The membrane topology of the a subunit of the F_{1}F_{0} ATP synthase from *Escherichia coli* has been probed by surface labeling using 3-(N-maleimidylpropionyl) biocytin. Subunit a has no naturally occurring cysteine residues, allowing unique cysteines to be introduced at the following positions: 8, 24, 27, 69, 89, 128, 131, 172, 176, 196, 238, 241, and 277 (following the COOH-terminal 271 and a hexahistidine tag). None of the single mutations affected the function of the enzyme, as judged by growth on succinate minimal medium. Membrane vesicles with an exposed cytoplasmic surface were prepared using a French pressure cell. Before labeling, the membranes were incubated with or without a highly charged sulfhydryl reagent, 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid. After labeling with the less polar biotin-N-maleimidylpropionyl) biocytin, the samples were solubilized with octyl glucoside/cholate and the subunit a was purified via the oligohistidine at its COOH terminus using immobilized nickel chromatography. The purified samples were electrophoresed and transferred to nitrocellulose for detection by avidin conjugated to alkaline phosphatase. Results indicated cytoplasmic accessibility for residues 69, 172, 176, and 277 and periplasmic accessibility for residues 8, 24, 27, and 131. On the basis of these and earlier results, a transmembrane topology for the subunit a is proposed.

The F_{1}F_{0} ATP synthase from *Escherichia coli* is typical of the ATP synthases found in mitochondria, chloroplasts, and many other bacteria (for recent reviews, see Refs. 1–4). It comprises five different subunits. Five different subunits are found in F_{1}: α, β, γ, δ, and ε. Predicted transmembrane spans (29, 30) and also details of the essential residue Asp^{61} and its local environment (32, 33). Hydropathy analysis (41) according to von Heijne (42) indicates five “certain” transmembrane spans and one “tentative” span. The mechanism by which an electrochemical proton gradient across the membrane drives ATP synthesis is slowly emerging (6), due in large part to success in recent years in obtaining structural information about the subunits of the enzyme. The crystallization of F_{1} from bovine mitochondria (7) led to a high resolution structure of the α_{3}β_{3} hexamer, plus parts of γ in the central core. Electron cryomicroscopic images have also contributed to an understanding of subunit arrangement (8, 9) and motion in F_{1} subunits (10). Subsequently, the hypothesis of rotation of γ relative to α_{3}β_{3} (11) has been supported by studies involving engineered disulfide-cross-linking of β to γ (12), a fluorescence technique termed “polarized absorption recovery after photobleaching” (13), and direct visualization of rotation of fluorescently labeled actin filaments covalently attached to γ (14). The three-dimensional structures of two other F_{1} subunits from *E. coli* have been determined: δ (15) and ε (16, 17). Both subunits are small, two-domain proteins. The ε subunit binds to γ through its NH_{2}-terminal domain at the “base” of F_{1} (18, 19) and interacts with α and β subunits through its COOH-terminal domain (20–22). Both γ and ε subunits have been cross-linked to c subunits (23, 24). The δ subunit interacts with α and β subunits at the “top” of F_{1} through its NH_{2}-terminal domain (25, 26), and probably with b subunits through its COOH-terminal domain (27, 28). The three-dimensional structure of a monomeric subunit c has also been determined by NMR (29, 30).

Lack of information about the tertiary and quaternary structure of F_{0} subunits has limited progress in understanding how F_{0} translocates protons and how it might drive rotation of γ and ε subunits in F_{1}. The b subunits seem to be embedded in the membrane via a span of hydrophobic amino acids at the NH_{2} terminus. A truncated, soluble form of subunit b has been shown to be extended and dimeric (31). NMR studies of subunit c have confirmed the α-helical hairpin structure of the two predicted transmembrane spans (29, 30) and also details of the essential residue Asp^{61} and its local environment (32, 33). Questions remain about the oligomeric structure of subunit c and how it interacts with subunits a and b. Mutagenesis has revealed that in addition to Asp^{61} of subunit c, three residues in subunit a seem to be important in proton translocation: Arg^{210}, Glu^{219}, and His^{245} (34–38). Knowledge of the relative location of these four residues could provide much insight into proton translocation and subunit movements.

Subunit a is an extremely hydrophobic protein of 271 amino acids. It cannot be expressed at high levels in *E. coli* (39) and has only been purified in the presence of trichloroacetate (40). Hydropathy analysis (41) according to von Heijne (42) indicates five “certain” transmembrane spans and one “tentative” span. Because the important residues in subunit a reside in the last two predicted transmembrane spans, and the uncertain span immediately precedes this region, at least two plausible arrangements exist. Such questions of membrane topology can be addressed by gene fusion experiments (43). In the case of subunit a, two groups (44, 45) have used phoA fusions to determine its membrane topology but failed to reach agreement. A more recent study used peptide-directed antibodies against polar regions of subunit a to determine membrane topology (46), but only three of the antibodies provided information.

To address the issues of how many transmembrane spans are in subunit a and how they are oriented, we have generated
Other identified restriction sites were used in mutagenesis. The gene black DNA to introduce new restriction sites by silent mutations are shown in black. The boundaries of these regions are identified by restriction sites. Other restricted sites used in mutagenesis. The gene coding for chloramphenicol resistance is also indicated (Cm).

FIG. 1. Plasmids used in this study. The gene for subunit a, uncB, is shown at the left. Regions that have been replaced with synthetic DNA to introduce new restriction sites by silent mutations are shown in black. The boundaries of these regions are identified by restriction sites. Other identified restriction sites used in mutagenesis. The gene coding for chloramphenicol resistance is also indicated (Cm).

a collection of subunit a mutants, each with a single unique cysteine residue. These mutants can be probed with sulfhydryl reagents in oriented membrane preparations to determine the surface accessibility of different residues.

**EXPERIMENTAL PROCEDURES**

**Materials—**Restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs. Materials for silver sequencing and plasmid minipreps were obtained from Promega Corp. Synthetic oligonucleotides were obtained from Operon Technologies or National Biociences. Urea was from International Biotechnologies, Inc. MPB and AMS were obtained from Molecular Probes. Nickel-nitrilotriacetic acid resin was obtained from Qiagen. Octyl glucoside was obtained from Sigma. Anti-a antibodies were provided by Dr. Karlheinz Altendorf (Osnabrücker). Immunoblotting reagents were obtained from Bio-Rad.

**Plasmids—**Plasmids were used for mutagenesis and expression are shown in Fig. 1. Mutations designated "His" code for 5 additional histidine residues following the natural carboxy-terminal Hisst of subunit a. Silent mutations have been introduced into various regions of uncB to facilitate cassette mutagenesis. While this project was under way, it was discovered that several plasmids had very poor expression: pSBV16 and its derivatives (47). It is thought that this was due to the loss of the tet promoter originally found in pACYC184. Therefore, many of the mutations were subcloned into new plasmids that contained the promoter. Plasmid pSW18His was constructed from pSBV18 (47) using the oligonucleotides that also introduced Cys277 (see Fig. 2). Plasmid pSW19His was constructed from pSBV18 (47) using the oligonucleotides that also introduced Cys277 (see Fig. 2). Plasmid pSW19His was constructed from pSBV18 (47) using the oligonucleotides that also introduced Cys277 (see Fig. 2).

**Preparation of Oriented Vesicles—**Inside out membrane vesicles were made from a 250-mL culture in LB medium grown to A660 = 1.0. Cells were resuspended in 5 ml of 50 mM Tris-HCl containing 10 mM MgSO4 (pH 8.0) and passed through the French press at 14,000 p.s.i. After a low speed spin to remove unbroken cells (7000 × g), the supernatant was centrifuged at 50,000 rpm for 1 h in a Beckman Ti70 rotor. To prepare oriented membrane vesicles (loss of Fp), the vesicles were resuspended in stripping buffer (1 mM Tris-HCl, pH 8.0, 0.5 EDTA, and 10% glycerol) and agitated at 4 °C overnight. The next day, the centrifugation was repeated, and the vesicles were resuspended in 200 mM Tris-HCl (pH 8.0). For nonstripped vesicles, the samples were resuspended directly in 200 mM Tris-HCl (pH 8.0).

**Chemical Labeling and Blocking—**The membrane vesicles were labeled in 200 mM Tris-HCl (pH 8.0) with MPB. The reaction was stoped by adding β-mercaptoethanol to a final concentration of 20 mM. The vesicles were then centrifuged at 50,000 rpm for 45 min and resuspended in the original buffer. Blocking with AMS was done in the same buffer, followed by centrifugation at 50,000 rpm for 45 min. The vesicles were resuspended in the same buffer, and labeled with MPB as described above.

**Purification of Subunit a—**After labeling, membrane vesicles were resuspended in 100 mM Tris-HCl (pH 8.0), 1.5% octyl glucoside, 0.1% deoxycholate, 0.5% cholate, 10 mM β-mercaptoethanol, 10 mM imidazole, and 1% Tween 20 (53). The samples were incubated with agitation for 1 h at 4 °C and centrifuged at 14,000 rpm (16,000 × g) for 10 min in a microcentrifuge (1.5 ml). The supernatant was added to 0.4 ml of nickel-nitrilotriacetic acid resin that had been previously incubated in the extraction buffer. The mixture was incubated with agitation for 45 min at room temperature, and centrifuged for 30 s at 14,000 rpm. The resin was washed three times with 1 ml of wash buffer, consisting of equal volumes of extraction buffer and 200 mM Tris-HCl (pH 8.0). Subunit a was eluted by adding 0.25 ml of elution buffer (extraction buffer containing 1 mM imidazole) to the mixture. The mixture was incubated at room temperature for 5 min and centrifuged at 16,000 × g for 30 s. The supernatant containing the purified subunit a was collected and stored at −20 °C.

**MPB Detection and Immunoblotting—**Samples of purified subunit a were subjected to SDS-polyacrylamide gel electrophoresis (13% acrylamide) and transferred to nitrocellulose membrane (0.2 μm) using a Trans-Blot apparatus (Bio-Rad) overnight at 16 V. The nitrocellulose membrane was blocked with 5% low fat powdered milk in 20 mM Tris-HCl, 500 mM NaCl, pH 7.5 (TBS) for 1 h and rinsed with TBS/Tween 20 (0.05% Tween 20) three times. For MPB detection, the blocked membrane was incubated with avidin-conjugated alkaline phosphatase for 2 h, rinsed three times in TBS/Tween 20, and developed with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium according to the manufacturer’s instructions. For subunit a detection, the blocked nitrocellulose membrane was incubated at room temperature for 2 h with rabbit anti-a serum, diluted 1:5000. After washing five times with TBS/Tween 20, it was incubated with goat, anti-rabbit IgG-alkaline phosphatase conjugate at a dilution of 1:1000 for 2 h. After another five washings with TBS/Tween 20, color was developed as described above.

**RESULTS**

Unique cysteine residues were introduced into subunit a at the following positions: 8, 24, 27, 69, 89, 128, 131, 172, 176, 196, 238, and 277 (COOH terminus). The resulting plasmids were transformed into RH305 (uncB205) and tested for growth on

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1 The abbreviations used are: MPB, 3-(N-maleimidylpropionyl) bicyclomycin; CAS, 4-actetamido-4’-maleimidylstilbene-2,2’-disulfonic acid; octyl glucoside, n-octyl-β-D-glucopyranoside; HA, the hemagglutinin H2 epitope (NPYDPYDVA).
succinate minimal medium. All grew as well as wild type, but
three other mutants grew poorly and were not further ana-
lyzed: 169 and two double mutants, 24/27 and 238/241.

Everted membrane vesicles were prepared using a French
pressure cell from cultures of *E. coli* cells bearing the unique
cysteine mutations. Membranes were reacted with various con-
centrations of MPB from 10 μM to 1 mM, at temperatures from
0 to 25 °C, and for various lengths of time from 10 min to 1 h.
The standard conditions chosen were the mildest under which
significant labeling was detectable, to minimize the penetra-
tion of the membrane by MPB. The labeling patterns were the
same whether or not the membranes were stripped of F1. The
HA was inserted at a SqiI site in pLN6His that precedes the His6. Along
with the oligohistidine, it generates a sequence following Ser268 of
YPYDVPDYASEEHHHHHH.

Membrane Topology of Subunit a

| Subunit a | Description |
|-----------|-------------|
| P8C       | BstEII      |
| R24C      | SpeI        |
| S27C      | HindIII     |
| S69C      | BstEII      |
| S89C      | HindIII     |
| Y128C     | BstEII      |
| E131C     | BstEII      |
| H132C     | BstEII      |
| K169C     | PstI        |
| G172C     | PstI        |
| K176C     | PstI        |
| E196C     | HindIII     |
| N238C     | AseI        |
| N238C     | AseI        |
| W241C     | BstXI       |
| 6-His NbeI| SapI        |
| 277C      | SapI        |

FIG. 2. Oligonucleotides used to construct mutations. The site of the
mutation is shown at the left. The restriction sites used in the cassette mutagen-
esis are also indicated. In some cases, an equal mixture of two bases was used at a
single site to generate multiple mutations (M, A plus C; K, G plus T, A plus T, Y,
C plus T, R, A plus G; S, G plus C). The oligohistidine construct was originally
made with 50% two stop codons, and 50% cysteine preceding a single stop codon.
The HA was inserted at a SqiI site in pLN6His that precedes the His6. Along
with the oligohistidine, it generates a sequence following Ser268 of
YPYDVPDYASEEHHHHHH.

To detect labeling, the subunit a was extracted from mem-
branes by solubilization with a mixture of detergents (cholate,
deoxycholate, and octyl glucoside). The subunit a was partially
purified via its oligohistidine using nickel affinity chromato-
graphy. The samples were electrophoresed, transferred to nitro-
cellulose, and probed with avidin-conjugated alkaline phospha-
tase. Results are presented in Fig. 4 for each of the cysteine
mutants. These are representative results of experiments per-
formed at least four times. The labeling experiments were
controlled for quantity of subunit a present by loading a second
panel of samples and transferring to the same sheet of nitro-
cellulose. The nitrocellulose was cut in half, and the second
panel was probed with anti-a antibody. These results are pre-
sented in Fig. 5. Subunit a could be detected for all of the
mutants except 277C, which was known to be expressed at low
levels. Nevertheless, labeling of 277 was relatively high.

The results presented in Fig. 4A show that the following
residues can be both labeled by MPB and blocked completely by
pretreatment with AMS, indicating a cytoplasm-facing location:
69, 172, 176, 277. In contrast, residues 8, 24, 27, and 131 can be labeled with
MPB but cannot be blocked completely by pretreatment with
AMS, indicating a location that does not face the cytoplasm.

FIG. 3. Sulphydryl reagents used. A, MPB; B, AMS.
DISCUSSION

The membrane topology of subunit a of the F₁F₀ ATP synthase from *E. coli* has been investigated previously, using several different experimental approaches (44–46). In this study, unique cysteine residues were introduced into subunit a and tested for accessibility to two maleimide reagents of differing water-solubility (54–56). A simple and reproducible method of preparation of oriented membrane vesicles was used, and multiple samples were probed in each region of subunit a.

The results indicated that residues 69, 172, 176, and 277 (COOH terminus) are accessible to the cytoplasmic face, because they can be labeled by MBP, and this labeling can be blocked completely by AMS. Among these sites, a great range of reactivity exists, indicating greater exposure of residues 69 and 277 than the region of 172 and 176. Also, nearby residues, 89 and 196, were even more resistant to labeling. The labeling pattern of residues 24, 27, and 131 suggests a periplasmic location for these residues, because although they are labeled by MBP, blocking by AMS was not complete. Again, there is a wide range of reactivity from the highly labeled residue 8 to the much less labeled 131. Again, a nearby residue, 128, was much more resistant to labeling. Finally, residue 238 could not be labeled at all. Such a result provides no topological information and could occur because of shielding by protein, including subunit b or c. Such interactions are discussed in the accompanying paper (48).

Two previous studies of the membrane topology of subunit a using the alkaline phosphatase gene fusion technique have been reported (44, 45). Such experiments are complementary to those described here, because high activity of alkaline phosphatase is indicative of a periplasmic location of the site of the fusion. Each of the two studies offered eight-transmembrane span models for subunit a but were largely incompatible with each other. With the results presented here, there is now more direct evidence that several regions are cytoplasmic, and it is possible to bring the models into agreement. One of the decisions to be made in analysis of fusion studies is to determine a threshold activity for assigning periplasmic location. If this value were to be set at 20% of the maximal activity in the results of Bjørkøk et al. (44) or at 25% in the results of Lewis et al. (45) using LB-glucose, the same two periplasmic regions would be established in each study: near residues 116 and 229–241 in the first study (44) and residues 110–132 and 230–246 in the second study (45). Our results support the first periplasmic assignment and are not in conflict with the second.

Recent MBP labeling experiments using permeabilized cells support this second periplasmic region. A discrepancy does exist when considering the results of Lewis et al. (45), using an LB growth medium lacking glucose. Under those conditions, they found two additional sites of high activity: at residue 200 (28% of maximum) and at the COOH terminus (83% of maximum). Similar results have been seen in other proteins when the site of fusion occurs at a cytoplasmic region without any positively charged residues to anchor COOH-terminal regions of the fusion protein to the cytoplasm (43). In subunit a, lysine normally occurs at position 203, and at the COOH terminus there are no positively charged residues except the final residue, His<sub>271</sub>.

In general, one might expect to encounter some difficulties when applying the alkaline phosphatase fusion technique to subunit a. It is now known by immunoblot analysis that subunit a is not found in membranes when either subunit b or c is missing (57). More recently, subunit a was discovered to be a specific target of the ftsH protease (58). Finally, a variety of results has indicated that the COOH-terminal region of subunit a is important in interactions with the other F<sub>0</sub> subunits (41, 48, 59, 60, 61). Therefore, it is not likely that subunit a fusions missing the COOH terminus will be able to assemble into an F<sub>0</sub> complex, and the resulting topology might not be meaningful. The presence of the ftsH protease adds another variable in interpretations of alkaline phosphatase activity levels of fusions with subunit a.

Hydropathy analysis of the sequence of subunit a (41, 62) reveals five potential transmembrane spans that are scored as “certain” by the method of von Heijne (42) and one, residues 152–202, that is scored as “tentative.” The labeling results presented here indicate that there should be an even number of transmembrane spans between residues 176 and the COOH terminus. If the region 230–240 is periplasmic, as both alkaline phosphatase fusion studies indicated, then the “tentative” span is likely to be largely on the cytoplasmic surface.

The most amino-terminal of the predicted transmembrane spans consists of residues 40–66. The labeling results indicate that residue 69 is cytoplasmic and that residues 8, 24, and 27 may be periplasmic, although seemingly significant levels of MBP labeling were blocked by AMS. The localization of the amino terminus to the periplasm is in disagreement with the results of studies using peptide-generated antibodies (Ref. 46; see also Ref. 3). At all other sites, those studies are in agreement with the labeling results presented here. Therefore, it

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2 T. Wada, J. C. Long, and S. B. Vik, unpublished results.
may be useful to consider alternative interpretations. Lewis and Simoni (63) analyzed the effect of a series of small deletions in subunit a on assembly and function. They found that deletions at the amino terminus, residues 2–35, had a great effect on the level of subunit a in the membrane and also on the alkaline phosphatase activities of fusions made at residue 125. They concluded that the amino terminus is important for targeting subunit a to the cytoplasmic membrane or for insertion of subunit a into the membrane. A sequence alignment of the amino-terminal portion of subunit a from E. coli and from several closely related organisms is shown in Fig. 6. A conserved region, residues 11–19, has been identified as capable of forming an amphipathic α-helix (62). Two possibilities might explain why the amino-terminal residues seem to show accessibility to both sides of the membrane. First, this region might have a function, such as to act as an “intramolecular chaperone” for the folding and membrane assembly of subunit a. As a consequence of this role, it might have increased accessibility on the cytoplasmic surface. Second, the physical properties of this region may lead to artificial exposure on the cytoplasmic surface during membrane preparation or may in some way contribute to its labeling. Experiments currently under way in our laboratory have demonstrated the accessibility this region of subunit a to the periplasm.

Finally, a model of the membrane topology of subunit a is presented in Fig. 7, based on results presented here, and also on this reinterpretation of the alkaline phosphatase fusion studies. Transmembrane spans are all modeled as containing 28 residues. This is consistent with the difficulty of labeling many of the residues that have been tested and with hydrophathy analysis (41, 62). Furthermore, these long spans would better match the length of the extremely hydrophobic subunits c, which have been shown by NMR analysis to contain similarly long α-helices (30). Extramembranous segments that are modeled as helical are those that were predicted to be amphipathic, surface α-helices (62). The implications of this membrane topology model on the function of F₀ are considered in the accompanying paper (48).

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