Interacting Helical Surfaces of the Transmembrane Segments of Subunits a and c’ of the Yeast V-ATPase Defined by Disulfide-mediated Cross-linking*

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Proton translocation by the vacuolar (H⁺)-ATPase (or V-ATPase) has been shown by mutagenesis to be dependent upon charged residues present within transmembrane segments of subunit a as well as the three proteolipid subunits (c, c’, and c”). Interaction between R735 in TM7 of subunit a and the glutamic acid residue in the middle of TM4 of subunits c and c’ or TM2 of subunit c’ has been proposed to be essential for proton release to the luminal compartment. In order to determine whether the helical face of TM7 of subunit a containing R735 is capable of interacting with the helical face of TM4 of subunit c’ containing the essential glutamic acid residue (Glu-145), cysteine-mediated cross-linking between these subunits in yeast has been performed. Cys-less forms of subunits a and c’ as well as forms containing unique cysteine residues were constructed, introduced together into a strain disrupted in both endogenous subunits, and tested for growth at neutral pH, for assembly competence and for cross-linking in the presence of cupric-phenanthroline by SDS-PAGE and Western blot analysis. Four different cysteine mutants of subunit a were each tested pairwise with ten different unique cysteine mutants of subunit c’. Strong cross-linking was observed for the pairs aS728C/cF143C, aA731C/cI142C, aA731C/cE145C, aA738C/cF143C, aA738C/cI147C, and aL739C/cL147C. Partial cross-linking was observed for an additional 13 of 40 pairs analyzed. When arrayed on a helical wheel diagram, the results suggest that the helical face of TM7 of subunit a containing Arg-735 interacts with the helical face of TM4 of subunit c’ centered on Val-146 and bounded by Glu-145 and Leu-147. The results are consistent with a possible rotational flexibility of one or both of these transmembrane segments as well as some flexibility of movement perpendicular to the membrane.

The vacuolar (H⁺)-ATPases (or V-ATPases)³ are ATP-driven proton pumps that acidify a variety of intracellular compartments in eukaryotic cells, including endosomes, lysosomes, clathrin-coated vesicles, Golgi-derived vesicles, secretory vesicles, and the central vacuoles of plants and fungi (1–8). Acidification of these intracellular compartments is important for many cellular processes, including receptor-mediated endocytosis, intracellular trafficking, protein processing, and degradation, coupled transport of small molecules and entry of viruses and toxins (1). V-ATPases also exist in the plasma membrane of certain cells, where they function in such processes as renal acidification, bone resorption, pH homeostasis, coupled potassium transport, and tumor invasion (9–13).

V-ATPases are composed of a peripheral V₁ domain responsible for ATP hydrolysis and an integral V₀ domain that carries out proton transport (1–8). V₁ is composed of eight different subunits (A–H) of molecular mass 70–13 kDa that are present in a stoichiometry of A,B,C,D,E,F,G,H₋₋₋₋ (14, 15) and that form a complex of ~640 kDa. Both the A and B subunits participate in nucleotide binding, with the catalytic sites located on the A subunits (16, 17). The V₀ domain is a 240-kDa complex composed of five different subunits (a, d, c, c’, and c”) of molecular mass 100–16 kDa that are present in a stoichiometry of a₃d₁c₁c’₁c” (14, 18). Both the a subunit (19) and the three proteolipid subunits (c, c’, and c”) (20, 21) have been shown to contain residues essential for proton transport.

The V-ATPases resemble the ATP synthases (or F-ATPases) responsible for ATP synthesis in mitochondria, chloroplasts, and bacteria (22–27). The F-ATPases have been shown to operate by a rotational mechanism in which ATP hydrolysis in F₁ drives rotation of a central stalk composed of the γ and ε subunits (28–30). The γ and ε subunits are in turn connected to a ring of proteolipid ϵ subunits in F₅ (31, 32), such that rotation of the central stalk drives rotation of the ε subunit ring relative to subunit a (33–35). Subunit a is held fixed relative to the α₃β₃ head by a peripheral stalk composed of the δ subunit and the soluble domains of subunit b (36, 37). It is movement of the ε subunit ring relative to subunit a that is thought to be responsible for unidirectional proton transport (38, 39).

Electron microscopy (40, 41) and cysteine-mediated cross-linking (42, 43) have revealed that, like the F-ATPases, the V-ATPases also contain a central and peripheral stalk, with the central stalk composed of the D and F subunits and the peripheral stalk containing the C, E, G, and H subunits, as well as the hydrophilic domain of subunit a (15, 42–44). ATP-driven rotation of the D and F subunits (45) as well as the ring of proteolipid subunits (46, 47), has recently been demonstrated. Thus the V-ATPases also appear to operate by a rotational mechanism. Because proton translocation through the V₀ domain is thought to be critically dependent upon the interaction between subunit a and the ring of proteolipid subunits, it is important to define the nature of the helical interactions that occur between these proteins. In order to obtain information about these interactions, we have carried disulfide-mediated cross-linking between subunit a and subunit c’ of the yeast V-ATPase.
**TABLE I**
Summary of growth phenotype of cysteine-substituted mutants at neutral pH

| a    | Ile-140 | Leu-141 | Ile-142 | Phe-143 | Ser-144 | Ser-144 | Glu-145 | Val-146 | Leu-147 | Gly-148 | Leu-149 | Cys-less |
|------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
| Ser-728 | +++    | +++     | +++     | +++     | +++     | +++     |        | +++     | +++     | +++     | +++     | +++     |
| Ala-731 | +++    | +++     | +++     | +++     | +++     | +++     |        | +++     | +++     | +++     | +++     | +++     |
| Ala-738 | +++    | +++     | +++     | +++     | +++     | +++     |        | +++     | +++     | +++     | +++     | +++     |
| Ile-739 | +++    | +++     | +++     | +++     | +++     | +++     |        | +++     | +++     | +++     | +++     | +++     |

EXPERIMENTAL PROCEDURES

**Materials and Strains—Zymolyase 100T was obtained from Seikagaku America, Inc. Protease inhibitors were from Roche Applied Science. The monoclonal antibody 3F10 (directed against the HA antigen) that is conjugated with horseradish peroxidase was also from Roche Molecular Biochemicals. The monoclonal antibody 8B1-F3 against the yeast V-ATPase A subunit (48) and the monoclonal antibody 10D7 against the 100 kDa a subunit (49) were from Molecular Probes. Escherichia coli and yeast culture media were purchased from Difco Laboratories. Restriction endonucleases, T4 DNA ligase and other molecular biology reagents were from Invitrogen, Promega, and New England BioLabs. Phenylmethylsulfonyl fluoride and most other chemicals were purchased from Sigma Chemical Co. Yeast strains lacking the VMA11, VPH1, and STV1 genes were constructed by replacing the entire coding region of VMA11 with the TRP gene and insertion of the LEU gene into the VPH1 gene and the LYS gene STV1 at the positions indicated by Mandson et al. (50) using the YPH500 yeast strain. YEPD buffered to pH 5.5 or pH 7.5 was used for selection of strains showing a vma phenotype.

**Transformation and Selection—** Yeast cells lacking functional endogenous Vph1p, Stv1p, and Vma11p were transformed using the lithium protoporphyrin (51). The transformants were isolated on histidine minus and uracil minus plates and growth phenotypes of the mutants were assayed on YEPP plates buffered with 50 mM KH$_2$PO$_4$ to pH 7.5. +++, indicates wild-type growth, +, partially defective growth, -, indicates severely defective growth, and -, indicates no growth (19).

**RESULTS**

Construction of the vma11::vph1::stv1::Triple Deletion Strain and Growth Properties of Yeast Containing Cys-less and Single Cysteine-containing Mutant Forms of Vma11p and Vph1p—To evaluate cysteine-mediated cross-linking between subunits a and c, a yeast strain disrupted in the two genes encoding subunit a (VPH1 and STV1) as well as the gene encoding subunit c (VMA11) was constructed. The VMA11 gene was replaced with the TRP gene (56) whereas the LEU and LYS genes were inserted into the VPH1 and STV1 genes, respectively, as previously described (50, 57). The VMA11 gene was then expressed in this strain using the pRS313 plasmid containing the HIS marker whereas the VPH1 gene was expressed using the pRS316 plasmid containing the URA marker.

We had previously shown that a Cys-less form of Vph1p supported wild-type growth of cells at pH 7.5 (58). It has been demonstrated that complexes retaining >20% of wild-type activity in vitro generally display a wild-type growth phenotype (59, 60). Cells expressing Cys-less Vph1p were next transformed with a Cys-less form of Vma11p and were found to also show wild-type growth at pH 7.5. These results indicate that there are no endogenous cysteine residues in either Vph1p or Vma11p that are absolutely required for V-ATPase activity.

We had previously demonstrated that Arg-735 in TM7 of Vph1p is essential for proton transport activity and suggested that this residue interacts with the buried carboxyl groups present in TM4 of subunits c and TM2 of subunit c (19). Using a helical wheel diagram, four a subunit residues that were predicted to lie on the same helical face of TM7 as Arg-735 were chosen for replacement with cysteine, including Ser-728, Ala-731, Ala-738, and Leu-739. When transformed into yeast expressing the Cys-less form of Vma11p, all four single cysteine-containing mutants of Vph1p gave wild-type growth at pH 7.5. We next constructed a series of ten single cysteine-containing mutants of Vma11p in which each of the residues in TM4 between Ile-140 and Leu-149 was replaced with cysteine. Because TM4 of Vma11p is predicted to extend from Val-135 to Ile-157 (56), the region subjected to cysteine mutagenesis corresponds to approximately the central half of this transmembrane segment surrounding Glu-145. Yeast strains were then constructed containing one of the four single cysteine-containing mutants of Vph1p and the Cys-less form or one of the ten single cysteine-containing mutants of Vma11p. The growth phenotype of each of the 44 resultant double mutant strains at pH 7.5 is shown in Table I. As can be seen, all of the double mutants containing the E145C mutation of subunit c failed to grow at pH 7.5, as predicted from previous results demonstrating that Glu-145 is an essential residue for proton transport by the V-ATPase (21). In addition, several of the double mutants containing the a subunit mutation L739C showed severely

**Disulfide Cross-linking of V-ATPase Subunits a and c′**

Growth of yeast strains expressing Vph1p (subunit a) and Vma11p (subunit c′) containing single cysteine residues at the indicated positions was assessed on YEPP plates buffered with 50 mM KH$_2$PO$_4$ to pH 7.5. +++, indicates wild-type growth, +, partially defective growth, -, indicates severely defective growth, and -, indicates no growth (19).
defective growth at neutral pH. The majority of the double mutants, however, showed normal (or near normal) growth at pH 7.5.

**Assembly of V\(\text{O}\) Complexes Containing Cysteine Mutations in Subunits a and c’**—To test the assembly competence of the V\(\text{O}\) complexes present in the double mutant strains, partially purified vacuolar membranes were subjected to SDS-PAGE and Western blot analyses using antibodies against Vph1p and the HA-tagged Vma11p (subunit c’). The presence of subunit a and the proteolipid subunits in isolated vacuoles indicates normal assembly of the V\(\text{O}\) domain (49). As can be seen in Fig. 1, all of the double mutants tested showed normal levels of both Vph1p and Vma11p relative to the wild-type strain, indicating normal assembly and targeting of the V\(\text{O}\) domain to the vacuole.

**Cysteine-mediated Cross-linking of Subunits a and c’ by Disulfide Bond Formation**—To test the proximity of the cysteine residues introduced into TM7 of subunit a and TM4 of subunit c’, vacuolar membrane vesicles were incubated in the absence or presence of cupric-phenanthroline for 30 min at room temperature to catalyze disulfide bond formation. The reaction was terminated by addition of EDTA and N-ethylmaleimide and the samples separated by SDS-PAGE followed by Western blot analysis using a horseradish peroxidase-conjugated monoclonal antibody against the HA epitope tag present on Vma11p. The monoclonal antibodies 10D7 (against Vph1p) or 3F10 (against HA) followed by a horseradish peroxidase-conjugated secondary antibody as described under “Experimental Procedures.”

![Fig. 1. Assembly competence of cysteine-substituted mutants of subunits a (Vph1p) and c’ (Vma11p).](image-url)

DISCUSSION

The integral V\(\text{O}\) domain of the V-ATPase contains three different proteolipid subunits (c, c’, and c”) that are required for activity (21, 61). Subunit c (Vma3p) and c’ (Vma11p) each contain four transmembrane helices, with a single essential glutamic acid residue buried in the middle of TM4 (21, 61). Subunit c” (Vma16p) was originally believed to contain an additional transmembrane helix at the N terminus of the protein, but recent studies have demonstrated that this subunit also contains four transmembrane helices, with an essential buried glutamate residue in TM2 (56). Interestingly, the region originally assigned to TM1 of subunit c” is not required for activity, and this subunit is oriented with the C terminus facing the cytosol, which is opposite to the orientation of subunit c (56, 62).

The V-ATPase proteolipid subunits are homologous to the F-ATPase subunit c, which contains two transmembrane helices in a helical hairpin structure, with an essential buried acidic residue present in TM2 (63). The F-ATPase c subunits have been shown to form a ten-membered ring (31, 65), although other stoichiometries have also been reported (66, 67). The V-ATPase proteolipid subunits also appear to form a ring structure (68) containing one copy each of subunits c and c’ and 4–5 copies of subunit c” (14, 18). The buried carboxyl groups on the ring of proteolipids for both the V and F-ATPases are believed to undergo reversible protonation and deprotonation during proton translocation through the integral domains. The proteolipid ring of the V\(\text{O}\) domain thus contains 6–7 essential buried carboxyl groups, whereas the F\(\text{O}\) domain contains 10 such groups. This difference has been suggested to account for the difference in \(H^+\)/ATP stoichiometry of the V and F-ATPases (69). Both classes of proteolipid ring have been shown to undergo rotation relative to subunit a during proton transport (33–35, 46, 47).

In addition to the proteolipid subunits, the other V\(\text{O}\) subunit shown to be essential for proton transport is subunit a. Based upon previous topological studies, subunit a of the V-ATPase is predicted to contain nine transmembrane helices, with the large hydrophilic N-terminal domain oriented toward the cytosolic side of the membrane and the C terminus oriented toward the lumen (58). The C-terminal domain was shown to contain multiple buried charged residues, including Glu-789, His-743, and Arg-799, whose mutation results in partial loss of proton transport activity (70, 71, 19). Only one a subunit residue, Arg-735 (predicted to reside in TM7), has been found to be absolutely essential for proton translocation (19). Thus, even...
the conservative substitution R735K completely inhibits proton transport (19). Based upon these findings, we proposed that Arg-735 of the V-ATPase subunit a is the functional homologue to Arg-210 of the *E. coli* F-ATPase subunit a, which has also been shown to be essential for proton transport (72).

Subunit a is thought to serve two functions in proton transport coupled to rotational catalysis by the F-ATPases (38, 39). The first is to provide the hemi-channels that allow access of protons from the cytoplasmic side of the membrane to the buried carboxyl groups on the proteolipid ring and from these buried groups to the luminal side of the membrane. The other function is to activate release of protons from the proteolipid carboxyl groups and to stabilize these groups in their negatively charged form. It is this latter function that is thought to be served by Arg-210 of the *E. coli* subunit a (24, 73, 74).

**TABLE II**  
Summary of disulfide-mediated cross-linking of cysteine-substituted mutants of subunits a and c'  

|        | aTM7 | cTM4 |
|--------|------|------|
| Ser-728| –    | +    |
| Ala-731| –    | +    |
| Ala-738| –    | ++   |
| Leu-739| –    | +    |

**FIG. 2**  
Cysteine-mediated cross-linking of cysteine-substituted mutants of subunits a and c'. Vacuum-enriched membranes isolated from strains expressing the indicated mutant forms of subunits a and c' were washed and incubated in the absence or presence of 2.5 mM Cu (1,10-phenanthroline),SO₄ for 30 min at room temperature followed by quenching with EDTA and N-ethylmaleimide, separation by SDS-PAGE on 7.5% acrylamide gels and Western blot analysis using the monoclonal antibody 3F10 against the HA epitope tag and a horseradish peroxidase-conjugated secondary antibody as described under “Experimental Procedures.” The positions of the monomeric subunit c' and the heterodimeric ac' cross-linked product are indicated to the left of each panel.

**FIG. 3**  
Helical wheel diagram depicting the location of the major and minor cross-linked products obtained using cysteine-substituted mutants of subunits a and c'. Each panel corresponds to the cross-linked products obtained with one of the single cysteine-containing mutants of subunit a, S728C (panel a), A731C (panel b), A738C (panel c), and L739C (panel d). The major cross-linked bands are depicted with a heavy dashed line (i.e. aS728C/c/H142C) whereas the lower yield cross-linked products are shown with a thin dashed line (i.e. aS728C/c/H142C).

**TABLE II**  
Summary of disulfide-mediated cross-linking of cysteine-substituted mutants of subunits a and c'  

Disulfide-mediated cross-linking of cysteine-substituted mutants of Vphlp (subunit a) and Vmal1p (subunit c') was performed using cupric-phenanthroline as described in the legend to Fig. 2. ++, indicates the presence of a strong cross-linked band, +, indicates the presence of a weaker cross-linked band, –, indicates the presence of a faint cross-linked band and –, indicates no detectable cross-linking. n.d., cross-linking could not be performed because of the inability of this strain to grow at neutral or acidic pH.

|        | a  | c  |
|--------|----|----|
|        | Ile-140 | Leu-141 | Ile-142 | Phe-143 | Ser-144 | Glu-145 | Val-146 | Leu-147 | Gly-148 | Leu-149 | Cys-less |
| Ser-728| –  | –    | ++   | –      | –      | +      | –      | –       | –      | –       | –       | –        |
| Ala-731| –  | –    | +    | ++     | –      | ++     | –      | –       | –      | –       | –       | –        |
| Ala-738| –  | –    | ++   | +      | –      | ++     | –      | –       | –      | –       | –       | –        |
| Leu-739| –  | –    | +    | –      | –      | n.d.   | +      | +       | –      | –       | –       | –        |
cally oriented hemi-channel of subunit a. Following rotation of the protonated carboxyl groups through the lipid bilayer, release of protons from the c subunit carboxyls into the luminal orientation of the hemi-channel is activated through interaction with the positively charged arginine residue of subunit a. For the V-ATPases, the critical arginine residue thought to activate release of protons from the proteolipid subunits is Arg-735 in TM7 (19). The buried charged residues Glu-789, His-743, and Arg-799 in TM7 and TM9 are postulated to contribute to the proton access channels in the V-ATPase subunit a (19, 70, 71).

To further test whether this model of proton translocation applies to the V-ATPases, we wished to determine whether the a subunit helix containing Arg-735 (TM7) is in close enough proximity to Glu-145 in TM4 of subunit c to undergo disulfide-mediated cross-linking. The identification of five strongly cross-linked species and thirteen weaker cross-linked bands using pairs of single cysteine-containing mutants of subunits a and c clearly indicate that TM7 of subunit a and TM4 of subunit c are in proximity in V0. This is the first evidence concerning the arrangement of helices in the V0 domain and suggests that these two segments make contact during rotational catalysis by the V-ATPase.

The next question concerns the helical faces of these subunits that are in contact. The results of our cross-linking studies (summarized in Fig. 3) are not really consistent with a unique orientation of these two helical segments with respect to each other. Thus, for example, although the A731C mutant of subunit a forms a strong cross-linked product with E145C of subunit c, a weaker cross-linked band is also observed for L147C of subunit c, which is predicted to be on the opposite helical face of TM4 of c. Similar results were obtained for a subunit cysteine residues at positions Ala-738 and Leu-739. Only for the a subunit mutant S728C did the two cross-linking sites observed at Ile-142 and Val-146 cluster together closely on the same helical face of TM4 of subunit c. Nevertheless, virtually all of the cross-links observed between the Arg-735-containing face of subunit a and TM4 of subunit c occur on the helical face of subunit c centered on Val-146 and bordered by Glu-145 and Leu-147 (Fig. 3). This includes all of the strong cross-linked products as well as almost all of the weaker ones. These results suggest that it is a helical face of TM4 of subunit c that is primarily oriented toward the Arg-735-containing face of TM7 of subunit a. It is interesting to note that mutation of residues corresponding to Phe-143 and Tyr-150 in the Neurospora c subunit confer resistance to the specific inhibitor bafilomycin (75). Because these residues are predicted to lie near the interface of the a subunit and the proteolipid ring (Fig. 4), bafilomycin may act to interfere with the rotation of the ring past subunit a, as previously suggested (75).

There are several possible explanations for the radial diversity of cross-linking observed between these two subunits. One possible explanation is that, during the course of rotational catalysis, the orientation of these two helical segments with respect to one another changes. Even if these helices are held rigidly within each subunit, as the subunits move past one another the relative proximity of cGlu-145 and cLeu-147 relative to aA738 will change. Another possible explanation is that either TM7 of subunit a or TM4 of subunit c or both rotate with respect to the other helices in their respective subunits. In fact, such rotation of the corresponding helices in both subunits a and c of Fv has been proposed (65, 76, 77) and is supported by both disulfide mediated cross-linking (65) and the NMR structure of subunit c (77). Thus, TM2 of subunit c changes in orientation with respect to TM1 following protonation or deprotonation of Asp-61 (77). This rotation of helices in subunits a and c has been proposed to be integral to the mechanism by which proton translocation through Fv occurs (76, 77). Analysis of the orientation of TM4 of the Nephrops subunit c by intramolecular cross-linking has led to a model in which the helical face containing the residue corresponding to Val-146 is oriented toward the interior of the proteolipid ring (64). Helical rotation in the proteolipid subunits may thus occur for the V-ATPases as well.

It should be noted that there is also considerable variation in the depth in the membrane of the observed cross-links between subunits a and c (Fig. 4). Thus, although strongly cross-linked pairs aSer-728/cIle-142 and aAla-731/cGlu-145 are at nearly the same depth in the membrane, other pairs (such as aAla-738/cPhe-143) are at fairly different depths. This may reflect some movement of either TM4 of subunit c or TM7 of subunit a perpendicular to the membrane. This variation in depth of cross-linked residues was also observed for subunits a and c of the F-ATPase (65). The possible importance of such movement for proton translocation is not known.

While most of the double mutant strains tested showed wild-type growth at pH 7.5, suggesting substantial V-ATPase activity (>20%) of the resultant complexes (59, 60), a number showed partial or complete growth defects, including some
which resulted in strong cross-linking (such as aA731C/c’E145C and aL739Cl/c’L147C). This was also observed for cross-linking of the F-ATPase a and c subunits, where three of the six double mutants showing the strongest cross-linking also displayed no growth, indicating a completely inactive complex. Because many of these mutations are likely being introduced at a critical interface necessary for rotational catalysis, it is not surprising that mutations at this interface may, in many cases, perturb activity. Nevertheless, the demonstration that the mutant strains tested do show normal assembly of the V_0 domain (as assayed by Western blot for subunits a and c’) indicates that useful structural information can be derived from analysis of the cross-linking patterns observed.

In summary, disulfide-mediated cross-linking of subunits a and c’ of the yeast V-ATPase indicate that TM7 of subunit a (which contains an arginine residue essential for activity) and TM4 of subunit c’ (which contains an essential glutamic acid residue) are in close proximity in the V_0 structure. The helical face of TM7 of subunit a containing the critical arginine residue makes contact with the helical face of TM4 of subunit c’ centered on Val-146 and bounded by Glu-145 and Leu-147, although rotation of one or both of these helices may account for the diversity of sites that can be cross-linked between these subunits.

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