The yeast vacuolar ATPase (V-ATPase) contains three proteolipid subunits: c (Vma3p), c′ (Vma11p), and c" (Vma16p). Each subunit contains a buried glutamate residue that is essential for function, and these subunits are not able to substitute for each other in supporting activity. Subunits c and c′ each contain four putative transmembrane segments (TM1–4), whereas subunit c" is predicted to contain five. To determine whether TM1 of subunit c" serves an essential function, a deletion mutant of Vma16p was constructed lacking TM1 (Vma16p-ΔTM1). Although this construct does not complement the loss of Vma3p or Vma11p, it does complement the loss of full-length Vma16p. Vacuoles isolated from the strain expressing Vma16p-ΔTM1 showed V-ATPase activity and proton transport greater than 80% relative to wild type and displayed wild type levels of subunits A and a, suggesting normal assembly of the V-ATPase complex. These results suggest that TM1 of Vma16p is dispensable for both activity and assembly of the V-ATPase. To obtain information about the topology of Vma16p, labeling of single cysteine-containing mutants using the membrane-permeable reagent 3-(N-maleimidylpropionyl)biocytin (MPB) and the impermeable reagent 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (AMS) was tested. Both the Cys-less form of Vma16p and eight single cysteine-containing mutants retained greater than 80% of wild type levels of activity. Of the eight mutants tested, two (SSC and S178C) were labeled by MPB. MPB-labeling of SSC was blocked by AMS in intact vacuoles, whereas S178C was blocked by AMS only in the presence of permeabilizing concentrations of detergent. In addition, a hemaggulwin epitope tag introduced into the C terminus of Vma16p was recognized by an anti-hemaggulwin antibody in intact vacuolar membranes, suggesting a cytoplasmic orientation for the C terminus. These results suggest that subunit c" contains four rather than five transmembrane segments with both the N and C terminus on the cytoplasmic side of the membrane.

The vacuolar H⁺-ATPases (or V-ATPases)¹ are found in a variety of intracellular compartments that function in both endocytic and secretory pathways (1–8). Acidification of these compartments is essential for many cellular processes, including receptor-mediated endocytosis, intracellular targeting, protein processing and degradation, and coupled transport. V-ATPases are also present in the plasma membrane of certain specialized cells, including osteoclasts (9), renal intercalated cells (10), and neutrophils (11), where they function in such processes as bone resorption, renal acidification, and pH homeostasis, respectively.

The V-ATPases from fungi, plants, and animals are structurally very similar and are composed of two domains (1–8). The V₀ domain is a 570-kDa peripheral complex composed of eight different subunits of molecular mass of 70 to 14 kDa (subunits A–H) that is responsible for ATP hydrolysis. The V₁ domain is a 260-kDa integral complex composed of five subunits of molecular mass 100 to 17 kDa (subunits a, d, c, c′, and c″) that is responsible for proton translocation. The overall structure of the V-ATPase is therefore similar to that of the FₐFₒ-ATP synthase (or F-ATPase) that functions in ATP synthesis in mitochondria, chloroplasts, and bacteria (12–15). Sequence homology between these classes of ATPase has been identified for both the nucleotide-binding subunits (16, 17) and the proteolipid subunits (18, 19).

Unlike the F-ATPases, however, which contain a single type of proteolipid subunit (subunit c), the V-ATPases contain three different proteolipid subunits (c, c′, and c″). All three proteolipid subunits are highly hydrophobic proteins, and all three are essential for V-ATPase function (19). In yeast, subunits c, c′, and c″ are encoded by the VMA3, VMA11, and VMA16 genes, respectively. The V-ATPase proteolipid subunits are homologous both to each other and to the F-ATPase subunit c, from which they appear to have been derived by gene duplication and fusion (18). Thus, the F-ATPase subunit c is an 8-kDa protein containing two transmembrane segments with an essential aspartate residue present in TM2 (14). Subunits c and c′ of the V-ATPase are 16-kDa proteins containing four putative transmembrane segments with an essential glutamate residue present in TM4 (18, 19). The N- and C-terminal halves of subunits c and c′ are homologous to each other. Subunit c has been shown recently (20) to contain the binding site for the specific V-ATPase inhibitor bafilomycin.

By contrast, subunit c″ of the V-ATPase is a 21-kDa protein predicted to have five transmembrane helices (19). Although TM2 to TM5 of subunit c″ are homologous to subunits c and c′, TM1 is not similar to anything in these proteins. Interestingly, the essential glutamate residue of subunit c″ is located in TM3 (19). Previously, we demonstrated that the C terminus of mouse subunit c is present on the luminal side of the mem-

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The abbreviations used are: V-ATPase, vacuolar proton-translocating adenosine triphosphatase; F-ATPase, FₐFₒ-ATP synthase; HA, influenza hemagglutinin; TM, transmembrane segment; Vma16p-ΔTM1, the Vma16p protein lacking putative transmembrane segment 1;
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**Analysis of Subunit Expression and V-ATPase Assembly**—Vacuolar membrane proteins were separated by SDS-PAGE on 8–12.5% acrylamide gels. The expression of Vma16p and Vma16p–TM1 was detected by Western blotting using the horseradish peroxidase-conjugated monoclonal antibody 3F10 against HA, whereas Vph1p or Vma1p was detected by monoclonal antibody 10D7 and 8B1-F3 (Molecular Probes, OR), respectively, followed by a horseradish peroxidase-conjugated secondary antibody (Bio-Rad). Assembly of the V-ATPase was assessed by measurement of the amount of subunit A present on isolated vacuolar membranes (23, 26). Blots were developed using a chemiluminescent detection method obtained from Kirkegaard & Perry Laboratories (Gaithersburg, MD).

**Immunoprecipitation of the HA-tagged Vma16p from Intact or Detergent-solubilized Vacuolar Membrane Vesicles**—To determine whether the C terminus of Vma16p is exposed on the cytoplasmic side of the membrane, the accessibility of the HA epitope tag introduced at the C terminus in intact vacuolar vesicles was determined. Anti-HA antibody was added to intact vacuolar membrane vesicle (100 µg) and incubated for 2 h at 4 °C. Vacular membranes were washed with the overlay buffer (20 mM MES-Tris, pH 7.6, 0.25 mM MgCl₂, 1.1 M glycerol) and solubilized with phosphate-buffered saline containing 1% CH₃SO₄ and 0.1% Triton X-100. The proteins were blotted by immunoprecipitation of the Vma16::HA with protein G-Sepharose. As a control, the anti-HA antibody was added to C₄₀₅₃-solubilized vacuolar membranes following precipitation of the Vma16::HA with protein G-Sepharose. Samples were separated by SDS-PAGE and transferred to nitrocellulose, and Vma16p was detected using a peroxidase-conjugated anti-HA antibody (Roche Molecular Biochemicals) and the Supersignal ULTRA chemiluminescent system (Pierce).

**Chemical Labeling and Blocking of Introduced Cysteine Residues**—Chemical labeling of introduced cysteine residues by the membrane-permeant sulphydryl reagent MPB and blocking by the membrane-impermeant reagent AMS was performed using a modification of the procedure described previously (27). Briefly, vacuolar membrane vesicles were washed using labeling buffer (10 mM MES-Tris, pH 7.0, 0.25 mM MgCl₂, 1.1 M glycerol) and divided into two tubes. AMS (100 µM) was added to one tube, and both samples were incubated for 5 min at 15 °C. Samples were then transferred to ice and diluted 5-fold with labeling buffer followed by immediate addition of 250 µM MPB and incubation for 15 min at 25 °C. This labeling reaction was then stopped by addition of 15 mM 2-mercaptoethanol. After MPB labeling, vesicles were pelleted and solubilized in ice-cold phosphate-buffered saline containing 1% C₄₀₅₃ and 0.1% Triton X-100. The V₀ domain was immunoprecipitated using the mouse monoclonal antibody 10D7 specific for Vph1p plus protein G-Sepharose. Samples were then subjected to SDS-PAGE on 10% acrylamide gels and transferred to nitrocellulose membranes. The blots were probed with horseradish peroxidase-conjugated NeutrAvidin and developed using the Supersignal ULTRA chemiluminescent system (Pierce).

**Other Procedures**—Protein concentrations were determined by the Lowry method (28). ATPase activity was measured using a coupled spectrophotometric assay in the presence or absence of 1 mM concanamycin, as described previously (29). ATP-dependent proton transport was measured in transport buffer (25 mM MES-Tris, pH 7.2, 0.2 M NaCl) using the fluorescence probe ACMA (9-amino-6-chloro-2-methoxycridine) in the presence or absence of 1 mM concanamycin, as described previously (29). SDS-PAGE was carried out as described by Laemmli (30).

**RESULTS**

**Transmembrane Helix 1 Is Not Essential for Vma16p Function**—Fig. 1 shows the sequence alignment of the three proteolipid subunits of the yeast V-ATPase: subunit c (Vma3p),
ATP-dependent proton transport was measured for vacuoles. Open bars: D Vma16p was 0.49 mol of ATP/min/mg of protein at 30 °C and 0.5 mM ATP, which corresponds to ~70% of the activity measured for the full-length untagged Vma16p.

![Diagram](Image)

**Fig. 2. Deletion of the first transmembrane region of Vma16p does not alter V-ATPase function.**

(a) Schematic illustration of the domain structure of Vma3p, Vma11p, and Vma16p. Putative transmembrane regions are indicated as boxes, and the positions of the essential glutamate residues in each subunit are indicated as open circles. The glutamate residue shown in the shaded circle in TM5 of Vma16p has been shown not to be essential for function (19). b, growth phenotype at pH 7.5 of yeast strains disrupted in the indicated proteolipid genes (host cell) upon introduction of the indicated full-length genes or the Vma16p-TM1 construct. c, Western blot analysis of vacuoles isolated from a vma16Δ strain expressing HA-tagged forms of Vma16p or the Vma16p-TM1 construct. Western blotting was performed using antibodies against the HA epitope, the V1 subunit Vma1p, or the V0 subunit Vph1p as described under "Experimental Procedures." d, concanamycin-sensitive ATPase activity (hatched bars) or ATP-dependent proton transport (open bars) was measured for vacuoles isolated from the vma16Δ strain expressing the HA-tagged forms of Vma16p or Vma16p-TM1. The specific activity of the ATPase in the vacuoles isolated from the strain expressing the full-length HA-tagged Vma16p was 0.49 μmol of ATP/min/mg of protein at 30 °C and 0.5 mM ATP, which corresponds to ~70% of the activity measured for the full-length untagged Vma16p.

Subunit c (Vma11p), and subunit c′ (Vma16p). As can be seen, subunits c and c′ both contain four putative transmembrane helices and share significant sequence homology with each other and with transmembrane segments 2–5 of subunit c′. By contrast, TM1 of subunit c′ is not homologous to any other sequence in the other two proteolipid subunits. The location of the buried glutamate residue critical for function of each of the proteolipid subunits is depicted in Fig. 2a. To address the functional role of TM1 of subunit c′, a deleted form of subunit c′ was constructed (Vma16p·ΔTM1) lacking amino acid residues 2–41 that contain TM1 (Fig. 1). We first tested the ability of this construct to complement the phenotype of yeast strains disrupted in each of the proteolipid genes. It has been shown previously that yeast lacking any of the V-ATPase genes (or the V0 domain) and with transmembrane segments 2–41. The positions of the essential glutamate residues in each subunit are indicated as open circles. The glutamate residue shown in the shaded circle in TM5 of Vma16p has been shown not to be essential for function (19). b, growth phenotype at pH 7.5 of yeast strains disrupted in the indicated proteolipid genes (host cell) upon introduction of the indicated full-length genes or the Vma16p-TM1 construct. c, Western blot analysis of vacuoles isolated from a vma16Δ strain expressing HA-tagged forms of Vma16p or the Vma16p-TM1 construct. Western blotting was performed using antibodies against the HA epitope, the V1 subunit Vma1p, or the V0 subunit Vph1p as described under "Experimental Procedures." d, concanamycin-sensitive ATPase activity (hatched bars) or ATP-dependent proton transport (open bars) was measured for vacuoles isolated from the vma16Δ strain expressing the HA-tagged forms of Vma16p or Vma16p-TM1. The specific activity of the ATPase in the vacuoles isolated from the strain expressing the full-length HA-tagged Vma16p was 0.49 μmol of ATP/min/mg of protein at 30 °C and 0.5 mM ATP, which corresponds to ~70% of the activity measured for the full-length untagged Vma16p.

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Previous results (33, 34) have suggested that retention of ~20% of wild type V-ATPase activity is sufficient to confer on cells a wild type growth phenotype. It is therefore necessary to measure directly V-ATPase activity and proton transport in vacuoles isolated from cells expressing the Vma16p-TM1 construct in order to determine quantitatively the effect of removal of TM1 of Vma16p on V-ATPase function. We first wished to test the stability of the Vma16p-TM1 protein and its ability to assemble with other V-ATPase subunits. To accomplish this, an HA epitope tag was inserted at the C-terminal end of both the full-length Vma16p and the Vma16p-TM1. This was necessary because of the lack of available antibodies against the native Vma16p protein. As can be seen in Fig. 2c, although Western blots of vacuoles isolated from the strain expressing Vma16p·ΔTM1 showed somewhat reduced reactivity with the anti-HA antibody relative to vacuoles isolated from the strain expressing the full-length Vma16p, both Vma1p (subunit A of the V1 domain) and Vph1p (subunit a of the V0 domain) were present at normal levels on the vacuolar membrane. It has been shown previously (19, 23) that the absence of any of the proteolipid subunits results in the failure of V1 to assemble onto the vacuolar membrane and aberrant assembly and targeting of the V0 domain. These results thus suggest that TM1 of subunit c′ is dispensable for normal assembly of the V-ATPase complex. The lower level of antibody staining of the Vma16p-TM1 construct in isolated vacuoles may reflect partial proteolytic removal of the HA tag or altered reactivity of the HA epitope in this construct. Finally, measurement of both concanamycin-sensitive ATPase activity and ATP-dependent proton transport (as assessed by quenching of ACMA fluorescence) in isolated vacuoles (Fig. 2d) indicates that V-ATPase complexes containing Vma16p-TM1 have nearly the same activity and coupling as complexes containing the full-length Vma16p. These results confirm that TM1 of Vma16p is not necessary for V-ATPase function.

Accessibility of C Terminus of HA-tagged Vma16p in Intact and Detergent-solubilized Vacuolar Membrane Vesicles—Previously, we demonstrated that the C terminus of the mouse subunit c′ appears to be facing the cytoplasmic side of the membrane in COS-1 cells transfected with a HA-tagged form of the mouse Vma16p homologue (21). To confirm this result for the yeast protein, we compared the accessibility of an HA epitope tag attached at the C terminus of Vma16p in intact versus solubilized vacuolar membrane vesicles. One sample of vacuolar membranes was incubated with an anti-HA antibody followed by washing, detergent solubilization, and immunoprecipitation of the complexes containing the bound HA antibody. As a control, vacuolar membranes were solubilized with detergent first before addition of the anti-HA antibody and immunoprecipitation. Both samples were then subjected to SDS-PAGE and Western blotting using the anti-HA antibody. As shown in Fig. 3, the amount of anti-HA antibody binding to Vma16p:HA is similar whether the immunoprecipitating antibody was added before or after detergent solubilization. The somewhat higher level of antibody binding observed in the right lane of Fig. 3 may be due to some change in interaction between the subunits upon detergent solubilization of the complex or to the loss of some antibody during washing of the.
membranes in the case where antibody is added before detergent solubilization. This result indicates that the antibody-binding site at the C terminus of Vma16p:HA is exposed in intact vacuolar membrane vesicles and hence resides on the cytoplasmic side of the membrane.

Labeling of Single Cysteine-containing Mutants of Vma16p by Membrane-permeant and -impermeant Maleimides—In order to obtain additional information about the topology of subunit c', we employed cysteine mutagenesis and covalent modification by the membrane-permeant sulfhydryl reagent MPB and the membrane-impermeant sulfhydryl reagent AMS. This method has been employed previously to study the membrane folding of subunit a of the F$_1$F$_0$-ATP synthase of E. coli (35, 36), and we have used this method to study the topology of subunit a of the yeast V-ATPase (27). In order to apply this method to subunit c', we first constructed a Cys-less form of Vma16p by replacing each of the three endogenous cysteine residues present at positions 67, 105, and 159 with serine (Fig. 1). Site-directed mutagenesis was performed on the HA-tagged form of Vma16p described above in order to facilitate detection of the expressed proteins by Western blot. As can be seen in Fig. 4a, the Cys-less form of the HA-tagged Vma16p was expressed at normal levels in isolated vacuoles relative to the wild type HA-tagged Vma16p. Moreover, both Vma1p and Vph1p were present at normal levels in isolated vacuoles, suggesting that removal of the endogenous cysteine residues of Vma16p did not perturb assembly of the V-ATPase. Finally, measurement of concanamycin-sensitive ATPase activity and ATP-dependent proton transport indicated that the Cys-less form of Vma1p gave rise to V-ATPase complexes possessing wild type levels of both ATPase activity and proton transport (Fig. 4b).

By using this Cys-less, HA-tagged form of Vma16p as the starting point, seven single-cysteine containing mutants were constructed by replacement of the endogenous residues at positions Ser-5, Ser-11, Ser-55, Ser-135, Ser-137, Ser-178, and Ser-210 with cysteine by site-directed mutagenesis. An additional mutant (Q213C) of the untagged form of Vma16p was also constructed. Because this mutant contained a cysteine residue at the very C terminus, it was felt that it would be better to use an untagged form of Vma16p to analyze labeling because of the possibility of interference with access of the labeling reagents by the presence of the C-terminal HA tag. These mutant forms of Vma16p were expressed in the VMA16 deletion strain, and the growth phenotype was analyzed at pH 7.5 and 5.5. All mutants displayed a wild type growth phenotype at both pH values (data not shown). Western blot analysis of vacuoles isolated from the mutant strains (Fig. 4a) revealed wild type levels of the HA-tagged Vma16p protein for six of the seven tagged mutants (only S210C showed somewhat lower labeling than wild type). In addition, all of the mutants (including Q213C) showed normal levels of Vma1p and Vph1p on the vacuolar membrane. Finally, vacuoles isolated from each of the mutant strains showed at least 80% of wild type levels of activity for both concanamycin-sensitive ATPase activity and ATP-dependent proton transport (Fig. 4b). These results indicate that the cysteine substitutions in Vma16p do not significantly compromise stability, assembly, or activity of the V-ATPase complex.

We next determined the ability of each of the introduced cysteine residues to react with the membrane-permeant reagent MPB. Vacular membrane vesicles isolated from each of the mutant strains were reacted with 250 μM MPB for 15 min at 25 °C followed by detergent solubilization and immunoprecipitation of the V$_0$ complexes with the monoclonal antibody 10D7 directed against subunit a. Previous studies have shown that this antibody only recognizes its epitope on subunit a in the free V$_0$ domain and not in the intact V$V_0$ complex (23). The immunoprecipitated proteins were then separated by SDS-PAGE, and Western blot analysis was performed using horse-radish peroxidase-conjugated NeutrAvidin (Pierce). As shown in Fig. 5a, of the eight single cysteine-containing mutants of Vma16p, only S5C and S178C showed significant labeling by MPB, with S5C showing much stronger labeling than S178C. The remaining cysteine residues appear to be inaccessible to labeling by MPB, possibly due to shielding of these sites by other subunits in the V$_0$ domain.

We next tested whether pretreatment of the vacuoles containing the S5C and S178C mutants with the membrane-impermeant sulfhydryl reagent AMS was able to prevent labeling of these proteins by MPB. As can be seen in Fig. 5b, whereas MPB labeling of S5C was effectively blocked by pretreatment with AMS, labeling of S178C was not. By contrast, permea-
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Fig. 5. Labeling of single cysteine-containing mutants of Vma16p by MPB and protection by AMS. a, vacuolar membrane vesicles (100 μg of protein) isolated from the vma16Δ strain expressing the Cys-less form of HA-tagged Vma16p or the indicated single cysteine-containing mutants were incubated with 250 μM MPB for 15 min at 23 °C. The membranes were then solubilized with C12E9, and the V0 complexes were immunoprecipitated with the monoclonal antibody 10D7 against subunit a and protein A-Sepharose. The samples were separated by SDS-PAGE and transferred to nitrocellulose, and the biotinylated bands were identified using horseradish peroxidase-conjugated NeutrAvidin and the Supersignal Western blotting system (Pierce). The exposure time is the same for all samples in this experiment. The asterisks mark the position of Vma16p. b, vacuolar membrane vesicles isolated from the S5C and S178C mutants of Vma16p were incubated in the presence or absence of 100 μM AMS for 5 min at 15 °C followed by 5-fold dilution and labeling with MPB, SDS-PAGE, and Western blot analysis as described above. Where indicated (ZW+), 0.25% Zwittergent 3-14 was included during treatment with AMS.

zilation of the vacuoles isolated from the S178C mutant with low concentrations of the detergent Zwittergent 3-14 resulted in effective blocking of MPB labeling by pretreatment with AMS. Quantitation of the extent of labeling using scanning densitometry (Alpha Imager 2200) revealed that AMS treatment of S5C reduced labeling of Vma16p by 98%, whereas AMS treatment of S178C reduced labeling by only 87%. After detergent permeabilization of the vacuolar membrane, however, AMS reduced labeling of the S178C mutant by 87%. These results suggest that the cysteine residue in the S5C mutant has a cytoplasmic orientation, whereas the cysteine residue in the S178C mutant is oriented toward the luminal side of the membrane. The values obtained for AMS protection of Vma16p mutants are similar to those observed previously (27) for single cysteine-containing mutants of Vph1p. Thus, for Vph1p residues interpreted as having a luminal orientation (S602C and S840C), AMS pretreatment reduced MPB labeling of subunit a by 50 and 27%, respectively, similar to the 45% observed for the S178C mutant of Vma16p. By contrast, for the seven cysteine residues interpreted as having a cytoplasmic orientation in Vph1p, the protection by AMS of MPB labeling ranged from 72 to 98%, with an average value of 87% (±9%), similar to the 80% observed for the S5C mutant of Vma16p.

DISCUSSION

The proteolipid subunits of the V- and F-ATPases have been shown by mutagenesis studies to play a critical role in proton translocation through the integral domains of these complexes (14, 19, 37). NMR analysis of the single F-ATPase proteolipid subunit (subunit c) reveals a helical hairpin structure with the essential glutamate residue present in the middle of the second transmembrane helix (38). X-ray crystallography (39) and cross-linking studies (40) indicate the presence of 10 copies of the c subunit in a ring structure, although this number may be species-dependent (41, 42). The critical aspartate residue is thought to undergo reversible protonation and deprotonation during proton translocation through F0 (14), and the orientation of this residue is believed to change during rotary catalysis (43).

Unlike the F-ATPases, the V-ATPases require three distinct proteolipid subunits for function (19). Hydropathy analysis suggests that subunits c and c' both contain four putative transmembrane helices, whereas subunit c" contains five (19, 37). Quantitation of the subunit stoichiometry of the bovine-coated vesicle V-ATPase suggests the presence of 5–6 copies of subunits c plus c' and a single copy of subunit c" (44). Immunoprecipitation of epitope-tagged forms of the yeast proteolipid subunits suggests the presence of single copies of both subunits c and c' and multiple copies of subunit c" (45). Combining the results from both of these studies suggests a subunit stoichiometry for the V-ATPase proteolipid ring of c-c'-c", although other studies have suggested that there may be two copies of subunit c" (46). Assuming the former subunit stoichiometry, the proteolipid ring of V0 would contain 25–29 transmembrane helices in comparison to the 20 transmembrane helices for F0. Because there is only one critical glutamate residue present in each of the V-ATPase proteolipid subunits (19, 37), the total number of such residues per V0 domain (6–7) is smaller than that for F0 (10). This difference has been suggested as an explanation for the lower H+/ATP stoichiometry of the V-ATPases relative to the F-ATPases (47).

Why the V-ATPases require three distinct proteolipid subunits is unclear, but previous results (19) have indicated (and it is confirmed in the current study) that they are not able to complement each other’s loss. Because of the unique structure predicted for subunit c", we were interested in determining whether TM1 played an important functional role in activity or assembly of the V-ATPase. It has been suggested, for example, that the fifth transmembrane segment might serve as a “plug” in the center of the proteolipid ring of V0 (45). It also appeared possible that removal of TM1 might allow the truncated Vma16p to substitute for one of the other proteolipid subunits in supporting activity. It was therefore surprising that although the Vma16p-ΔTM1 was not able to substitute for either subunits c or c', it was able to replace the full-length subunit c" in supporting both assembly and activity. This result is in conflict with that reported by Gibson et al. (46) who found that removal of TM1 of subunit c" led to a vma− phenotype. Although the basis for this difference in results is not certain, it should be noted that in their study the mutant and wild type forms of Vma16p were overexpressed in a vma16-deficient strain using a galactose-inducible promoter (46). Because replacement of glucose in the medium with galactose causes partial dissociation of the V-ATPase complex (48), it is possible
that the vma— phenotype observed for the strain expressing the Vma16p-3TM construct is the result of somewhat greater dissociation of the V-ATPase complex containing the deleted form of Vma16 relative to the full-length protein upon glucose substitution. It should also be noted that the region removed in the Gibson et al. (46) study was from residue Leu-12 to Ser-55 (inclusive), whereas the region removed in the present study extended from residue Asn-2 to His-41. It is thus possible that removing the residues Gly-42 to Ser-55 (which is outside the region predicted for TM1) accounts for the observed vma— phenotype. It is in any case clear from the present study that TM1 of subunit c' is dispensable for both assembly and activity of the V-ATPase complex.

Because TM1 of Vma16p did not appear to serve a crucial functional role in V-ATPase activity, we wished to determine whether this region of the protein actually corresponds to a transmembrane segment. To address this question, a series of mutants of Vma16p were constructed containing unique cysteine residues in the N and C terminus of the protein as well as in three of the predicted loop regions. The reactivity of these cysteine residues toward the membrane-impermeant maleimide AMS and the membrane-permeant maleimide MPB was then determined in intact vacuolar membrane vesicles. This same approach was used to address the membrane topology of subunit a of the V-ATPase (27) as well as subunit a of the F-ATPase (35, 36). The results presented in the current study show that the N terminus of subunit c' is present on the cytoplasmic side of the membrane, whereas the loop between TM4 and TM5 containing Cys-178 is present on the luminal side of the membrane. Previous work (21) from our laboratory on the mammalian homologue of Vma16p indicates that the C terminus of the mouse subunit c', in contrast to the C terminus of subunit c, is present on the cytoplasmic side of the membrane. The results presented in Fig. 3 of the present paper confirm that the C terminus of the yeast Vma16p is also exposed on the cytoplasmic side of the membrane. Interestingly, the least conserved part of subunit c' is located in the putative TM1 region (21). Because both the N and C terminus of subunit c' appear to be exposed to the cytoplasmic side of the membrane, the polypeptide chain likely crosses the membrane an even number of times. This suggests that subunit c' contains four rather than five transmembrane segments and is consistent with the luminal labeling observed for the SI78C mutant. A model depicting the folding of subunit c' is shown in Fig. 6. The failure of the remaining 6 introduced cysteine residues to react with MPB likely reflects the inability of this reagent to access these sites. This limited access may be due to shielding of these parts of the polypeptide chain by other portions of Vma16p itself or by other subunits in the V_{0} domain. In particular, both subunit d (a peripheral protein that remains tightly bound to V_{0} upon dissociation of V_{1}) and the N-terminal domain of subunit a (which is both cytoplasmic (27) and appears from electron microscopy to fold down on the membrane (49)) are likely candidates for this inter-subunit shielding effect. Because immunoprecipitation of the labeled complexes was performed using a monoclonal antibody against subunit a that only recognizes its epitope in the free V_{0} domain, the observed shielding of these sites is most likely not due to interference by the V_{1} domain.

These results suggest that the TM1 region of subunit c' does not correspond to an actual membrane spanning segment but instead represents a somewhat hydrophobic domain located on the cytoplasmic side of the membrane. What might the function of this region be? As mentioned above, this region is not well conserved between species, with only 8 of 55 residues identical between the yeast and mouse sequences (21). Because of its location on the cytoplasmic side of the membrane, it is oriented to participate in the interaction between the V_{1} and V_{0} domains, although no obvious perturbation in the stability of the V-ATPase complex is observed upon its removal. Alternatively, it may function in the regulated interaction between V_{1} and V_{0}, which has been shown to play an important role in controlling V-ATPase activity in vivo (48). Additional studies will be required to elucidate the role of this region of subunit c'.

The model shown in Fig. 6 for the topology of subunit c' suggests that, like subunit a and presumably subunit c1, subunit c' also contains four transmembrane helices, giving a V_{0} structure having a subunit stoichiometry of c_{4}c'_{4}c_{2}, a total of 24 transmembrane helices. Because the critical glutamate residue in subunit c' is present in TM2 (new model) rather than in TM4 of subunit c and c' (19), there is an asymmetry to the V_{0} proteolipid ring even beyond the presence of three different proteolipids. Based upon current models of the arrangement of c subunits in the proteolipid ring of F_{0} (50), accommodating such an asymmetric placement of the critical carboxyl in the ring of proteolipid subunits should not be problematic. It should also be noted, however, that the orientation of the transmembrane segment containing the critical glutamate residue (that is the N- to C-terminal direction) is opposite for subunit c' relative to subunits c and c' (21). Whether these structural asymmetries are important for the functional properties of the V_{0} domain, such as the inactivity of native V_{0} (in contrast to F_{0}) to conduct protons (51), remains to be determined.

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