TM2 but not TM4 of Subunit c” Interacts with TM7 of Subunit a of the Yeast V-ATPase as Defined by Disulfide-mediated Cross-linking

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Running title: Disulfide-mediated cross-linking of V-ATPase subunits a and c”
Abstract

The vacuolar (H\(^{+}\))-ATPase (or V-ATPase) is an ATP-dependent proton pump which couples the energy released upon ATP hydrolysis to rotational movement of a ring of proteolipid subunits (c, c’ and c’’ ) relative to the integral subunit a. The proteolipid subunits each contain a single buried acidic residue that is essential for proton transport, with this residue located in TM4 of subunits c and c’ and TM2 of subunit c’’. Subunit c’’ contains an additional buried acidic residue in TM4 that is not required for proton transport. The buried acidic residues of the proteolipid subunits are believed to interact with an essential arginine residue (Arg735) in TM7 of subunit a during proton translocation. We have previously shown that the helical face of TM7 of subunit a containing Arg735 interacts with the helical face of TM4 of subunit c’ bordered by Glu145 and Leu147 (Kawasaki-Nishi, et al. (2003) J.Biol.Chem. 278, 41908-41913). We have now analyzed interaction of subunits a and c’’ using disulfide-mediated cross-linking. The results indicate that the helical face of TM7 of subunit a containing Arg735 interacts with the helical face of TM2 of subunit c’’ centered on Ile105, with the essential glutamic acid residue (Glu108) located near the opposite border of this face compared to TM4 of subunit c’. By contrast, TM4 of subunit c’’ does not form strong cross-links with TM7 of subunit a, suggesting that these transmembrane segments are not normally in close proximity. These results are discussed in terms of a model involving rotation of interacting helices in subunit a and the proteolipid subunits relative to each other.
The vacuolar (H\textsuperscript{+})-ATPases (or V-ATPases\textsuperscript{1}) are a family of ATP-dependent proton pumps that function in both intracellular compartments and the plasma membrane (1-8). Within intracellular compartments such as lysosomes, endosomes, the Golgi and secretory vesicles, V-ATPases function in a variety of processes, including protein degradation, receptor-mediated endocytosis, viral entry, intracellular membrane traffic, protein processing and neurotransmitter uptake (1). Plasma membrane V-ATPases have been shown to function in acid secretion in the kidney, bone degradation by osteoclasts, pH homeostasis in macrophages and neutrophils, sperm maturation in the vas deferens, K\textsuperscript{+} transport by insect goblet cells and invasion by tumor cells (9-13).

Subunits of the V-ATPase are organized into a peripheral domain (V\textsubscript{1}) responsible for ATP hydrolysis and an integral domain (V\textsubscript{0}) that carries out proton translocation (1-8). ATP hydrolysis in the V\textsubscript{1} domain occurs at nucleotide binding sites located at the interface of the A and B subunits (14,15), which form the hexameric structure observed in electron microscopic images of the V-ATPase (16). ATP hydrolysis has been shown to drive rotation of a central stalk, composed of the D and F subunits (17,18), which in turn drives rotation of a ring of proteolipid subunits (c, c’ and c”) relative to subunit a (19,20). Subunit a is an integral membrane protein possessing an amino-terminal hydrophilic domain located on the cytoplasmic side of the membrane and a hydrophobic carboxyl-terminal domain containing nine transmembrane segments (21). Subunit a is held fixed relative to the hydrolytic head of V\textsubscript{1} by a peripheral stalk composed of subunits C, E, G, H and the hydrophilic domain of subunit a (18,22,23).

The membrane integral domain of subunit a contains a number of buried charged residues, including Glu789, His743 and Arg799, whose mutation results in partial
inhibition of proton transport (24-26). The only a subunit residue absolutely required for proton translocation is Arg735 located in TM7 (26). Even conservative replacement of this residue with lysine results in complete loss of proton transport (26). Arg735 has been postulated to function in displacement of protons bound to buried acidic residues on the ring of proteolipid subunits, analogous to the function of Arg210 in proton transport by the F-ATPases (27-29).

The V-ATPases contain three different proteolipid subunits (c, c’ and c”) which are present in a stoichiometry of one copy each of c’ and c” and 4-5 copies of c (30,31). Subunits c and c’ are composed of four transmembrane segments (32,33) whereas subunit c” contains four or five transmembrane segments (34,35). Each proteolipid subunit contains a single glutamic acid residue buried in the middle of one of these segments that is essential for proton transport (33). For subunits c and c’, the essential glutamic acid residue is present in the last transmembrane segment, whereas for subunit c” the essential glutamic acid residue is present near the middle of the molecule (32-35). It is these essential glutamate residues that are believed to undergo reversible protonation and deprotonation during rotary catalysis and that are thought to interact with Arg735 of subunit a to activate proton release. It is therefore important to define the helical interactions that occur between the proteolipid subunits and subunit a within the V₀ domain.

We have previously demonstrated by cysteine-mediated cross-linking that TM7 of subunit a is in close proximity to TM4 of subunit c’ and have identified the helical faces of these subunits that interact (36). In the present study we have extended this analysis to the interaction of subunit a and subunit c”, whose topology and location of essential
residues make it unique among the proteolipid subunits of both the V and F-ATPases.
Experimental Procedures

*Materials and Strains*—Zymolyase 100T was obtained from Seikagaku America, Inc. Protease inhibitors and the monoclonal antibody 3F10 (directed against the HA antigen) that is conjugated with horseradish peroxidase were from Roche Molecular Biochemicals. The monoclonal antibody 10D7 against the yeast 100 kDa a subunit Vph1p (37) was 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 Fisher and New England Biolabs. Phenylmethylsulfonyl fluoride and most other chemicals were purchased from Sigma Chemical Co. Yeast strains lacking the *VMA16*, *VPH1*, and *STV1* genes were constructed by replacing the entire coding region of *VMA16* with the *TRP* gene and insertion of the *LEU* gene into the *VPH1* gene and the *LYS* gene *STV1* gene at the positions indicated by Manolson et al. (38) using the YPH500 strain (*MAT alpha ade2, ura3, leu2, his3, trp1, lys2*). The proteolipid Vma16p was tagged at the carboxyl terminus with the 9-amino acid epitope (YPYDVPDYA) from influenza hemagglutinin (HA) as described previously (34). YEPD buffered to pH 5.5 or pH 7.5 was used for selection of strains showing a vma− phenotype.

*Transformation and Selection*—Site directed mutants of Vph1p and Vma16p were constructed using the Altered Sites II *in vitro* mutagenesis system (Promega) and the presence of the mutations was verified by sequencing the entire length of subcloned DNA. Plasmids carrying mutations of a and c′′ are co-transformed into yeast cells lacking functional endogenous Vph1p, Stv1p, and Vma16p by the lithium acetate method (39). The transformants were selected on histidine minus and uracil minus plates and
growth phenotypes of the mutants were assessed on YEPD plates buffered with 50 mM 
KH$_2$PO$_4$ or 50 mM succinic acid to either pH 7.5 or pH 5.5.

*Analysis of Subunit Expression and V-ATPase Assembly*—Yeast vacuolar membranes 
and whole cell lysates were prepared using the protocol described previously (40,41). 
Whole cell lysates and vacuolar membrane enriched fractions were separated by SDS-
PAGE on 4-15% gradient acrylamide gels. The presence of Vma16p on vacuolar 
membranes was detected by Western blotting using a horseradish peroxidase-conjugated 
monoclonal antibody 3F10 against HA, while Vph1p was detected using the monoclonal 
antibody 10D7 (Molecular Probes, OR), followed by a horseradish peroxidase-conjugated secondary antibody (Bio-Rad) (36). Blots were developed using a 
chemiluminescence detection method obtained from Kirkegaard & Perry Laboratories 
(Gaithersburg, MD).

*Cross-linking of Subunits a and c’’ by Cu(1,10-phenanthroline)$_2$SO$_4$ (CuP).*—Cross-
linking between cysteine residues introduced into subunits a and c’’ was performed using 
the protocol described previously by Jiang and Fillingame (42). Vacuolar membrane 
vesicles (approximately 50µg of protein) were washed in labeling buffer (5 mM Tris-
Mes, pH 7.5, 0.25 mM MgCl$_2$ and 1.1 M glycerol). 2.5 mM Cu(1,10-
phenanthroline)$_2$SO$_4$ was added and samples were incubated for 60 min at room 
temperature to catalyze disulfide bond formation. The reaction was terminated by 
addition of EDTA and N-ethylmaleimide to a final concentration of 15 mM and 20 mM, 
respectively. Samples were then subjected to SDS-PAGE on 4-15% acrylamide gels and 
transferred to nitrocellulose membranes. The blots were probed with horseradish
peroxidase-conjugated monoclonal antibody 3F10 against the HA epitope tag (36). Blots were developed using the Supersignal ULTRA chemiluminescent system (Pierce).

**Other Procedures**-- ATPase activity was measured using a coupled spectrophotometric assay and ATP-dependent proton transport was measured by fluorescence quenching using the fluorescence probe ACMA as described previously (43). All assays were carried out in the presence or absence of 1 μM concanamycin A, a specific inhibitor of the V-ATPase (44). SDS-polyacrylamide gel electrophoresis was carried out as described by Laemmli (45).
Results

Growth Phenotype of Yeast Strains Expressing Single Cysteine-containing Mutant Forms of Vma16p and Vph1p – To study the interaction between subunits a and c”, a yeast strain disrupted in the two genes that encode subunit a (VPH1 and STV1) as well as the gene encoding subunit c” (VMA16) was constructed as follows. Using the parental strain YPH500, the VMA16 gene was replaced with the TRP gene, and the LEU and LYS genes were inserted into the VPH1 and STV1 genes, respectively, as previously described (38,46). The VMA16 gene was expressed in this strain using the pRS413 plasmid containing the HIS marker whereas the VPH1 gene was expressed using the pRS316 plasmid containing the URA marker. A Cys-less form of Vph1p has previously been shown to support wild type growth at pH 7.5 (21), suggesting it forms a V-ATPase complex possessing at least 20% of wild type levels of activity in vitro (47,15). Cells co-expressing Cys-less forms of both Vph1p and Vma16p also displayed wild type growth at pH 7.5, indicating that none of the endogenous cysteine residues in either protein are essential for activity.

TM7 of Vph1p contains a buried arginine residue (Arg-735) that is absolutely required for proton transport by the V-ATPase and that has been proposed to interact with the buried carboxyl groups on the proteolipid subunits (26). Unique cysteine residues were therefore introduced at nine different positions along TM7 of Vph1p, including sites predicted to reside on the same helical face as Arg-735 (Ser-728, Ala-731, Ser-732, Ala-738, Leu-739 and Ala-742), sites adjoining this helical face (Leu734 and Leu736) and sites on the opposite helical face (Tyr-733). Unique cysteine residues were also introduced into the two transmembrane helices of Vma16p that contain buried acidic
residues. Assuming a four transmembrane segment model of Vma16p (34), these correspond to TM2 and TM4 (see Discussion). In TM2 of Vma16p, which contains the essential glutamic acid residue Glu-108, cysteine residues were introduced at Ser-103, Ile-104, Ile-105, Phe-106, Ser-107, Glu-108, Val-109, Val-110, Ala-111 and Ile-112. These residues correspond to approximately the central half of TM2 of Vma16p and represent all of the helical faces of this segment (33). In TM4 of Vma16p, which contains a buried glutamic acid reside (Glu-188) that is not essential for proton transport (33), cysteine residues were introduced at Ile-184, Leu-185, Val-186, Ile-187, Glu-188, Ile-189, Phe-190, Gly-191, Ser-192 and Ile-193. The yeast strain disrupted in VMA16, VPH1 and STV1 was then transformed with plasmids bearing the Cys-less form or one of the single cysteine-containing mutants of Vma16p as well as one of the single cysteine-containing mutants of Vph1p. The growth phenotype of the resultant 189 double-replacement strains at pH 7.5 is shown in Table I. As expected from previous results, strains bearing mutations at Glu-108 of Vma16p showed no growth at neutral pH whereas most strains expressing the E188C mutant of Vma16p displayed a wild type growth phenotype (33). For mutations in the a subunit, the only mutation to seriously compromise growth at pH 7.5 was Y733C. The majority of the 189 double mutants tested showed normal (or near normal) growth at neutral pH, suggesting that the resultant V-ATPase complexes possessed significant activity in vivo.

Assembly of V0 Complexes Containing Cysteine Mutations in Subunits a and c” – To assess the effects of the mutations in Vma16p and Vph1p on assembly of the V0 domain, partially purified vacuolar membranes were subjected to SDS-PAGE and Western blot analysis was performed using antibodies against both Vph1p and the HA-tagged
Vma16p. As can be seen in Fig.1, vacuolar membranes from all of the double replacement strains tested showed wild type levels of both Vph1p and Vma16p, suggesting normal assembly and targeting of the V₀ domain (37).

While proton transport and ATPase activities have not been measured for vacuoles from the 189 double mutant strains analyzed, it was felt that useful information about the arrangement of V₀ subunits could be obtained even from complexes lacking substantial activity, as for those displaying a vma- phenotype (Table I). This is similar to previous studies of contacts between subunits α and c’ of the V-ATPase (36) and subunits α and c of the F-ATPase (42), where many of the strongest cross-links were observed for complexes lacking activity as assessed by their growth phenotype.

ATPase and Proton Transport Activity of E108Q and E188Q Mutants of Subunit c” - It had previously been reported that the mutations E108Q in TM2 but not E188Q in TM4 of subunit c” led to a vma- phenotype (33). In addition, the E108Q mutation resulted in loss of 99% of wild type ATPase activity in isolated vacuolar membranes and qualitative loss of vacuolar acidification as assessed by quinacrine staining of vacuoles in vivo (33). By contrast, the effect of the E188Q mutation on ATPase activity was not determined and the effect of this mutation on proton transport was not quantitated. We therefore measured both proton transport and concanamycin-sensitive ATPase activity in vacuolar membranes isolated from the wild type strain and strains expressing the E108Q or E188Q mutations in subunit c” as described under Experimental Procedures. In agreement with the previous findings (33), vacuolar membranes from the strain expressing the E108Q mutant of subunit c” possessed no detectable ATP-dependent proton transport or concanamycin-sensitive ATPase activity. Interestingly, vacuolar membranes from the
strain expressing the E188Q mutant had 87 \pm 7\% of the wild type ATPase activity but only 64 \pm 4\% of wild type proton transport activity. These results suggest a partial uncoupling of the E188Q mutant and a possible function of this residue in proton transport.

**Cysteine-mediated Cross-linking of Subunits a and c” Using Cupric-phenathroline** – To determine the proximity of cysteine residues introduced into TM7 of subunit a to cysteine residues introduced into TM2 and TM4 of subunit c”, vacuolar membranes were isolated from each of the strains expressing single cysteine-containing forms of Vph1p and Vma16p followed by cross-linking using cupric-phenathroline, as described under Experimental Procedures. Samples were separated by SDS-PAGE and Western blotting was performed using the horseradish peroxidase-conjugated monoclonal antibody 3F10 directed against the HA epitope tag introduced at the carboxyl-terminus of Vma16p. Cross-linking was tested for all 189 strains expressing single cysteine-containing forms of Vph1p and Vma16p, but results are shown in Fig. 2 for only those mutants in which cross-linked products were detected. As can be seen, strong cross-linked products of molecular mass approximately 125 kDa (corresponding to a heterodimer of subunits a and c”) were observed for the pairs aS728C/c”E108C, aA731C/c”I112C and aA738C/c”I105C. Bands of intermediate intensity were observed for the pairs aS728C/c”F106C and aS728C/c”V109C and very faint cross-linked products were observed for the pairs aS728C/c”I104C, aS732C/c”V109C and aA742C/c”I112C. All of these products involved cross-linking between TM7 of subunit a and TM2 of subunit c”. Of the ninety strains expressing single cysteine mutations in TM4 of subunit c”, only a single very faint cross-linked product was observed for the pair aS728C/c”I189C (Fig.2).
These results are summarized in Table II and the strong and intermediate cross-linked products are depicted in Fig.3. The left panel of Fig.3 is based on a four transmembrane helix model of subunit c” (34), which places the N-terminus of the helix containing Glu108 on the lumenal side of the membrane. The right panel of Fig.3 is based on a five transmembrane helix model (35) which places the N-terminus of this helix on the cytoplasmic side of the membrane (see Discussion).

It should be noted that in all single cysteine-containing mutants of subunit c” (but not in the Cys-less form), a strongly reactive band of molecular mass approximately 40 kDa was observed (Fig.2). Because subunits c and c’ contain their endogenous cysteine residues in these strains (six in subunit c and five in subunit c’), and because this 40 kDa species was abolished following reduction of samples with 20% 2-mercaptoethanol (data not shown), it is likely that this 40 kDa species corresponds to a cross-linked heterodimer of either subunits c and c” or subunits c’ and c”.
Discussion

Subunit c” has a number of properties that make it unique among the proteolipid subunits of the V and F-ATPases (33-35). First, it contains an amino-terminal hydrophobic region that was originally postulated to correspond to the first of five transmembrane segments, giving it one more than subunits c and c’ (33). Initial topological studies suggested that both the N and C-terminus of subunit c” are exposed on the cytoplasmic side of the membrane, and that the N-terminal hydrophobic region is dispensable for function (34). Thus, subunit c”, as for the other two proteolipid subunits of the V-ATPase, would contain four transmembrane segments instead of five. The orientation of these segments relative to the cytosolic and lumenal sides of the membrane in this model would be opposite to that of subunits c and c’ (34). Recent studies using protease sensitivity of epitope tags introduced at the N and C-termini of subunit c”, however, suggest that the N and C-termini are on opposite sides of the membrane, consistent with a five transmembrane helix model (35).

Subunit c” is also unique in the location of the essential buried acidic residue (33). Unlike subunits c and c’, which contain an essential glutamic acid residue in the last transmembrane segment, the essential glutamate in subunit c” is located in TM2 (Glu108) (assuming a four TM model) or TM3 (assuming a 5 TM model). A glutamic acid residue present in the last transmembrane segment of subunit c” (Glu188) has been shown not to be required for function (33). Although the stoichiometry of subunits in V₀ is a₁d₁c₄₅c’₁c”₁ (30,31), the arrangement of these subunits has not been defined.

Because proton translocation through the V₀ domain is thought to occur at the interface of the a subunit and the ring of proteolipid subunits, it is important to define the
helical interactions which occur at this interface. We have previously demonstrated that TM7 of subunit a containing the essential arginine residue Arg735 is in close proximity to TM4 of subunit c' containing the critical glutamic acid residue Glu145 (36). Moreover, these two helices do not appear to adopt a single relative orientation, but rather to expose somewhat different helical faces to each other. Thus, strong cross-linked products were observed for both aL739C/c’L147C and aA731C/c’E145C (36) (see Fig.4), which requires an approximately 90° rotation of TM7 of subunit a and a greater than 180° rotation of TM4 of subunit c’. These results suggest a rotation of these helices relative to one another and are consistent with evidence for helical rotation within F₀ (29,48). Recent studies characterizing the bafilomycin and concanamycin binding sites on the V-ATPase indicate that these inhibitors bind at the interface of helices of the c subunit and may block activity by preventing helical rotation within or between c subunits (49,50).

Because of the unique properties of subunit c”, we wished to determine whether the transmembrane helices of this subunit adopt a similar relative orientation to TM7 of subunit a. In particular, we wished to compare the helical interactions of the two transmembrane segments of subunit c” that contain buried glutamic acid residues (TM2 and TM4 in the four TM model). Fig.4 compares the helical surfaces that can be cross-linked to subunit a for both subunits c’ and c” of the V-ATPases and subunit c of the F-ATPases, assuming the four TM model of subunit c”. As can be seen, TM2 of subunit c” is capable of forming a number of strong and intermediate cross-links with TM7 of subunit a, suggesting that these two helices are in close proximity. Moreover, the radial distribution of cross-links suggests rotation of these helices relative to one another. It
should be noted that a number of a subunit residues were included in this analysis that are predicted to be on the sides (Leu734, Leu736) or back (Tyr733) of TM7 relative to Arg735, and that no cross-linking to subunit c” was observed at any of these sites. We have also analyzed five additional a subunit positions for cross-linking to subunit c’, including His729, Thr730, Ser732, Leu734 and Leu736 (Fig.4), and found no cross-linking of cysteines at these positions to any of the ten cysteine residues introduced into subunit c’ (data not shown).

Helical rotation is postulated to bring the arginine residue in subunit a into close proximity to the acidic residue on the proteolipid subunit to facilitate proton release (29,48). As can be seen from Fig.4, the minimal movement of TM2 of subunit c” required to bring Glu108 into close proximity to Arg735 involves a counter-clockwise rotation, whereas for TM4 of subunit c’ and TM2 of the F-ATPase subunit c, a clockwise rotation is required. It should be noted, however, that for the five TM model of subunit c”, the helical rotation required to bring Glu108 into contact with Arg735 would be clockwise as predicted for the other proteolipids. Moreover, the orientation of the transmembrane segment containing Glu108 relative to subunit a would be very similar to that of TM4 of subunit c’ (compare Fig.3, right panel and Fig.4). It is thus easier to explain the cross-linking results in the present study based upon a model of subunit c” containing five rather than four transmembrane segments.

By contrast with TM2, only one very weak cross-link was observed between TM4 of subunit c” and TM7 of subunit a. Although such a negative result is not conclusive, it suggests that these two transmembrane segments are not typically in close proximity. From the crystal structure of the partial F-ATPase complex containing a ring of 10
protolipid subunits (51), it might be predicted that TM2 and TM4 of the V-ATPase proteolipids would occupy equivalent positions relative to subunit a. The cross-linking results obtained, however, suggest that for subunit c” the preferred point of contact with subunit a is TM2 rather than TM4. If the transmembrane segments of subunits c and c’ containing the essential glutamate residues are likewise preferred points of contact with subunit a (as suggested by previous results (36)), the position in the proteolipid ring occupied by subunit c” may form a gap in which these preferred contacts are not present on every other helical segment (see Fig.5, top panel). This assumes that the arrangement of the four transmembrane helices in the three proteolipid subunits is the same. Such a gap might constitute an energy barrier to rotation that prevents passive proton translocation through V0. In fact, the isolated V0 domain, unlike the F0, domain, has been shown to not normally catalyze passive proton translocation (52). On the other hand, if the arrangement of the four transmembrane helices is different for subunit c” relative to subunits c and c’ (Fig.5, bottom panel), no gap in the proteolipid ring would occur at the position of subunit c”. For a 5 TM model of subunit c”, the first transmembrane segment could be accommodated within the proteolipid ring (see box in Fig.5). As noted above, however, rotation of the TM containing the critical glutamic acid residue in subunit c” would occur in the opposite direction to that depicted in Fig.5.

Based upon lipid accessibility and intra-subunit cross-linking of the V-ATPase c subunit, Harrison, et al. (53,54) have proposed a significantly different model for the arrangement of transmembrane segments in the proteolipid ring as compared to the structure observed for F0 (51). In the Harrison model, TM4 is present at the outer surface of the proteolipid ring, TM2 and TM3 are present closer to the center of the ring and
TM1 forms the inner most boundary of the ring. Our data are not consistent with subunit c” adopting such a helical arrangement, since in that case TM4 rather than TM2 would show strong cross-linking to subunit a. It is possible, however, to arrange the transmembrane segments in such a way as to place TM2 of subunit c” on the outer most face of the proteolipid ring and TM3 on the inner most surface. While this would require a long cytoplasmic loop spanning TM2 and TM3, this is consistent with the available sequence data, in which TM2 and TM3 are connected by a 23 amino acid hydrophilic bridge (33,34).

The major cross-linked species observed between subunits a and c’ or c” have been arranged in Fig.6 to suggest a possible sequence of states the enzyme may occupy during rotational catalysis. Beginning with the upper left hand panel, Glu145 of subunit c’ is facing away from TM7 of subunit a and Arg735 is above the point of contact with TM4 of subunit c’. In going to state 2 (the second panel from the top), TM4 of c’ is postulated to undergo clockwise rotation of 120°, bringing this residue into closer proximity to TM7 of subunit a. From state 2 to state 3, TM4 of subunit c’ continues its clockwise rotation while TM7 of subunit a also undergoes clockwise rotation of 60°. These two events bring Glu145 and Arg735 into close proximity and thus cause Glu145 to lose a proton, which exits the membrane via a lumenal access channel in subunit a. From state 3 to state 4 and 5, the ring of proteolipid subunits undergoes rotation, replacing one proteolipid subunit with another having the same helical orientation as in state 1. In states 4 and 5, however, Arg735 is below the point of contact with TM4 of the proteolipid subunit, and must undergo counterclockwise rotation of 90° to “reset” to state 1. Not shown is the resetting of TM4 of the original proteolipid subunit, which requires
reprotonation from a cytoplasmically oriented hemichannel in subunit a. The transmembrane segment of subunit c” containing Glu108 is proposed to undergo a similar set of rotational states, involving either an initial counterclockwise rotation for the four TM model (Fig.6, right panels), or an initial clockwise rotation for the five TM model (not shown). It should be noted that the different cross-linked products observed may not correspond to specific sub-states in the proton translocation pathway but rather to the possible helical orientations that can be accessed through thermal movements. It will be of interest to determine whether inhibitors such as bafilomycin, which are proposed to block activity by inhibiting helical rotation in the proteolipid subunits (49,50), effect the pattern of cross-linking between these subunits and subunit a.

The mechanism depicted is similar to, although less detailed than, models proposed for proton transport through F0 (27,29,48,55). Although much less is know concerning the a subunit residues lining the aqueous access channels of the V-ATPase than of the F-ATPase (55), it is interesting to note that His729 and His743 of Vph1p are present on TM7 of subunit a above and below Arg735. Both of these residues (whose mutation leads to partial inhibition of proton transport (24,25)) would be expected to move during rotation of TM7. It is possible that rotation of TM7 may move these histidine residues in such a way that either the lumenal or cytoplasmically oriented hemichannels become open. Additional information concerning the arrangement of transmembrane helices in subunit a will be required to test this model.
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References

1. Nishi, T., and Forgac, M. (2002) Nat. Rev. Mol. Cell Biol. 3, 94-103.
2. Forgac, M. (1999) J. Biol. Chem. 274, 12951-12954.
3. Graham, L. A., Powell, B., and Stevens, T. H. (2000) J. Exp. Biol. 203, 61-70.
4. Kane, P. M., and Parra, K. J. (2000) J. Exp. Biol. 203, 81-87.
5. Bowman, E. J., and Bowman, B. J. (2000) J. Exp. Biol. 203, 97-106.
6. Nelson, N., Perzov, N., Cohen, A., Hagai, K., Padler, V., and Nelson, H. (2000) J. Exp. Biol. 203, 89-95.
7. Futai, M., Oka, T., Sun-Wada, G., Moriyama, Y., Kanazawa, H., and Wada, Y. (2000) J. Exp. Biol. 203, 107-116.
8. Sze, H., Li, X., and Palmgren, M. G. (1999) Plant Cell 11, 677-690.
9. Brown, D., and Breton, S. (2000) J. Exp. Biol. 203, 137-145.
10. Li, Y.-P., Chen, W., Liang, Y., Li, E., and Stashenko, P. (1999) Nat. Genet. 23, 447-451.
11. Nanda, A., Brumell, J. H., Nordström, T., Kjeldsen, L., Sengelov, H., Borregaard, N., Rotstein, O.D., and Grinstein, S. (1996) J. Biol. Chem. 271, 15963-15970.
12. Wieczorek, H., Grüber, G., Harvey, W. R., Huss, M., Merzendorfer, H., and Zeiske, W. (2000) J. Exp. Biol. 203, 127-135.
13. Sennoune SR, Bakunts K, Martinez GM, Chua-Tuan JL, Kebir Y, Attaya MN, Martinez-Zaguilan R. (2004) Am J Physiol 286, C1443-1452
14. Liu, Q., Kane, P.M., Newman, P.R. and Forgac, M. (1996) J. Biol. Chem. 271, 2018-2022.
15. MacLeod, K.J., Vasilyeva, E., Baleja, J.D. and Forgac, M. (1998) *J. Biol. Chem.* **273**, 150-156.

16. Gruber, G., Radermacher, M., Ruiz, T., Godovac-Zimmermann, J., Canas, B., Kleine-Kohlbrecher, D., Huss, M., Harvey, W.R., and Wieczorek, H. (2000) *Biochemistry.* **39**, 8609-8616.

17. Imamura, H., Nakano, M., Noji, H., Muneyuki, E., Ohkuma, S., Yoshida, M. and Yokoyama, K. (2003) *Proc. Natl. Acad. Sci. USA* **100**, 2312-2315.

18. Arata, Y., Baleja, J. D., and Forgac, M. (2002) *Biochemistry* **41**, 11301-11307.

19. Hirata, T., Iwamoto-Kihara, A., Sun-Wada, G. H., Okajima, T., Wada, Y. and Futai M. (2003) *J. Biol. Chem.* **278** 23714-23719.

20. Yokoyama, K., Nakano, M., Imamura, H., Yoshida M. and Tamakoshi, M. (2003) *J. Biol. Chem.* **278**, 24255-24258.

21. Leng, X. H., Nishi, T. and Forgac, M. (1999) *J. Biol. Chem.* **274**, 14655-14661.

22. Arata, Y., Baleja, J. D., and Forgac, M. (2002) *J. Biol. Chem.* **277**, 3357-3363.

23. Landolt-Marticorena, C., Williams, K. M., Correa, J., Chen, W. and Manolson, M. F. (2000) *J. Biol. Chem.* **275**, 15449-15457.

24. Leng, X. H., Manolson, M., Liu, Q. and Forgac, M. (1996) *J. Biol. Chem.* **271**, 22487-22493.

25. Leng, X. H., Manolson, M. and Forgac, M. (1998) *J. Biol. Chem.* **273**, 6717-6723.

26. Kawasaki-Nishi, S., Nishi, T. and Forgac, M. (2001) *Proc. Natl. Acad. Sci. USA* **98**, 12397-12402.

27. Vik, S.B., Long, J.C., Wada, T. and Zhang, D. (2000) *Biochim. Biophys. Acta* **1458**, 457-466.
28. Cain, B. D. (2000) *J. Bioenerg. Biomemb.* **32**, 365-371.

29. Fillingame, R. H., Angevine, C. M. and Dmitriev, O. Y. (2002) *Biochim. Biophys. Acta* **1555**, 29-36.

30. Arai, H., Terres, G., Pink, S. and Forgac, M. (1988) *J. Biol. Chem.* **263**, 8796-8802.

31. Powell, B., Graham, L. A. and Stevens, T. H. (2000) *J. Biol. Chem.* **275**, 23654-23660.

32. Mandel, M., Moriyama, Y., Hulmes, J. D., Pan, Y. C., Nelson, H., and Nelson, N. (1988) *Proc. Natl. Acad. Sci. USA.* **85**, 5521-5524.

33. Hirata, R., Graham, L. A., Takatsuki, A., Stevens, T. H., and Anraku, Y. (1997) *J. Biol. Chem.* **272**, 4795-4803.

34. Nishi, T., Kawasaki-Nishi, S. and Forgac, M. (2003) *J. Biol. Chem.* **278**, 5821-5827.

35. Flannery, A. R., Graham, L. A. and Stevens, T. H. (2004) *J. Biol. Chem.* **279** (in press).

36. Kawasaki-Nishi, S., Nishi, T. and Forgac, M. (2003) *J. Biol. Chem.* **278**, 41908-41913.

37. Kane, P. M., Kuehn, M. C., Howald-Stevenson, I., and Stevens, T. (1992) *J. Biol. Chem.* **267**, 447-454.

38. 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.

39. Gietz, D., St. Jean, A., Woods, R. A., and Schiestl, R. H. (1992) *Nucleic Acids Res.* **20**, 1425.

40. Horazdovsky, B. F. and Emr, S. D. (1993) *J. Biol. Chem.* **268**, 4953-4962.

41. Graham, L. A., Hill, K. J. and Stevens, T. H. (1998) *J. Cell Biol.* **142**, 39-49.

42. Jiang, W. and Fillingame, R. H. (1998) *Proc. Natl. Acad. Sci. USA* **95**, 6607-6612.
43. Feng, Y. & Forgac, M. (1992) *J. Biol. Chem.* **267**, 5817-5822.

44. Drose, S., Bindseil, K. U., Bowman, E. J., Siebers, A., Zeeck, A. & Altendorf, K. (1993) *Biochemistry* **32**, 3902-3906.

45. Laemmli, U. K. (1970) *Nature* **227**, 680-685.

46. Manolson, M. F., Proteau, D., Preston, R. A., Stenbit, A., Roberts, B. T., Hoyt, M., Preuss, D., Mulholland, J., Botstein, D. and Jones, E. W. (1992) *J. Biol. Chem.* **267**, 14294-14303.

47. Liu, J. and Kane, P. M. (1996) *Biochemistry* **35**, 10938-10948.

48. Rastogi, V. K. and Girvin, M. E. (1999) *Nature* **402**, 263-268.

49. Bowman, E. J., Graham, L. A., Stevens, T. H. and Bowman, B. J. (2004) *J. Biol. Chem.* **279** (in press).

50. Bowman, B. J. and Bowman, E. J. (2002) *J. Biol. Chem.* **277**, 3965-3972.

51. Stock, D., Lelie, A. G. and Walker, J. E. (1999) *Science* **286**, 1700-1705.

52. Zhang, J., Myers, M., Forgac, M. (1992) *J. Biol. Chem.* **267** 9773-9778.

53. Harrison MA, Murray J, Powell B, Kim YI, Finbow ME, Findlay JB. (1999) *J Biol Chem.* **274**, 25461-25470.

54. Harrison M, Powell B, Finbow ME, Findlay JB. (2000) *Biochemistry.* **39**, 7531-7537.

55. Angevine, C. M., Herold, K. A. G., and Fillingame, R. H., (2003) *Proc Natl. Acad. Sci. USA.* **100**, 13179-13183.
Footnotes

1. The abbreviations used are: V-ATPase, vacuolar proton-translocating adenosine triphosphatase; F-ATPase, $F_1F_0$ ATP synthase; HA, influenza hemagglutinin; TM, transmembrane segment; ACMA, 9-amino-6-chloro-2-methoxyacridine; YEPD, yeast extract peptone dextrose; PAGE, polyacrylamide gel electrophoresis.
Figure Legends

Figure 1. Assembly of cysteine-substituted mutants of subunits a and c”. To assess the ability of the indicated mutant forms of subunit a (Vph1p) and subunit c” (Vma16p) to assemble into V$_0$ complexes, vacuolar membrane fractions were isolated from each strain, subjected to SDS-PAGE and Western blotting was performed using the monoclonal antibodies 10D7 (against Vph1p) or 3F10 (against the HA tag introduced at the C-terminus of Vma16p) as described under Experimental Procedures.

Figure 2. Formation of a-c” cross-links by cupric phenanthroline treatment of vacuolar membranes isolated from yeast strains expressing mutant forms of subunits a and c”. Vacuolar membranes isolated from strains expressing the indicated mutants of subunits a and c” were cross-linked with 2.5 mM Cu (1,10-phenanthroline)$_2$SO$_4$ and separated by SDS-PAGE on 4-15% acrylamide gels. Western blot analysis was performed using the monoclonal antibody 3F10 against the HA epitope tag present on subunit c” as described under Experimental Procedures. The positions of the a-c” heterodimer and the c” monomer are indicated by the arrowheads.

Figure 3. Location of the major cross-links formed between TM7 of subunit a and TM2 of subunit c”. The strongest cross-linked product is indicated by the thick dashed line while the other strong and intermediate cross-links are shown with thinner dashed lines. The upper panels show helical wheel diagrams of transmembrane segments of Vph1p and Vma16p viewed from the cytoplasmic side of the membrane while the lower panels depict a side view of these helices oriented with the cytoplasmic side of the
membrane at the top. Also shown are the critical Arg735 in TM7 of subunit a and Glu108 in TM2 of subunit c”. The panels on the left assume a four transmembrane segment model of subunit c” (34) whereas the panels on the right assume a five transmembrane segment model, in which Glu108 is present in TM3 of subunit c” (35).

Figure 4. Comparison of the cross-linkable surfaces of subunit a and the proteolipid subunits of the V and F-ATPases. The upper panel shows the results obtained previously by Zhang and Fillingame for the F-ATPase subunits a and c (41), the second panel shows the results obtained by Kawasaki-Nishi, et al. for subunits a and c’ of the V-ATPase (35) and the lower two panels summarize the results reported in the present paper for TM7 of subunit a and TM2 or TM4 of subunit c”, respectively. A four TM model of subunit c” is assumed in the diagram shown. The critical arginine residue on subunit a and the critical acidic residue on the proteolipid subunits are depicted by filled circles. Residues showing strong and intermediate intensity intermolecular cross-linking are depicted in black, those showing weak cross-linking are depicted in dark gray, those showing no cross-linking are depicted in light gray and those not tested are indicated in white. All helices are viewed from the cytoplasmic side of the membrane.

Figure 5 – Possible helical arrangements of subunits in the proteolipid ring of the V-ATPase. The models shown assume the transmembrane segments of subunit c” adopt the same (top panel) or different (bottom panel) arrangement relative to subunits c and c’. A four transmembrane segment model of subunit c” is shown, with the N-terminal hydrophobic region of this subunit (not a TM in this model) shown as a box oriented towards the center of the proteolipid ring. The locations of the critical glutamic acid
residues are shown, assuming that they are buried within a four helix bundle corresponding to each subunit when not in contact with subunit a. The helical surfaces of subunits c’ and c” that can be cross-linked to TM7 of subunit a are shown in shading. Arrows indicate the proposed direction of helix rotation. The arrangement of proteolipid subunits within the ring is not known, but is shown for the purposes of this model with subunits c’ and c” at adjacent positions. All helices are viewed from the cytoplasmic side of the membrane.

Figure 6. Helical wheel diagrams depicting the location of the major cross-linked products obtained between subunit a and either subunit c’ or subunit c”. Major cross-links observed between subunits a and c’ are shown in the left hand panels while those between subunits a and c” are shown on the right. The critical arginine residue in subunit a and the critical glutamic acid residue in subunits c’ or c” are indicated by filled circles. The cross-linked products have been arranged in an order related to a possible mechanism of proton translocation occurring at the interface of subunit a and the proteolipid ring (see text). Helices are viewed from the cytoplasmic side. A four TM model of subunit c” is assumed in this diagram.
Table I. Growth Phenotypes of Cys-substituted mutants at pH 7.5

|       | S728 | A731 | S732 | Y733 | L734 | L736 | A738 | L739 | A742 |
|-------|------|------|------|------|------|------|------|------|------|
| Cys-less | +++  | +++  | ++   | +    | +++  | +++  | +++  | +++  | +++  |
| S103   | +++  | +++  | ++   | +    | +++  | +++  | +++  | ++   | +++  |
| I104   | +++  | +++  | ++   | +    | +++  | +++  | +++  | +++  | +++  |
| I105   | ++   | ++   | +    | +    | +++  | +++  | +++  | +++  | +++  |
| F106   | +++  | +++  | ++   | +    | +++  | +++  | +++  | +++  | +++  |
| S107   | +++  | +++  | ++   | +    | +++  | +++  | +++  | +++  | +++  |
| E108   | -    | -    | -    | -    | -    | -    | -    | -    | -    |
| V109   | +++  | +++  | ++   | +    | +++  | +++  | +++  | +++  | +++  |
| V110   | +++  | +++  | ++   | +    | +++  | +++  | +++  | +++  | +++  |
| A111   | +++  | +++  | ++   | +    | +++  | +++  | +++  | +++  | +++  |
| I112   | +++  | +++  | ++   | +    | +++  | +++  | +++  | +++  | +++  |
| I184   | +++  | +++  | ++   | +    | +++  | +++  | +++  | +++  | +++  |
| L185   | +++  | +++  | ++   | +    | +++  | +++  | +++  | +++  | +++  |
| V186   | +++  | +++  | ++   | +    | +++  | +++  | +++  | +++  | +++  |
| I187   | +++  | +++  | ++   | +    | +++  | +++  | +++  | +++  | +++  |
| E188   | +++  | +++  | ++   | +    | +++  | +++  | +++  | +++  | +++  |
| I189   | +++  | +++  | ++   | +    | +++  | +++  | +++  | +++  | +++  |
| F190   | +++  | ++   | +    | -    | +++  | +++  | +++  | +++  | +++  |
| G191   | +++  | +++  | ++   | +    | +++  | +++  | +++  | +++  | +++  |
| S192   | +++  | +++  | ++   | +    | +++  | +++  | +++  | +++  | +++  |
| I193   | +++  | +++  | ++   | +    | +++  | +++  | +++  | +++  | +++  |

Growth of yeast strains expressing Vph1p (subunit a) and Vma16p (subunit c”) containing single cysteine residues in TM2 or TM4 at the indicated positions was assessed on YEPD plates buffered with 50 mM KH₂PO₄ to pH 7.5. +++ indicates wild-type growth, ++, indicates partially defective growth, +, indicates severely defective growth, and - indicates no growth.
Table II. Summary of disulfide-mediated cross-linking between subunits a and c”.

|      | S728 | A731 | S732 | Y733 | L734 | L736 | A738 | L739 | A742 |
|------|------|------|------|------|------|------|------|------|------|
| Cys-less | -    | -    | -    | -    | -    | -    | -    | -    | -    |
| S103 | -    | -    | -    | -    | -    | -    | -    | -    | -    |
| I104 | ++   | -    | -    | -    | -    | -    | -    | -    | -    |
| I105 | -    | -    | -    | -    | -    | -    | -    | +++  | -    |
| F106 | ++   | -    | -    | -    | -    | -    | -    | -    | -    |
| S107 | -    | -    | -    | -    | -    | -    | -    | -    | -    |
| E108 | +++  | -    | -    | -    | -    | -    | -    | -    | -    |
| V109 | ++   | -    | -    | -    | -    | -    | -    | -    | -    |
| V110 | -    | -    | -    | -    | -    | -    | -    | -    | -    |
| A111 | -    | -    | -    | -    | -    | -    | -    | -    | -    |
| I112 | -    | +++  | -    | -    | -    | -    | -    | -    | +    |
| I184 | -    | -    | -    | -    | -    | -    | -    | -    | -    |
| L185 | -    | -    | -    | -    | -    | -    | -    | -    | -    |
| V186 | -    | -    | -    | -    | -    | -    | -    | -    | -    |
| I187 | -    | -    | -    | -    | -    | -    | -    | -    | -    |
| E188 | -    | -    | -    | -    | -    | -    | -    | -    | -    |
| I189 | +    | -    | -    | -    | -    | -    | -    | -    | -    |
| F190 | -    | -    | -    | -    | -    | -    | -    | -    | -    |
| G191 | -    | -    | -    | -    | -    | -    | -    | -    | -    |
| S192 | -    | -    | -    | -    | -    | -    | -    | -    | -    |
| I193 | -    | -    | -    | -    | -    | -    | -    | -    | -    |

Cupric phenanthroline-mediated cross-linking of cysteine-substituted mutants of subunits a and c” was performed as described under Experimental Procedures. Relative yield of cross-linked products is as follows: ++++, indicates the strongest cross-linked product, ++++, indicates the presence of a strong cross-linked band, ++, indicates the presence of a cross-linked band of intermediate intensity, +, indicates the presence of a faint cross-linked band and -, indicates no detectable cross-linking.
TM2 but not TM4 of subunit c" interacts with TM7 of subunit a of the yeast
V-ATPase as defined by disulfide-mediated cross-linking
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