Definition of Membrane Topology and Identification of Residues Important for Transport in Subunit a of the Vacuolar ATPase*

Masashi Toei†, Satoko Toei‡, and Michael Forgac§

From the Graduate Program in Cell and Molecular Physiology, Sackler School of Graduate Biomedical Sciences, and the Department of Molecular Physiology and Pharmacology, School of Medicine, Tufts University, Boston, Massachusetts 02111

Background: Subunit a of the V-ATPase is thought to contribute to proton-conducting hemichannels within the integral V0 domain.

Results: We have identified transport-important residues and further defined the topology of subunit a.

Conclusion: We propose a model for the proton-conducting hemichannels in V0.

Significance: This represents the first proposed mechanism for proton transport through the V-ATPase.

Subunit a of the vacuolar H+-ATPases plays an important role in proton transport. This membrane-integral 100-kDa subunit is thought to form or contribute to proton-conducting hemichannels that allow protons to gain access to and leave buried carboxyl groups on the proteolipid subunits (c, c′, and c″) during proton translocation. We previously demonstrated that subunit a contains a large N-terminal cytoplasmic domain followed by a C-terminal domain containing eight transmembrane (TM) helices. TM7 contains a buried arginine residue (Arg-73S) that is essential for proton transport and is located on a helical face that interacts with the proteolipid ring. To further define the topology of the C-terminal domain, the accessibility of 30 unique cysteine residues to the membrane-permeant reagent N-ethylmaleimide and the membrane-impermeant reagent polyethylene glycol maleimide was determined. The results further define the borders of transmembrane segments in subunit a. To identify additional buried polar and charged residues important in proton transport, 25 sites were individually mutated to hydrophobic amino acids, and the effect on proton transport was determined. These and previous results identify a set of residues important for proton transport located on the cytoplasmic half of TM7 and TM8 and the luminal half of TM3, TM4, and TM7. Based upon these data, we propose a tentative model in which the cytoplasmic hemichannel is located at the interface of TM7 and TM8 of subunit a and the proteolipid ring, whereas the luminal hemichannel is located within subunit a at the interface of TM3, TM4, and TM7.

V-ATPases3 are ATP-driven proton pumps present in intracellular compartments, including endosomes, lysosomes, and secretory vesicles, as well as in the plasma membrane of specialized cells, such as osteoclasts and renal intercalated cells (1–4). Intracellular V-ATPases function in a variety of normal and disease processes, including receptor-mediated endocytosis, intracellular membrane traffic, protein degradation, neurotransmitter loading, and the entry of many enveloped viruses and toxins (1, 5). Plasma membrane V-ATPases are involved in urinary acidification (4), bone resorption (6), and sperm maturation (7). They have also been suggested to play an important role in tumor cell invasion (8, 9).

V-ATPases are multisubunit complexes composed of two domains (2, 3). The peripheral V1 domain consists of eight subunits (A–H) and carries out ATP hydrolysis. The integral V0 domain (in yeast) contains subunits a, c, c′, c″, d, and e and transports protons from the cytoplasm to the lumen. The proteolipid subunits (c, c′, and c″) form a ring in which each subunit contains a single buried glutamic acid residue that undergoes reversible protonation during proton transport (10). ATP hydrolysis in the V1 domain drives rotation of a central rotary complex that includes the ring of proteolipid subunits, which rotates relative to subunit a (part of the stator complex) (11, 12). It is rotation of the proteolipid ring relative to subunit a that drives unidirectional transport of protons across the membrane (2).

Subunit a is a 100-kDa integral membrane protein composed of two domains. The 50-kDa N-terminal domain is cytoplasmic and forms part of the peripheral stalk that connects V1 and V0 and stabilizes the complex during rotary catalysis (13–15). The 50-kDa C-terminal domain is membrane-embedded, contains eight transmembrane helices, and is involved in proton transport (16, 17). We have identified a number of buried polar and charged residues in the C-terminal domain of subunit a whose mutation leads to partial or complete loss of proton transport.

* This work was supported, in whole or in part, by National Institutes of Health Grant GM34478 (to M. F.). This work was also supported by a Postdoctoral Fellowship from the Northeast Affiliate of the American Heart Association (to M. T.).

§ The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

† Both authors contributed equally to this work.

‡ To whom correspondence should be addressed: Dept. of Molecular Physiology and Pharmacology, Tufts University School of Medicine, 136 Harrison Ave., Boston, MA 02111. Tel.: 617-636-6939; Fax: 617-636-0445; E-mail: michael.forgac@tufts.edu.

§ The abbreviations used are: V-ATPase, vacuolar proton-translocating adenosine triphosphatase; F-ATPase, F0F1 ATP synthase; PEG-Mal, polyethylene glycol maleimide; NEM, N-ethylmaleimide; TM, transmembrane segment; YEPD, yeast extract peptone dextrose.

3 The abbreviations used are: V-ATPase, vacuolar proton-translocating adenosine triphosphatase; F-ATPase, F0F1 ATP synthase; PEG-Mal, polyethylene glycol maleimide; NEM, N-ethylmaleimide; TM, transmembrane segment; YEPD, yeast extract peptone dextrose.
(17–20). These residues have been postulated to form part of proton-conducting hemichannels in V₀ (2). The idea of proton-conducting hemichannels was first proposed as a mechanism of proton translocation through the integral FₐFₒ domain of the FₐFₒ ATP synthase (21–23). For the V-ATPases, a proton is thought to first enter the membrane through a cytoplasmically oriented hemichannel that allows the proton to reach the buried carboxyl group on one of the proteolipid subunits (2). Following protonation of this carboxylate, ATP-driven rotation of the proteolipid ring brings the protonated carboxyl group into contact with the luminal hemichannel. Interaction between the carboxyl group on the proteolipid subunit and a buried arginine residue in subunit a displaces the proton into the lumenal hemichannel, thereby creating a deprotonated carboxylate available to bind another proton. In support of this model, we have shown that TM7 of subunit a containing the critical arginine residue comes into close proximity to TM4 of subunit c' and TM3 of subunit c" that each contain a buried glutamic acid residue essential for proton transport (24, 25). Although previous mutagenesis studies have identified several buried polar and charged residues in subunit a that have been postulated to contribute to the proton-conducting hemichannels in V₀ (17–20), little information is available concerning their relative proximity or location within the membrane.

To elucidate the structure of the proton-conducting hemichannels in V₀, we have first carried out a more comprehensive mutagenesis scan of buried polar and charged residues in subunit a. The results have identified four additional residues whose mutation causes a dramatic (>70%) loss of both proton transport and ATPase activity and an additional three residues where mutation causes substantial (50–70%) loss of activity. In order to place these and previously identified residues at the appropriate depth within the membrane, we have also further defined the cytoplasmic and luminal borders of transmembrane helices in the C-terminal domain of subunit a using accessibility of introduced cysteine residues to membrane-permeant and -impermeant reagents (16). These results are combined to construct a first working model of the proton-conducting hemichannels in the integral domain of the V-ATPases.

**EXPERIMENTAL PROCEDURES**

**Materials**—Zymolase 20T was obtained from Seikagaku America, Inc. and MP Biomedical LLC. Protease inhibitors were purchased from Roche Applied Science. N-Ethylmaleimide (NEM), ATP, phenylmethylsulfonyl fluoride, concanamycin A, and most other chemicals were purchased from Sigma. Polyethylene glycol maleimide (PEG-Mal, 5 kDa) was purchased from SunBio Inc. 9-Amino-6-chloro-2-methoxyacridine was purchased from Molecular Probes.

**Strains and Culture Conditions**—Yeast strain MM112 (MATa Δvph1::LEU2 Δstv1::LYS2 his3-Δ200 leu2 lys2 ura3-52) lacking the endogenous Vph1p and Stv1p subunit a isoforms was used to study all Vph1p mutants (26). Yeast cells were grown in Ura⁻ S.D. minimal medium or YEPD medium buffered with 50 mM phosphate/succinate to either pH 7.5 or 5.5 (16).

**Protein Preparation, SDS-PAGE, and Western Blot Analysis**—Vacuolar membrane vesicles were isolated as described previously (28). Vacuolar proteins were separated by SDS-PAGE on 4–15% gradient acrylamide gels (29). The presence of Vph1p (subunit a, 100 kDa) or Vma2p (subunit B, 60 kDa) was detected by Western blotting using the monoclonal antibodies 1D7 and 13D11, respectively, from Molecular Probes, followed by a horseradish peroxidase-conjugated secondary antibody (Bio-Rad). Blots were developed using the Amersham Biosciences ECL Western blotting analysis system from GE Healthcare.

**Sulphydryl Modification with PEG-Maleimide**—3 μg of vacuolar membrane vesicles were diluted into 50 μl of phosphate-buffered saline (PBS) containing 137 mM NaCl, 1.2 mM KH₂PO₄, 15.3 mM Na₂HPO₄, 2.7 mM KCl, pH 7.4, and then incubated with 1 mM PEG-Mal for 1 h at 23 °C. Samples were quenched with a buffer containing 100 mM dithiothreitol for 10 min and separated by SDS-PAGE on 7.5% acrylamide gels. Samples were transferred to nitrocellulose and blotted with the monoclonal antibody against Vph1p (1D7). Reaction of an accessible single cysteine residue on Vph1p with PEG-Mal shifts the apparent mobility of the protein by 10–25 kDa (16).

**Sulphydryl Modification with NEM**—The accessibility of cysteine residues to NEM was detected indirectly by PEG-Mal modification in the presence of SDS following NEM treatment. 12.5 μg of vacuolar membrane vesicles were diluted into 200 μl of PBS buffer, incubated with 1 mM NEM for 30 min at room temperature, and centrifuged at 16,000 × g for 5 min. Pellets were washed by sedimentation with 1 ml of ice-cold PBS. The samples were suspended in 50 μl of PBS and then incubated with 2% SDS and 1 mM PEG-Mal for 1 h at 23 °C. Samples were quenched with sample buffer containing 100 mM dithiothreitol for 10 min. SDS-PAGE and Western blot were performed as described above.

**ATPase and Protein Transport Activity**—ATP hydrolysis was measured using a coupled spectrophotometric assay as described previously (30). Vacuolar membranes were incubated with DMSO or 1 μM concanamycin A (in DMSO) for 5 min prior to measurement of ATPase activity. ATP-dependent proton transport was measured by the initial rate of ATP-dependent fluorescence quenching using the fluorescence dye 9-amino-6-chloro-2-methoxyacridine, as described previously (30). All reactions were carried out at 30 °C.

**Other Methods**—Protein concentration was determined by the method described by Lowry et al. (31).

**RESULTS**

**Identification of Buried Polar and Charged Residues in Subunit a That Are Important for Proton Transport**—Previous studies from our laboratory had identified a number of buried
TABLE 1

Growth phenotypes of subunit a mutants containing mutations in buried polar and charged residues

| Strain | Growth at pH 7.5 | Strain | Growth at pH 7.5 |
|--------|------------------|--------|------------------|
| Wild type | ++ | Q580A | ++ |
| Vector | – | S587F | ++ |
| N480F | ++ | H635F | ++ |
| N480A | ++ | R672F | ++ |
| D481F | – | N725A | ++ |
| D481A | – | S732F | ++ |
| S484F | ++ | Y733F | ++ |
| S484A | ++ | S740F | ++ |
| K485F | ++ | S740A | ++ |
| S534F | ++ | T770F | ++ |
| S534A | ++ | T791F | ++ |
| Y353F | ++ | S792F | ++ |
| K536F | ++ | S792A | + |
| K536A | ++ | H796F | – |
| T549F | ++ | H796A | ++ |
| Y550F | ++ | S797F | ++ |
| S551F | ++ | H801F | + |
| Y552A | ++ | H801A | ++ |

were isolated, and Western blot analysis was performed using antibodies against both Vph1p and subunit B. Because subunit B is part of the V$_r$ domain, its presence on vacuolar membranes is a measure of the degree of assembly of the mutants relative to wild type complexes. As can be seen in Fig. 1, two mutations (N480F and S484F) cause loss of stability of Vph1p or its ability to assemble into stable complexes. These mutants were not further pursued. For mutations that affect assembly, it is not possible to determine the role of the corresponding residue in activity. It should be noted, however, that alanine substitutions of Asn-480 and Ser-484 are without effect on assembly (Fig. 1) or activity (data not shown). This suggests that polar residues at these positions are not required for transport. A more complete analysis of the concanamycin-sensitive ATPase activity and proton transport was then performed on the remaining mutants. As shown in Fig. 2, six mutations dramatically reduced proton transport and ATP hydrolysis (activity is <30% of wild type) while having little or no effect on V-ATPase assembly, including D481F, D481A, S792F, H796F, H796A, and

polar and charged residues in subunit a whose mutation led to significant or complete loss of proton transport (17–20). Arg-735 in TM7 is essential for transport because mutation to any residue, including the conservative lysine substitution, leads to complete loss of proton transport (17). In addition, non-conservative replacements of Glu-721, Asn-725, Ser-728, His-729, Glu-733, and His-743 in TM7 and Glu-789 and Arg-799 in TM8 lead to substantial loss of activity (17–20). In order to obtain a more comprehensive picture of the buried polar and charged residues in subunit a that are important for proton transport by the V-ATPase, site-directed mutagenesis was performed on a total of 25 sites within the C-terminal domain of Vph1p that encodes one of the two isoforms of subunit a in yeast. These mutant constructs were then expressed in a strain disrupted in both Vph1p and Stv1p (the second a subunit isoform in yeast). Residues were mutated to either alanine or phenylalanine or both to determine the importance of the presence of a charged or polar side chain at that position. Each mutant strain was first tested for its growth phenotype. Yeast strains expressing V-ATPase complexes possessing activity that is substantially lower than wild type (<20%) are unable to grow at pH 7.5 but are able to grow at pH 5.5 (referred to as a vma phenotype) (32). As shown in Table 1, most mutant strains showed normal growth at pH 7.5, suggesting that the V-ATPase complexes in these strains possessed substantial activity relative to complexes containing wild type Vph1p. Preliminary measurements of concanamycin-sensitive ATPase activity and ATP-dependent proton transport on vacuolar membranes isolated from each mutant strain confirmed that for most of the mutants, the V-ATPase possessed at least 50% of the wild type level of activity (data not shown). Mutations at a number of predicted transmembrane sites, however, led to either a severe or mild vma growth phenotype, including Asn-480, Asp-481, and Ser-484 in TM3 and Ser-792, His-796, and His-801 in TM8.

In order to obtain a more precise picture of how the introduced mutations affected V-ATPase activity, V-ATPase assembly was assessed for each strain showing either a vma phenotype or a greater than 50% loss of activity. Vacuolar membranes

FIGURE 1. Analysis of stability of V-ATPase complexes containing phenylalanine and alanine mutants of Vph1p by Western blot of isolated vacuolar membranes using antibodies against Vph1p and subunit B. Data are shown for mutants that, from preliminary measurements, have V-ATPase activity less than 50% of wild type as well as N480A and S484A. Vacuolar membranes were isolated from the yeast strain MM112 (disrupted in the endogenous VPH1 and STV1 genes) expressing either a wild type form of Vph1p (WT) or the indicated single mutant forms of Vph1p. Samples of vacuolar membranes (0.1 mg of protein) were separated by SDS-PAGE followed by transfer to nitrocellulose and Western blot using mouse monoclonal antibodies against subunit a (10D7) or subunit B (13D11), as described under “Experimental Procedures.”

FIGURE 2. Effect of phenylalanine and alanine mutations in Vph1p on concanamycin-sensitive ATPase activity and ATP-dependent proton transport. Vacuolar membranes were isolated from the yeast strain MM112 (disrupted in the endogenous VPH1 and STV1 genes) expressing either a wild type form of Vph1p (WT) or the indicated single mutant forms of Vph1p. ATPase activity (black bars) was measured using a coupled spectrophotometric assay, and ATP-dependent proton transport (open bars) was measured as the initial rate of quenching of the fluorescence of the pH-sensitive dye ACMA in the presence or absence of 1 µM concanamycin, as described under “Experimental Procedures.” Concanamycin-sensitive ATPase activity for wild-type vacuolar membranes was 0.57 µmol of ATP/min/mg of protein. All values are normalized to the wild type. Values of mutants represent the average of at least two measurements on each of two independent vacuolar preparations, with the error bars corresponding to the S.E.
H801F. In addition, three mutations caused a substantial loss of activity (retaining 30–50% of wild type), including S534F, K536F, and S740F. These results suggest that these seven residues are important for proton transport by the V-ATPase. Our results, together with those obtained from previous studies, are summarized in the model of subunit a shown in Fig. 3.

Defining the Borders of Transmembrane Helices of Subunit a—Although we have previously shown that the C-terminal domain of subunit a possesses eight transmembrane helices with both the N and C termini located on the cytoplasmic side of the membrane (16), the borders of most of the transmembrane helices remain poorly defined. In order to better localize the transmembrane helix borders in subunit a, we have employed accessibility of introduced cysteine residues to membrane-permeant and -impermeant sulfhydryl reagents. As a membrane-permeant reagent, we have used NEM, whereas as a membrane-impermeant reagent, we have used PEG-Mal (16).

The protocol used in these experiments is described in detail below. Thirty unique cysteine residues were introduced into a Cys-less form of Vph1p and expressed in the strain MM112. We have previously shown that the Cys-less form of Vph1p gives rise to V-ATPase complexes possessing nearly wild type levels of both ATPase activity and proton transport (16). We first tested the growth phenotype of the mutants at pH 5.5 and pH 7.5. As shown in Table 2, most of the 30 mutants showed normal growth at pH 7.5, indicating the ability of the mutant Vph1p to form V-ATPase complexes having substantial (~20%) activity. Three of the cysteine mutants (K536C, E721C, and A742C) showed a mild vma- growth phenotype, suggesting that these complexes possessed activity close to the limit of

TABLE 2
Growth phenotypes of subunit a mutants containing single cysteine substitutions

| Strain     | Growth at pH 7.5 | Strain     | Growth at pH 7.5 |
|------------|-----------------|------------|-----------------|
| Wild type  | ++              | K593C      | ++              |
| Vector     | ++              | D597C      | ++              |
| Cys-less   | ++              | Q630C      | ++              |
| S472C      | ++              | Q683C      | ++              |
| I482C      | ++              | K632C      | ++              |
| T486C      | ++              | H655C      | ++              |
| E526C      | ++              | T659C      | ++              |
| S532C      | ++              | H660C      | ++              |
| S536C      | ++              | K663C      | ++              |
| S540C      | ++              | H714C      | ++              |
| S555C      | ++              | E718C      | ++              |
| S564C      | ++              | A738C      | ++              |
| S571C      | ++              | L739C      | ++              |
| L576C      | ++              | A742C      | ++              |
| S587C      | ++              | +           |                |
V-ATPase Subunit α Topology and Role in Transport

that required for wild type growth at neutral pH. We next assessed the effect of the mutations on assembly of the V-ATPase complex as described above. As shown in Fig. 4, all of the mutants show significant levels of both subunits on isolated vacuoles compared with wild type, suggesting substantial assembly of V-ATPase complexes. Note that the growth phenotype and assembly competence of mutants analyzed as described below (see Fig. 6) but not shown in Table 2 and Fig. 4 (including G602C, G620C, L638C, L648C, S670C, D710C, C723, L734C, and A744C) were previously reported (16).

In order to assess whether the introduced cysteine residues in Vph1p are exposed to the cytoplasmic or luminal side of the membrane, their reactivity toward the membrane-permeant reagent NEM and the membrane-impermeant reagent PEG-Mal was analyzed in isolated vacuolar membranes in which the cytoplasmic surface is exposed (16). In this system, cysteines exposed on the cytoplasmic side of the membrane are able to react with both PEG-Mal and NEM. By contrast, cysteines exposed on the luminal side of the membrane are able to react with NEM but not PEG-Mal because of the latter’s inability to cross the vacuolar membrane. Because reaction with either NEM or PEG-Mal requires the formation of a thiolate that is prevented within the hydrophobic phase of the membrane, cysteine residues located within the bilayer are unable to react with either NEM or PEG-Mal except under denaturing conditions (i.e. in the presence of SDS). Similarly, cysteine residues located within the folded protein structure or at subunit interfaces will be unable to react with either reagent under non-denaturing conditions. It should be noted that because NEM has a considerably smaller molecular radius than PEG-Mal, it would be expected to penetrate further into the core of an aqueous channel. Thus, we would predict that for residues located within such an aqueous channel, reactivity toward NEM but not PEG-Mal would be observed. Although this is the same pattern as is predicted for cysteine residues located within the lumen, it would be distinguishable from the latter by the presence of immediately adjacent cytoplasmic residues on one side and immediately adjacent membrane-embedded residues on the other. By contrast, a stretch of luminal residues would be expected to have membrane-embedded residues (i.e. non-reactive under native conditions) on both sides. It should also be noted that because the vacuolar membranes used in our labeling studies contain both assembled V1V0 complexes and free V0 domains, it is possible that accessibility of some of the cysteine residues may be affected by the assembly state of the enzyme. Nevertheless, the presence of V1 and V0 subunits at ratios similar to that observed for the wild type enzyme (Fig. 4) suggest that changes in accessibility of cysteine residues due to changes in assembly state are not occurring.

Isolated vacuolar membranes were treated using each of the following protocols. To determine accessibility of cysteine residues to PEG-Mal, membranes were reacted with 1 mM PEG-Mal for 60 min at 23 °C followed by SDS-PAGE and Western blot using the antibody 10D7 against Vph1p. Modification by PEG-Mal results in a shift in mobility of a fraction of the a subunit by 10–25 kDa (16). Because only cytoplasmic residues will be reactive to PEG-Mal, the labeling pattern shown in the top portion of Fig. 5 would be predicted. In order to determine accessibility of cysteine residues to NEM, vacuolar membranes are reacted with 1 mM NEM for 30 min at 23 °C. Because of its small size, NEM does not cause a detectable shift in mobility of the a subunit on SDS-PAGE, requiring NEM modification to be detected indirectly. Following removal of unreacted NEM by washing, vacuolar membranes were denatured by treatment with SDS, and 1 mM PEG-Mal was added, followed by incubation for 60 min at 23 °C. Samples were then analyzed for the PEG-Mal reaction as described above. PEG-Mal will only be able to modify cysteine residues that have not previously been blocked by reaction with NEM. As a result, the labeling pattern shown in the bottom portion of Fig. 5 would be predicted, with only cysteines that had been prevented from reaction with NEM by their presence within the membrane or in an otherwise shielded environment showing the shift following reaction with PEG-Mal. As noted above, residues at the cytoplasmic borders of transmembrane helices could be distinguished from those exposed on the luminal surface by the nature of the labeling pattern observed for closely adjacent regions. As a control, we have tested the ability of each introduced cysteine residue to react with PEG-Mal in the presence of SDS. Although PEG-Mal modified a large fraction of each of the cysteine mutants if membranes were reacted directly with PEG-Mal, some variation in the degree of modification was observed if a washing step was added prior to PEG-Mal treatment (supplemental Fig. 1). We have

FIGURE 4. Analysis of stability of V-ATPase complexes containing cysteine mutants of Vph1p by Western blot of isolated vacuolar membranes using antibodies against Vph1p and subunit B. Vacuolar membranes were isolated from the yeast strain MM112 (disrupted in the endogenous VPH1 and STV1 genes) expressing either a wild type form of Vph1p (WT), the pRS316 vector alone (Vector), or the indicated single cysteine-containing mutants of Vph1p. Samples of vacuolar membranes (0.1 μg of protein) were separated by SDS-PAGE followed by transfer to nitrocellulose and Western blot using mouse monoclonal antibodies against subunit a (10D7) or subunit B (13D11) as described under "Experimental Procedures."
considered this variation in labeling by PEG-Mal in our interpretation of the results, as discussed below.

We first investigated the region from Asp-710 to Ala-744 that encompasses TM7 containing the critical Arg-735 residue. Previous studies indicated that Asp-710 had a cytoplasmic orientation and that Ala-744 was luminal (16), but both the cytoplasmic and lumenal borders of this important region were poorly defined. As shown in Fig. 6A, both D710C and H714C show a cytoplasmic labeling pattern (i.e. are labeled by PEG-Mal), whereas H718C and E721C both show a pattern consistent with their presence in the cytoplasmic border region of TM7 (i.e. reaction with NEM but not PEG-Mal in the absence of SDS). By contrast, C723, L734C, L736C, A738C, L739C, and A742C all display a labeling pattern indicating their presence within the membrane or otherwise sequestered from reaction with either reagent under non-denaturing conditions. Finally, A744C again shows a pattern consistent with its presence on the luminal side of the membrane. This indicates that the TM6/7 loop is exposed on the cytoplasmic surface up until residue His-714, that the region His-718 to Glu-721 represents the cytoplasmic border of TM7 (accessible to NEM but not PEG-Mal), that TM7 extends from somewhere between Cys-723 and Leu-734 to Ala-742, and that the luminal border of TM7 coincides approximately with His-743.

We next investigated the region from Gly-620 to Ser-670 that includes TM6, whose borders once again were poorly defined based on previous studies (16). As shown in Fig. 6B, a luminal labeling pattern is observed for G620C, Q630C, and Q634C; a membrane-embedded pattern for L638C and L648C; a cytoplasmic border pattern for K652C and H655C; and a cytoplasmic pattern for T659C, H660C, K663C, and S670C. These results indicate that the luminal TM5/6 loop ends between residues Gln-634 and Leu-638, TM6 extends from at least Leu-638 to Leu-648, and the cytoplasmic border region of TM6 includes Lys-652 to His-655. The TM6/7 loop then begins at approximately Thr-659. Given the shortness of the sequence between 638 and 648 relative to that required to span the bilayer as an α-helix, the region from 649 to 658 probably extends a considerable way into the membrane.

Finally, we investigated the region from Ser-472 to Gly-602 that encompasses TM3, TM4, and TM5. As can be seen from the data in Fig. 6C, TM3 appears to include the region from Ser-472 to Thr-486, the TM3/4 loop extends from Phe-490 to Ser-532, and the inaccessible region of TM4 extends from Lys-536 to somewhere between Ser-540 and Phe-554. Although we detected no PEG-Mal-accessible loop between TM4 and TM5, we have previously shown that a Factor Xa cleavage site inserted after residue Leu-560 can be cleaved from the cytoplasmic side of the membrane (13). Thus, the region that includes Ser-555 to Asn-571 appears to encompass the cytoplasmic border regions of both TM4 and TM5. Finally, TM5 includes at least the residues between Leu-576 and Lys-593, with the residues Asp-597 and Gly-602 located in the TM5/6 luminal loop. The topological model of subunit a shown in Fig. 3 is based on the results of the present study together with results from our previous work.

Three cysteine mutants (S526C, G620C and H718C) showed very low modification by PEG-Mal in the presence of SDS (supplemental Fig. 1). This is not the case if the washing step prior to PEG-Mal modification is eliminated, suggesting that partial oxidation of the sulfhydryl is occurring during the wash. Because PEG-Mal modification of these residues is low, it is difficult to interpret the apparent lack of PEG-Mal modification of these residues in Fig. 6. For S526C, the adjacent mutants (F490C and S532C) are clearly luminal, making it likely that the S526C is also luminal. Similarly, for G620C, both G602C and Q630C clearly show a luminal labeling pattern. For H718C, H714C gives a cytoplasmic labeling pattern, whereas E721C appears to be in the cytoplasmic border region because it appears to react with NEM but not PEG-Mal. Given the low level of PEG-Mal modification of H718C under denaturing conditions, it is also possible that His-718 is cytoplasmic.

| Cysteine position | Cytoplasm | Cytoplasmic borders | TM | Lumen |
|-------------------|-----------|---------------------|----|-------|
| PEG-Mal           | accessible| not accessible      | not accessible | not accessible |
| NEM               | accessible| accessible          | not accessible | not accessible |
| Expected pattern on PEG-Mal test |          |                     |                |                  |
| Expected pattern on NEM test |          |                     |                |                  |
DISCUSSION

Transmembrane Topology of Subunit a—Our current model of the transmembrane topology of subunit a is shown in Fig. 3. It is based on results presented in the present paper as well as results from our previous topological studies (13, 16). The primary approach employed to analyze transmembrane topology in these studies is accessibility of introduced cysteine residues to the membrane-permeant reagent NEM and the membrane-impermeant reagent PEG-Mal, although we have also used the accessibility of introduced protease sites to cleavage by Factor Xa protease (13). One drawback of our current approach is that the labeling pattern obtained for lumenal residues and residues in the cytoplasmic border region that are accessible to NEM but not PEG-Mal is the same. NEM modification is detected indirectly by the absence of reaction with PEG-Mal after NEM treatment (Fig. 5). Nevertheless, as described above, these two classes of residues are distinguishable by the nature of the adjacent regions. Thus, lumenal residues are bordered on both sides...
by regions that show a labeling pattern consistent with a membrane-embedded or otherwise shielded location, whereas residues in the cytoplasmic border are flanked by a cytoplasmic region on one side and transmembrane or shielded region on the other. Our current model is consistent with our previously published model in showing the C-terminal domain of subunit a composed of eight transmembrane helices with both the N and C termini on the cytoplasmic side of the membrane (16).

Because it contains the critical Arg-735 residue as well as His-729 and His-743, which are both important for transport, our initial studies focused on TM7 of subunit a. Our previous work had shown that five cysteine mutants constructed between Ala-744 and Phe-761 were exposed to the lumenal side of the membrane and therefore were contained within the lumenal loop between TM7 and TM8 (16). However, the lumenal border of TM7 was not well defined because the first residue to show a labeling pattern consistent with a membrane localization was L734C. Based on the current results, we can now place the lumenal border of TM7 between Ala-742 and Ala-744 (Fig. 6). This places the critical Arg-735 ∼9 residues from the lumenal side of TM7 and thus near the middle of the membrane, where it is able to interact with the buried glutamic acid residues on the proteolipid ring. In addition, it places His-743 in a position to contribute to the mouth of a putative lumenal hemichannel (see below). Our previous work had also provided little information about the location of the cytoplasmic border of TM7, placing it somewhere between Asp-710 and Cys-723. We now show that the last PEG-Mal-accessible mutant is H714C but that NEM can continue to react with residues through Glu-721 (Fig. 6). We suggest that the residues between His-718 and Glu-721 form part of a cytoplasmic hemichannel that is restricted in diameter such that NEM but not PEG-Mal is able to penetrate. An alternate explanation for these results is that the region between His-718 and Glu-721 is located at an interface between the C- and N-terminal domain of subunit a or at the interface of subunit a and another subunit such that a cleft is formed that allows NEM but not PEG-Mal to reach the corresponding cysteine residue.

We next addressed the topology of TM6, whose cytoplasmic border had previously been localized between Leu-648 and Ser-670 and whose lumenal border was proposed to lie between Gly-620 and Leu-638 (16). Our current results indicate that T659C is the last PEG-Mal-accessible residue but that NEM can continue to penetrate to Lys-652. Because the labeling results now suggest that the lumenal border of TM6 is between Gln-634 and Leu-638 (Fig. 6), it appears that the completely inaccessible region of TM6 is actually quite short (Leu-638 to Leu-648). Thus, although the restricted accessibility of Lys-652 and His-655 could be due to their location at a subunit or domain interface, it would appear more likely that this region extends TM6 to the cytoplasmic side of the membrane.

The last region of interest was the portion of the sequence from Ser-472 to Gly-602, which was postulated to encompass TM3–TM5. The only information previously available concerning this region was the observation that Asn-447 and Asn-450 were accessible from the cytoplasm (from labeling studies), Leu-560 is also cytoplasmic (from Factor Xa cleavage studies), and Gly-602 and Gly-620 are lumenal (from labeling) (13, 16).

The present results now place the luminal border of TM3 between Thr-486 and Phe-490 and the luminal border of TM4 between Ser-532 and Lys-536 (Fig. 6). The extended region between Ser-555 and Asn-571 is NEM- but not PEG-Mal-accessible and is therefore labeled the “cytoplasmic border.” Because introduction of Factor Xa cleavage sites following Leu-560 results in a protein that can be cleaved from the cytoplasmic side of the membrane (13), it is unclear why proximal residues (Ser-564) are not accessible to PEG-Mal. As with the similarly reactive regions described above, this portion of the polypeptide chain may be located at a domain or subunit interface that prevents PEG-Mal reaction. Further analysis is required to better define the accessibility of this region. Because mutagenesis of polar and charged residues between Ser-540 and Tyr-592 (including the TM4/5 loop) is without effect on transport activity (Fig. 3) (data not shown), we think that this region does not play an important role in proton transport. Finally, TM5 extends from at least Leu-576 to Lys-593, with Asp-597 and Gly-602 located in the TM5/6 luminal loop. It should be noted that no topological experiments have been performed on TM1 and TM2 beyond showing that both Ser-266 preceding TM1 and Asn-447 following TM2 are both exposed to the cytoplasm (13). The borders of TM1 and TM2 shown in Fig. 3 are thus based strictly on hydrophytly analysis (13). The indicated lengths of these helices (19 and 17 amino acids, respectively) are both somewhat short for standard transmembrane helices, although the shortest transmembrane helix reported in the glycerol 3-phosphate transporter of *Escherichia coli* is 18 amino acids in length (33). It is possible that the charged residues shown as outside the membrane on the cytoplasmic side of TM1 and TM2 are actually at the cytoplasmic borders of the helices or that the helices only penetrate part of the way across the membrane, as has been shown for some ion channels (34).

Identification of Subunit a Residues Important for Proton Transport—Our laboratory had previously demonstrated that Arg-735 in TM7 of subunit a is essential for proton transport because even the conservative R735K mutation is completely inactive for proton transport (17). In addition, mutations at a number of positions were previously shown to significantly reduce activity without affecting assembly, including Glu-721, Asn-725, Ser-728, His-729, and His-743 in TM7 and Glu-789 and Arg-799 in TM8, suggesting an important but not essential role of these residues in proton transport (17–20). In the present study, we identified three additional residues in TM8 (Ser-792, His-796, and His-801) whose mutagenesis significantly affects activity, whereas substitution of phenyalanine for Ser-740 in TM7 reduces activity by 50–60%. For many of these residues, including Glu-721, His-729, His-743, Glu-789, His-796, and Arg-799, the charged character of the side chain appears to be important for transport activity. By contrast, for the remaining residues, it appears that a charged or polar character is not required for transport. We postulate that in these latter cases (and for others described below), the introduction of a bulky phenyalanine side chain may prevent the formation of a continuous water channel. Alternatively, the phenyl ring may turn the helix so as to misalign other important polar and charged residues from participating in proton transport.
As shown in Fig. 3, four of the six important residues on TM7 are on the cytoplasmic side of Arg-735. Similarly, all five of the important polar and charged residues on TM8 are on the cytoplasmic half of TM8. Moreover, as shown in Fig. 7, all of the cytoplasmically oriented residues on TM7 and all but one of the cytoplasmically oriented residues on TM8 are clustered on the same face of their respective helix. We thus propose that these helical faces of TM7 and TM8 interact to form a cytoplasmic hemichannel (shaded in yellow in Fig. 7, right).

In addition to the residues noted above, the present study identified a number of residues present in other regions of subunit a that appear important for proton transport. These include Asp-481 in TM3 and Ser-534 and Lys-536 in TM4. All of these residues are present on the luminal half of the membrane, as are Ser-740 and His-743 on TM7. We postulate that these residues may therefore contribute to formation of a luminal hemichannel at the interface of TM3, -4, and -7 (shaded in blue in Fig. 7, left). It should be noted that the relative orientation of TM7 of subunit a and TM3 of subunit c” shown in the left panel of Fig. 7 is consistent with the major disulfide-mediated cross-links that can be formed between these helices (25). It should also be noted that all of the residues shown in red in Fig. 3 (whose mutation leads to loss of >70% of activity) as well as most of the residues shown in orange (whose mutation leads to loss of 50–70% of activity) are perfectly conserved between the two a subunit isoforms in yeast (Vph1p and Stv1p) (26) and nearly perfectly conserved when compared with a subunit isoforms from other species.

Because the helical faces of TM7 containing the important residues contributed to the proposed cytoplasmic and luminal hemichannels partly overlap, it is not possible to optimally align TM7 to contribute to both hemichannels simultaneously, assuming that the hemichannels are not stacked on top of one another. Instead, we propose that TM7 undergoes “helical swiveling” in going between the states described by the left and right panels of Fig. 7. Such a model has the advantage that the two hemichannels open sequentially, possibly coordinated with the movement of the rotation of the proteolipid ring. In fact, we have obtained evidence for helical swiveling within subunit a from cysteine-mediated cross-linking studies. Thus, cross-linking of TM7 in subunit a and TM4 of subunit c” or TM3 of subunit c” can occur over a significant rotational angle of each helix (24, 25), suggesting that helices in both subunit a and the proteolipid subunits have considerable rotational mobility. It should be noted, however, that an alternate model in which the two hemichannels are nearly stacked on top of one another would not require a swiveling of TM7.

FIGURE 7. Proposed model of proton-conducting hemichannels in V0. Each helical wheel diagram represents the indicated transmembrane segments viewed from the cytoplasmic side of the membrane. The left panel shows a model of the luminal hemichannel (shaded blue) with residues contributed from TM3, TM4, and TM7 (also blue). Note that TM7 is oriented so as to optimize the interaction between Ser-740 and His-743 on TM7 (both on the luminal side of Arg-735) with the luminaly oriented residues on TM3 and TM4. The major disulfide-mediated cross-links that can be formed between TM7 of subunit a and TM3 of subunit c” (25) are shown by the solid black lines. Following release of a proton from the glutamic acid on the proteolipid subunit into the luminal hemichannel and stabilization of the negative charge by Arg-735, ATP-driven rotation of the proteolipid ring (shown by the clockwise arrow) brings this glutamic acid into contact with the cytoplasmic hemichannel (shaded yellow in the right panel). Coincident with this, swiveling of TM7 of subunit a (shown by the counterclockwise arrow) aligns the residues on TM7 that contribute to the cytoplasmic hemichannel (Ser-728, Glu-721, Asn-725, and His-729) with the residues on TM8 that contribute to this hemichannel (Arg-799, Ser-792, His-796, and Glu-789), all shown in yellow. The residues of the luminal hemichannel are simultaneously misaligned, disrupting the ability of this hemichannel to conduct protons. The cycle is completed by reprotonation of the proteolipid glutamic acid from the cytoplasmic hemichannel.
Proposed Mechanism of Proton Translocation through V₀—
Based on our current results and those of previous studies, we propose that a proton-conducting hemichannel oriented toward the luminal side of the membrane is located at the interface of TM3, TM4, and TM7. Interaction between the protonated glutamate residue on one of the proteolipid subunits and Arg-735 of subunit a displaces the proton into this luminal hemichannel, stabilizing the deprotonated state of the glutamate through charge interactions with the arginine (Fig. 7, left). Clockwise rotation of the proteolipid ring (as viewed from the cytoplasm) driven by ATP hydrolysis in V₀, concurrent with swiveling of TM7 of subunit a, disrupts this luminal hemichannel and now creates a cytoplasmically oriented hemichannel formed at the interface of TM7 and TM8 (Fig. 7, right). The deprotonated glutamate residue is then able to reacquire a proton from the cytoplasmic compartment. Further ATP-driven rotation of the proteolipid ring would again bring the protonated glutamate into contact with the luminal hemichannel, where the cycle could be repeated.

Relationship to Proton Conduction through F₀—The F₁F₀ ATP synthase operates by a rotary mechanism similar to the V-ATPases and contains a 23-kDa subunit a corresponding to the 100-kDa subunit a of the V-ATPases (23). The F₀ subunit contains five transmembrane helices with a critical arginine residue (Arg-210) present in the penultimate helix (TM4) (21–23). Based upon cross-linking, sensitivity to Ag⁺, and suppressor analysis, Fillingame’s group (23, 35–38) has proposed a model for the F₀ domain in which a proton-conducting cytoplasmic hemichannel exists at the interface of TM4, TM5, and the proteolipid ring. This is similar to the model proposed here in which the cytoplasmic hemichannel is formed by TM7, TM8, and the proteolipid ring. By contrast, a luminal hemichannel is suggested to be formed in F₀ from a four-helix bundle composed of TM2–TM5 (23, 35–38). This is quite different from our model for V₀, in which the luminal hemichannel is proposed to contain residues from TM3, TM4, and TM7. Because TM7 and TM8 of the V-ATPase subunit a correspond to TM4 and TM5 of the F-ATPase subunit a, a model based upon the F-ATPase data would imply the involvement of TM5, TM6, TM7, and TM8 in a luminal hemichannel. The fact that different sets of transmembrane helices appear to participate in the formation of a luminal hemichannel in the V- and F-ATPases may contribute to differences in the passive proton conducting properties of the two classes of enzymes. Thus, whereas the free F₀ domain is able to passively conduct protons upon dissociation of F₁ (41), the free V₀ domain is silent with respect to passive proton conductance (42). This property is essential for the V-ATPases because significant amounts of free V₀ exist within the cell as a result of reversible dissociation of the V₁V₀ complex in vivo (43). Without silencing of the passive proton transport properties of free V₀, it would be difficult to maintain steady state proton gradients across intracellular membranes. The altered structure of the proton-conducting hemichannels in V₀ may allow inhibition of passive proton transport. It should also be noted that helical swiveling within subunit a has previously been proposed to control proton conduction of the hemichannels in F₀ (37).

In summary, we have identified a number of buried polar and charged residues in subunit a that appear to participate in proton transport by the V-ATPases. These residues are clustered on the cytoplasmic half of TM7 and TM8 and the luminal half of TM3, TM4, and TM7. Based upon these data and the refinement of the topology of subunit a, we propose the first tentative model for the location of proton-conducting hemichannels within V₀. Additional work will be required to test and refine this model.

Acknowledgments—We thank Drs. Regina Saum, Sarah Bond, and Ayana Hinton as well as Joseph Capecci and Rachel Nager for many helpful discussions.

REFERENCES
1. Toei, M., Saum, R., and Forgac, M. (2010) Biochemistry 49, 4715–4723
2. Forgac, M. (2007) Nat. Rev. Mol. Cell Biol. 8, 917–929
3. Kane, P. M. (2006) Microbiol. Mol. Biol. Rev. 70, 177–191
4. Brown, D., Paunescu, T. G., Breton, S., and Marshansky, V. (2009) J. Exp. Biol. 212, 1762–1772
5. Gruenberg, J., and van der Goot, F. G. (2006) Nat. Rev. Mol. Cell Biol. 7, 495–504
6. Toyomura, T., Murata, Y., Yamamoto, A., Oka, T., Sun-Wada, G. H., Wada, Y., and Futai, M. (2003) J. Biol. Chem. 278, 22023–22030
7. Shum, W. W., Da Silva, N., Brown, D., and Breton, S. (2009) J. Exp. Biol. 212, 1753–1761
8. Hinton, A., Sennoune, S. R., Bond, S., Fang, M., Reuveni, M., Sahagian, G. G., Jay, D., Martinez-Zaguilan, R., and Forgac, M. (2009) J. Biol. Chem. 284, 16400–16408
9. Sennoune, S. R., Bakunts, K., Martinez, G. M., Chua-Tuan, J. L., Kebir, Y., Attaya, M. N., and Martinez-Zaguilan, R. (2004) Annu. J. Physiol. Cell Physiol. 286, C1443–C1452
10. Hirata, R., Graham, L. A., Takatsuki, A., Stevens, T. H., and Anraku, Y. (1997) J. Biol. Chem. 272, 4795–4803
11. Imamura, H., Nakano, M., Noji, H., Muneyuki, E., Okajima, T., Okomu, S., Yoshida, M., and Yokoyama, K. (2003) Proc. Natl. Acad. Sci. U.S.A. 100, 2312–2315
12. Hirata, T., Iwamoto-Kihara, A., Sun-Wada, G. H., Okajima, T., Wada, Y., and Futai, M. (2003) J. Biol. Chem. 278, 23714–23719
13. Leng, X. H., Nishi, T., and Forgac, M. (1999) J. Biol. Chem. 274, 14655–14661
14. Inoue, T., and Forgac, M. (2005) J. Biol. Chem. 280, 27986–27993
15. Landoldt-Marticorena, C., Williams, K. M., Correa, J., Chen, W., and Manolson, M. F. (2000) J. Biol. Chem. 275, 15449–15457
16. Wang, Y., Toei, M., and Forgac, M. (2008) J. Biol. Chem. 283, 20696–20702
17. Kawasaki-Nishi, S., Nishi, T., and Forgac, M. (2001) Proc. Natl. Acad. Sci. U.S.A. 98, 12397–12402
18. Leng, X. H., Manolson, M. F., Liu, Q., and Forgac, M. (1996) J. Biol. Chem. 271, 22487–22493
19. Leng, X. H., Manolson, M. F., and Forgac, M. (1998) J. Biol. Chem. 273, 6717–6723
20. Wang, Y., Inoue, T., and Forgac, M. (2005) J. Biol. Chem. 280, 40481–40488
21. Vik, S. B., and Antonio, B. J. (1994) J. Biol. Chem. 269, 30364–30369
22. Cain, B. D. (2000) J. Bioenerg. Biomembr. 32, 365–371
23. Fillingame, R. H., Angevine, C. M., and Dmitriev, O. Y. (2002) Biochim. Biophys. Acta 1555, 29–36
24. Kawasaki-Nishi, S., Nishi, T., and Forgac, M. (2003) J. Biol. Chem. 278, 41908–41913
25. Wang, Y., Inoue, T., and Forgac, M. (2004) J. Biol. Chem. 279, 44628–44638
26. Manolson, M. F., Wu, B., Proteau, D., Taillon, B. E., Roberts, B. T., Hoyt, M. A., and Jones, E. W. (1994) J. Biol. Chem. 269, 14064–14074
27. Gietz, D., St Jean, A., Woods, R. A., and Schiestl, R. H. (1992) Nucleic Acids Res. 20, 1425
28. Uchida, E., Ohsumi, Y., and Anraku, Y. (1985) J. Biol. Chem. 260, 1090–1095
29. Laemmli, U. K. (1970) Nature 227, 680–685
30. Vasilyeva, E., Liu, Q., MacLeod, K. I., Baleja, J. D., and Forgac, M. (2000) J. Biol. Chem. 275, 255–260
31. Lowry, O., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
32. Nelson, H., and Nelson, N. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 3503–3507
33. Huang, Y., Lemieux, M. J., Song, J., Auer, M., and Wang, D. N. (2003) Science 301, 616–620
34. Roux, B., and MacKinnon, R. (1999) Science 285, 100–102
35. Angevine, C. M., and Fillingame, R. H. (2003) J. Biol. Chem. 278, 6066–6074
36. Steed, P. R., and Fillingame, R. H. (2008) J. Biol. Chem. 283, 12365–12372
37. Moore, K. J., and Fillingame, R. H. (2008) J. Biol. Chem. 283, 31726–31735
38. Angevine, C. M., Herold, K. A., and Fillingame, R. H. (2003) Proc. Natl. Acad. Sci. U.S.A. 100, 13179–13183
39. Schwem, B. E., and Fillingame, R. H. (2006) J. Biol. Chem. 281, 37861–37867
40. Angevine, C. M., Herold, K. A., Vincent, O. D., and Fillingame, R. H. (2007) J. Biol. Chem. 282, 9001–9007
41. Suzuki, T., Ueno, H., Mitome, N., Suzuki, J., and Yoshida, M. (2002) J. Biol. Chem. 277, 13281–13285
42. Zhang, J., Myers, M., and Forgac, M. (1992) J. Biol. Chem. 267, 9773–9778
43. Kane, P. M. (1995) J. Biol. Chem. 270, 17025–17032