrane vesicles (Fig. 4B).

The C-terminal end of the fifth, and last, transmembrane span was probed by cysteine substitution at positions 257, 260, 263, and 265, and the results are shown in Fig. 5. None of these residues was labeled in PMBN-permeabilized cells (Fig. 5A). All but 257 were labeled in inside-out membrane vesicles (Fig. 5B), indicating cytoplasmic exposure for approximately twelve residues at the C terminus of subunit α.

**DISCUSSION**

A new method for determining the orientation of inner membrane proteins in *Escherichia coli* has been described. This method is based upon a procedure described by Matos et al. (27), in which *E. coli* cells were partially permeabilized with EDTA to permit periplasmic labeling of cysteine mutants of an inner membrane transport protein. In this study, PMBN (22) has been used in place of EDTA for more efficient labeling of subunit α of the ATP synthase. PMBN is not essential for the labeling of some residues by MPB, e.g., E131C. This suggests that MPB has a limited, intrinsic permeability with respect to the outer membrane. In addition, the detection of labeled subunit α after labeling in whole cells requires a reliable purification procedure, to eliminate background labeling. That is an essential step because subunit α cannot be expressed to high levels. These results demonstrate that immunoprecipitation of subunit α, tagged with the HA epitope, by the commercial mouse anti-HA provides a cleaner background than does a procedure using Ni-NTA chromatography.

The results indicate that during permeabilization of *E. coli* outer membrane by PMBN, only residues of subunit α that are facing the periplasm are exposed to the sulphydryl reagent MPB. For example, mutant S69C, which labels strongly in inside-out membrane vesicles prepared by French press, is not labeled in whole cells permeabilized by PMBN. This indicates that the concentration of the reagent MPB does not build up inside the cell to levels that are high enough for significant reaction with S69C. This feature distinguishes this method from other methods in which labeling of membrane proteins occurs in oriented membrane vesicles. In such preparations, residues that are labeled strongly in one orientation, are generally labeled in the opposite orientation, to a lesser extent. Also, this method does not seem to label within transmembrane spans, as indicated by the lack of labeling in residues 233–246. Therefore, the method introduced here allows a more definitive analysis of the orientation of membrane proteins in *E. coli*, and eliminates the need for the preparation of right-side-out membrane vesicles.

The results presented here provide support for the five-transmembrane model of subunit α of the ATP synthase, presented recently (18, 19). They also define better, the periplasmic loops and the cytoplasmic ends of the first and last transmembrane spans. These results are summarized in Fig. 6.

The first transmembrane span is well defined by the labeling results, indicating that it occurs between residues 37 and 64. The first cytoplasmic loop is highly exposed in a region extending from residue 64 to 70. The first periplasmic loop is not as highly exposed, but more work will be necessary to define this region. Residues following 131 have not been tested, and so the start of the third transmembrane span remains uncertain. Previous labeling of residues G172C (18) and K169C (19) indicates the probable cytoplasmic end of the third span. The second periplasmic loop has been extensively examined in this study. Of twelve residues tested, only three consecutive residues, 230–232, could be labeled. Furthermore, the extent of labeling of each was low, compared with residues near the N terminus, indicating rather limited exposure in this region of the protein. The cytoplasmic end of the fifth transmembrane span was defined by the labeling of residues 260, 263, and 265, but not 257. Previous work had identified residue 266 as cytoplasmic (19).

For function, the only essential residue of subunit α seems to be Arg210, which is thought to interact with the essential Asp61 of subunit c. This has been supported (14) by analysis of engineered disulfide cross-links formed between subunit α and subunit c, which indicated that a face of subunit α between residues 207 and 225 lies opposed to residues 55–73 in subunit c. The results presented here suggest that Arg210 is located closer to the cytoplasmic surface than the periplasmic surface of the protein. This should be verified by more labeling studies in the region of the protein preceding residue 210. Results so far indicate that residue 196 can be labeled, but not residue 202 (19).

Two other residues have been implicated in the function of subunit α, His245 and Glu219. Glu219 can tolerate a limited range of amino acid substitutions, and cysteine mutants of both Glu219 and His245 can be suppressed by second-site mutations in subunit α, A145E and D119H, respectively (15). Results

![Fig. 2. Labeling of residues 62–70 in subunit α.](image1)

![Fig. 3. Labeling of residues 127–131 in subunit α.](image2)
Residues 8, 27, and 37 are clearly labeled in PMBN permeable Cys are indicated by number above each subunit of subunit a is indicated to the right. The residues changed to Cys are indicated by number above each lane. The double mutant S233C,Q234C is indicated by 233/4. Panel A shows labeling of cysteine residues by MPB in PMBN-permeabilized cells, as detected by avidin-conjugated alkaline phosphatase color assay. Panel B shows labeling of cysteine residues by MPB in inside-out membrane vesicles, as detected by the same color assay. Panel C shows an immunoblot of the samples from panel A, using rat anti-HA for detection of subunit a.

FIG. 5. Labeling of residues 257–265 in subunit a. The first lane on the left contains molecular mass standards, 28 and 36 kDa. The migration of subunit a is indicated to the right. The residues changed to Cys are indicated by number above each lane. Panel A shows labeling of cysteine residues by MPB in inside-out membrane vesicles, as detected by the same color assay. Panel B shows labeling of cysteine residues by MPB in inside-out membrane vesicles, as detected by the same color assay. Panel C shows an immunoblot of the samples from panel A, using rat anti-HA for detection of subunit a.

The residues changed to Cys are indicated by number above each lane. Panel A contains molecular mass standards, 28 and 36 kDa. The migration of subunit a is indicated to the right. The residues changed to Cys are indicated by number above each lane. Panel A shows labeling of cysteine residues by MPB in PMBN-permeabilized cells, as detected by avidin-conjugated alkaline phosphatase color assay. Panel B shows labeling of cysteine residues by MPB in inside-out membrane vesicles, as detected by the same color assay. Panel C shows an immunoblot of the samples from panel A, using rat anti-HA for detection of subunit a. The residues changed to Cys are indicated by number above each lane. Constructs for Y263C and S265C do not contain the HA-epitope.

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