Cysteine-directed Cross-linking to Subunit B Suggests That Subunit E Forms Part of the Peripheral Stalk of the Vacular H\(^{+}\)-ATPase*

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Yoichiro Arata‡, James D. Balej‡, and Michael Forgac‡¶
From the Departments of Physiology and Biochemistry, Tufts University School of Medicine, Boston, Massachusetts 02111

We have employed a combination of site-directed mutagenesis and covalent cross-linking to identify subunits in close proximity to subunit B in the vacuolar H\(^{+}\)-ATPase (V-ATPase) complex. Unique cysteine residues were introduced into a Cys-less form of subunit B, and the V-ATPase complex in isolated vacuolar membranes from each mutant strain was reacted with the bifunctional, photoactivable maleimide reagent 4-(N-maleimido)benzophenone. Photoactivation resulted in cross-linking of the unique sulfhydryl groups on subunit B with other subunits in the complex. Four of the eight mutants constructed containing a unique cysteine residue at Ala\(^{15}\), Lys\(^{45}\), Glu\(^{494}\), or Thr\(^{501}\) resulted in the formation of cross-linked products, which were recognized by Western blot analysis using antibodies against both subunits B and E. These products had a molecular mass of 84 kDa, consistent with a cross-linked product of subunits B and E. Molecular modeling of subunit B places Ala\(^{15}\) and Lys\(^{45}\) near the top of the V\(_{1}\) structure (i.e. farthest from the membrane), whereas Glu\(^{494}\) and Thr\(^{501}\) are predicted to reside near the bottom of V\(_{1}\), with all four residues predicted to be oriented toward the external surface of the complex. A model incorporating these and previous data is presented in which subunit E exists in an extended conformation on the outer surface of the A\(_{3}\)B\(_{3}\) hexamer that forms the core of the V\(_{1}\) domain. This location for subunit E suggests that this subunit forms part of the peripheral stalk of the V-ATPase that links the V\(_{1}\) and V\(_{0}\) domains.

The vacuolar H\(^{+}\)-ATPases (V-ATPases)\(^1\) are a family of ATP-dependent proton pumps that are responsible for acidification of intracellular compartments in eukaryotic cells. The V-ATPases are present in a variety of intracellular compartments in eukaryotic cells. The V-ATPase complex contains eight different subunits with molecular masses of 70 to 14 kDa and is responsible for ATP hydrolysis. The nucleotide-binding sites are located on two subunits of the V\(_{1}\) domain: the 69-kDa A subunit and the 57-kDa B subunit. The V\(_{0}\) domain is a 260-kDa integral complex composed of five subunits with molecular masses of 100 to 17 kDa and is responsible for proton translocation.

The V-ATPases are structurally and evolutionarily related to the ATP synthases (or F-ATPases) of mitochondria, chloroplasts, and bacteria (12–17). Thus, the nucleotide-binding subunits of the V-ATPase (A and B) are homologous to the corresponding \(\beta\) and \(\alpha\) subunits of F\(_{1}\) (18, 19), and the proteolipid subunits of the two complexes are also homologous (20, 21). The structure of the peripheral F\(_{1}\) domain of the mitochondrial F-ATPase has been determined by x-ray crystallography and shown to consist of a hexamer of alternating \(\alpha\) and \(\beta\) subunits surrounding a central cavity containing the highly \(\alpha\)-helical \(\gamma\) subunit (22–24). F\(_{1}\) is attached to the F\(_{0}\) domain via both a central stalk, which includes both the \(\gamma\) and \(\epsilon\) subunits (24, 25), and a peripheral stalk, composed of the \(\delta\) subunit and the soluble portion of subunit b (26, 27). The F\(_{0}\) domain contains a ring of c subunits with the a and b subunits to one side (14, 28).

The mechanism of proton transport by the V-ATPases has been proposed to be similar to that of the F-ATPases, which are believed to operate by a rotary mechanism (29, 30). In the rotary mechanism, ATP hydrolysis within the \(\alpha_{3}\)\(\beta_{3}\) head of the F\(_{1}\) domain drives rotation of a central \(\gamma\) subunit (31–33), which is tightly linked to a ring of c subunits in the F\(_{0}\) domain. Rotation of the ring of c subunits relative to the a subunit of F\(_{0}\) (which is held fixed relative to the \(\alpha_{3}\)\(\beta_{3}\) head by a peripheral stator) (34–36) in turn leads to unidirectional proton transport. Like the F\(_{1}\) and F\(_{0}\) domains (37), the V\(_{1}\) and V\(_{0}\) domains have been shown to be connected by both a central and a peripheral stalk (38, 39), although the subunit composition of these connections remains uncertain.

Although no subunits in the V-ATPase complex show homology to the \(\gamma\) subunit of F\(_{1}\), two subunits (D and E) are predicted from sequence analysis to have a similarly high \(\alpha\)-helical content (40, 41). Mutations have been identified in subunit D that lead to changes in coupling of proton transport and ATP hydrolysis (42), similar to mutations in the \(\gamma\) subunit of F\(_{1}\) that cause altered coupling efficiency (43). Such mutations do not conclusively show that subunit D is the \(\gamma\) subunit homolog, however, because mutations altering the coupling efficiency of
the F-ATPase have also been identified in the δ subunit of F₁ (44). Moreover, subunit b, which forms part of the peripheral stalk of the F-ATPases (26, 27), has also been shown to be highly α-helical (45, 46). In addition, differential protease sensitivity and release by chaotropic agents have been used in support of a model in which subunit E, rather than subunit D, functions as the central rotator (47).

To begin to address which subunits in the V-ATPase complex form part of the central stalk and which form part of the peripheral stalk, we have employed a combination of site-directed mutagenesis of subunit B to introduce unique cysteine residues and chemical cross-linking using a photoactivatable sulfhydryl reagent. A molecular model for subunit B was constructed based upon sequence homology between the B and α subunits, the available x-ray coordinates of the α subunit (22), and energy minimization. We have identified four cysteine residues predicted to be oriented toward the outer surface of V₁ that give rise to photoactivated cross-linking with subunit E upon covalent modification using a bifunctional maleimide reagent. These results suggest that subunit E is located on the outer surface of the V₁ complex rather than in the interior of the A₃B₃ hexamer and is therefore a likely candidate to function as part of the peripheral stator connecting the V₁ and V₀ domains.

**EXPERIMENTAL PROCEDURES**

**Materials and Strains—**Zymolyase 100 T was obtained from Seikagaku America, Inc. Concanamycin A was purchased from Fluka Chemical Corp. 9-Amino-6-chloro-2-methoxyacridine was obtained from Molecular Probes, Inc. SDS, nitrilotriacetic acid (0.45-μm pore size), Tween 20, horseradish peroxidase-conjugated goat anti-rabbit IgG, and horseradish peroxidase-conjugated goat anti-mouse IgG were from Bio-Rad. 4-(N-Maleimido)benzophenone (MBP) and most common chemical cross-linkers were obtained from Sigma. The chemiluminescence substrate for horseradish peroxidase was from KPL Laboratories.

The yeast VMA2-deleted strain SF383-5AV1 (MATα·A1::LEU2) and pCY41 (VMA2 in pBluescript) are described previously (48, 49) and were kind gifts from Dr. Patricia Kane (State University of New York Upstate Medical University).

**Antibodies—**The anti-Vma2p antibody was purchased from Molecular Probes, Inc. The anti-Vma4p antibody was a generous gift from Dr. Daniel Kionsky (University of Michigan).

**Construction of Mutants—**Site-directed mutants were constructed using the Altered Sites II in vitro mutagenesis system (Promega) following the manufacturer's protocol. The full-length VMA2 gene was cloned into pAlter-1 using SacI and KpnI. The mutant form of VMA2 encoding the Cys-less form of subunit B (C188S) is described previously (50). Site-directed mutants were constructed by the method of Kunkel (51). After electrophoresis, the samples were electrochemically transferred to nitrocellulose membranes for 4 h at 100 V. The blots were then cut into strips and analyzed by Western blotting using a mouse monoclonal antibody against Vma2p (subunit B) and a rabbit polyclonal antibody against Vma4p (subunit E) as previously described (52).

**Modeling of the V-ATPase B Subunit—**A model for the A₃B₃ hexamer of the V₁ domain was created from the x-ray crystal structure of bovine mitochondrial F₁-ATPase (50). Alignment of the amino acid sequences was carried out using the Genetics Computer Group sequence analysis software package, and energy minimization was performed using X-PLOR.

**Other Methods—**ATPase activity was measured using a coupled spectrometric assay as previously described (50). ATP-dependent proton transport was measured by fluorescence quenching using the probe 9-amin-6-chloro-2-methoxyacridine as described previously (50) with a PerkinElmer Life Sciences LS50B spectrofluorometer. Activities were measured in the absence or presence of 1 μM concanamycin A, a specific inhibitor of the V-ATPase (53). Protein concentrations were determined by the method of Bradford (54).

**RESULTS**

**Construction of vma2 Mutants Containing Unique Cysteine Residues—**The approach employed to distinguish between V-ATPase subunits located on the periphery of the V₁ domain and subunits located in the interior of the complex was site-directed introduction of cysteine residues, followed by photoactivated cross-linking. Subunit B was selected for introduction of unique cysteine residues for several reasons. First, we had previously constructed a Cys-less form of subunit B by replacing the single endogenous cysteine residue at position 188 with serine and had shown that the V-ATPase complex containing the C188S form of B subunit had >90% of the wild-type levels of ATPase activity and proton transport (50). Second, a molecular model of subunit B had been constructed based upon sequence homology between subunit B and the α subunit of F₁, the available x-ray coordinates of the bovine mitochondrial F₁, the docking (22), and energy minimization (50). This model had been shown to accurately predict the location of the single cysteine encoded by the non-catalytic nucleotide-binding site on subunit B based upon nucleotide-protectable labeling of unique subunit B cysteine residues by biotin-maleimide (50). Thus, it was felt that this model could be used with some confidence in the selection of sites predicted to be oriented toward the interior or exterior of the complex.

**Location of Subunit E within the V-ATPase Complex**

GACTAGTAGATCCCCGG-3

for subcloning as described below using the primer 5'-H11032 BamI site, was constructed as described previously (50).

**Electrophoresis and Immunoblotting—**For analysis of cross-linked products, proteins (15 μg/lane) were separated by SDS-PAGE using 10% acrylamide gels according to the method of Laemmli (51). After electrophoresis, the samples were electrotransferred to nitrocellulose membranes for 4 h at 100 V. The blots were then cut into strips and analyzed by Western blotting using a mouse monoclonal antibody against Vma2p (subunit B) and a rabbit polyclonal antibody against Vma4p (subunit E) as previously described (52).
The results in decreased levels of V1 subunits present on isolated V-ATPase assembly by loss of any of the V-ATPase subunits vector alone. It has previously been shown that disruption of wild-type of the V-ATPase complex, bly expressed and competent for assembly with the remainder mutant and each of the double mutants containing unique Thr270, Asp199, Glu494, and Thr501 (see Fig. 1) are shown on the right, whereas those predicted to face the interior of the A3B3 hexamer (Asp341 and Lys417) are on the left. In Fig. 1b, the molecule is oriented such that the external surface of V1 is shown on the right, whereas the region oriented toward the interior of the V1 complex is shown in the left. In a, the molecule is oriented such that the external surface is facing out of the figure. Shown in dark shading are the residues selected for mutation to cysteine that are oriented toward the external surface of V1, whereas those shown in light shading are predicted to be facing the interior of V1, Ala15 is not shown because the structure for this portion of the α subunit was not available, although it is likely to reside near the top of the molecule. The remainder of the A3B3 hexamer is not shown for clarity, although the entire hexamer was used in the energy minimization.

Fig. 1 shows the molecular model of subunit B and the location of seven of the eight sites at which unique cysteine residues were introduced by site-directed mutagenesis of the VMA2 gene. The molecule is oriented such that the top is located farthest from the membrane. In Fig. 1a, residues predicted to be located on the outer surface of V1 (including Lys45, Thr270, Asp199, Glu494, and Thr501) are shown on the right, whereas those predicted to be facing the interior of the A3B3 hexamer (Asp414 and Lys417) are on the left. In Fig. 1b, the molecule is rotated 90° such that the residues predicted to be facing the outer surface of V1 are coming out of the figure. Ala15 is not shown because the available x-ray coordinates of the F1 α subunit do not extend to this position; however, it is likely to be located near the top of the molecule.

The mutant VMA2 genes were subcloned into the yeast shuttle vector pRS316 and expressed in a vma2Δ strain in which the VMA2 gene was deleted. Thus, in each double mutant, Cys185 has been converted to a serine residue, and a unique cysteine has been introduced at the indicated position.

**Effect of VMA2 Mutations on V-ATPase Assembly, Activity, and Proton Transport**—To determine the effects of the mutations on the stability of subunit B and the assembly of the V-ATPase complex, Western blot analysis was performed on vacuoles isolated from the vma2Δ strain transformed with the wild-type VMA2 gene, each of the mutant forms, or the pRS316 vector alone. It has previously been shown that disruption of V-ATPase assembly by loss of any of the V-ATPase subunits results in decreased levels of V1 subunits present on isolated vacuolar membranes (55). As shown in Fig. 2, both the C188S mutant and each of the double mutants containing unique cysteine residues showed wild-type levels of subunit B on the vacuolar membrane, indicating that mutant proteins were stably expressed and competent for assembly with the remainder of the V-ATPase complex.

To determine the effects of the subunit B mutations on V-ATPase complex activity, concanamycin A-sensitive proton transport and ATPase activities were measured on isolated vacuoles using 9-amino-6-chloro-2-methoxyacridine quenching and a continuous spectrophotometric assay, respectively. As shown in Fig. 3, vacuoles isolated from the C188S mutant had >90% of the wild-type levels of both proton transport and ATPase activities, consistent with our previous results (50). In addition, each of the double mutants containing unique subunit B cysteine residues had >40% of the wild-type activity. This is consistent with the ability of each of these mutant strains to grow on plates buffered to both pH 5.5 and 7.5 (data not shown), as previously reported for strains expressing wild-type V-ATPase (56).

**Photoactivated Cross-linking of V-ATPase Complexes Containing Subunits Bearing Unique Cysteine Residues**—To determine the V-ATPase subunits in proximity to the cysteine residues introduced into subunit B, photoactivated cross-linking was carried out using the photoreactive sulfhydryl reagent MBP (52, 57). This reagent is capable of reacting with the sulfhydryl groups of cysteine residues via the maleimide moiety and, upon ultraviolet irradiation, generating a reactive species capable of insertion into nearby bonds. The linker arm connecting the two reactive species is ~10 Å in length (57).

To determine whether MBP was capable of cross-linking subunit B to other subunits in the V-ATPase complex as a result of reaction with sulfhydryl groups on other subunits, vacuoles isolated from a strain expressing the C188S mutant of subunit B were reacted with MBP in the dark, followed by irradiation with ultraviolet light. The proteins were then separated by SDS-PAGE on a 10% acrylamide gel and probed by Western blotting with a monoclonal antibody against Vma2p. As shown in Fig. 4a, no higher molecular mass species containing subunit B were observed upon reaction of the V-ATPase containing the C188S mutant with MBP, suggesting that although the V-ATPase likely contains other cysteine residues capable of reacting with MBP, these do not lead to cross-linking of subunit B with other V-ATPase subunits. Thus, any cross-linked products containing subunit B observed for the single cysteine-containing mutants of subunit B should be due to reaction of MBP with the unique subunit B cysteine residues.

To identify subunits in proximity to each of the cysteine residues introduced into subunit B, vacuoles from cells expressing each of the mutant forms of subunit B were reacted with MBP in the dark, followed by irradiation with ultraviolet light. SDS-PAGE, and Western blotting as described above. As shown in Fig. 4b, an additional band with a molecular mass of 84 kDa that was reactive with the antibody against Vma2p was observed in the presence of MBP for the A15C, K45C, E494C, and T501C mutants, but not for the D199C, T270C, D341C, and K417C mutants. This 84-kDa band was observed in the presence, but not the absence, of MBP (Fig. 4b) and was not
observed for samples that had been reacted with MBP, but not irradiated with ultraviolet light (data not shown).

Because subunit E (product of the VMA4 gene) has a molecular mass of 27 kDa in yeast and subunit B has a molecular mass of 57 kDa, the observed molecular mass of 84 kDa for the cross-linked product suggested that this species might correspond to a B-E heterodimer. To test this possibility, Western blotting was carried out on the products of the cross-linking reaction using a polyclonal antibody against subunit E (Vma4p). As shown in Fig. 4b, an 84-kDa species was also observed to react with the anti-Vma4p antibody for the A15C, K45C, E494C, and T501C mutants, but not for the remaining mutants. Formation of this 84-kDa cross-linked product was again dependent upon the presence of MBP (Fig. 4a), was not observed for the C188S mutant (Fig. 4a), and was not observed in the absence of ultraviolet irradiation (data not shown). These results suggest that subunits B and E are in close proximity on the outer surface of the V₁ complex, both near the top of subunit B and in a region closer to the membrane, but not within the A₃B₃ hexamer.

It should be noted that the efficiency of cross-linking varied between the cysteine-containing mutants, with the A15C and K45C mutants showing better cross-linking to subunit E than the E494C and T501C mutants. This may be due to differences in the initial reaction with MBP or in the ability of MBP to cross-link the two subunits. It should also be noted that a number of additional higher molecular mass species capable of reacting with the anti-Vma2p antibody were observed for some of the cysteine mutants (for example, K45C, D341C, K417C, and T501C). These species, however, have not yet been identified using subunit-specific antibodies and were therefore not further characterized.

DISCUSSION

An important issue regarding the V-ATPases is the arrangement of subunits in the V-ATPase complex. Thus, although the mechanism of the V-ATPases has been proposed to
operate by a rotary mechanism similar to that of the F-ATPases (29, 30). In the rotary mechanism, the subunits can be described as belonging to one of two subcomplexes: a rotary subcomplex and a stationary subcomplex. For the *Escherichia coli* F-ATPase, the rotary subcomplex is composed of the γ and ε subunits together with the ring of c subunits in the F₃ domain, whereas the stationary subcomplex includes the α₂β₂ hexamer of F₅ connected to subunit a of F₄₀ by a peripheral stalk that includes the two b subunits and the δ subunit. ATP hydrolysis by the α₂β₂ hexamer drives rotation of the central γ subunit (31–33), which in turn causes the ring of c subunits to rotate relative to subunit a (34–36). The c subunits possess protonatable groups that carry the protons during rotation, whereas subunit a is postulated to provide the hemichannels that allow for access of protons to and from these sites (29, 30). It is rotation of the subunit c ring relative to subunit a that is believed to be the essential step in driving proton transport. The subunits composing the central and peripheral stalks thus serve equally important functions in the coupling of ATP hydrolysis to proton movement.

Previous cross-linking studies have suggested that subunit D may serve as the γ subunit homolog, whereas subunit E was suggested to form part of the peripheral stator (52). This model was based upon the observation that although subunit D shows a quite restricted cross-linking pattern using the bifunctional amino reagent disuccinimidyl glutarate (cross-linking mainly to subunit F), subunit E can be cross-linked to at least four other subunits, including subunits C, G, and H of the V₁ domain and subunit a of the V₀ domain (52). Because subunit a is thought to form part of the stationary complex, this makes it less likely that subunit E is part of the central rotor. Moreover, two of the subunits to which subunit E becomes cross-linked (subunits C and H) can readily be removed from the V-ATPase (58, 59), suggesting that they are likely to occupy a more exposed position in the complex, consistent with a role as part of the peripheral stator.

In further support of the role of subunit D as the γ subunit homolog, mutants of the VMA8 gene encoding subunit D have been isolated that show altered coupling of proton transport and ATP hydrolysis (42). These mutations clustered in two regions of the molecule (between Val⁷¹ and Gly⁴⁰ and between Lys⁸⁰ and Mete²²¹), with synergetic effects observed for mutations in these two regions, suggesting that, like the γ subunit of F₅ (22–24), subunit D may fold back upon itself in a coiled-coil structure (42). Mutations affecting coupling of ATP hydrolysis and proton transport have also been identified at a number of sites within the γ subunit, including Mete²²¹, Asn²⁶⁹, and Thr²⁷³ (43, 60); and several additional regions, including residues 80–90 and 236–246, have been shown to make contact with the conserved DELSEED sequence of the β subunit (22, 25, 31). Because mutations affecting coupling of the F-ATPases have also been identified in the δ subunit, however (44), such mutations do not provide definitive evidence for a functional relationship between the D and γ subunits.

In contrast to the results described above, studies on the V-ATPase from *Manduca sexta* have suggested that subunit E, rather than subunit D, is the γ subunit homolog (47). Thus, treatment of the V₁ domain with trypsin resulted in relatively rapid cleavage of subunit D, suggesting a more accessible location within the complex than would be expected for a γ subunit homolog. In addition, a difference in CuCl₂-induced cross-linking of subunits to subunit E was observed in the presence or absence of CaATP, suggesting a nucleotide-dependent change in subunit contacts of subunit E (47). Conflicting results thus exist concerning the locations of subunits D and E.

In an effort to further define the location of subunits within the V-ATPase complex, unique cysteine residues were introduced into a Cys-less form of subunit B to serve as sites of attachment of the photoactivable cross-linking reagent MBP. Subunit B was selected because previous studies had indicated that its structure is well predicted by a molecular model constructed on the basis of the high resolution crystal structure of F₁, sequence homology between the B and α subunits, and energy minimization (50). Thus, many residues predicted to be located near the nucleotide-binding site on subunit B showed nucleotide-protectable labeling by biotin-maleimide upon mutation to cysteine (50).

The results presented in this study have identified four residues within subunit B that, upon mutation to cysteine, give rise to cross-linking with subunit E using the photoactivable maleimide reagent MBP. Two of these residues are predicted to be near the top of subunit B in a position farthest from the membrane, whereas the other two are predicted to be located near the bottom of subunit B. All four are predicted to be oriented toward the outer surface of the V₁ complex. By contrast, no cross-linking was observed between subunits B and E upon introduction of cysteine residues at sites predicted to be oriented toward the central cavity of the AₐB₃ hexamer. These results suggest that subunit E is located near the periphery of the V₁ complex and are consistent with a role of subunit E in formation of a peripheral stalk connecting V₁ and V₀. Two additional cysteines (at positions 199 and 270), which are also predicted to be located on the periphery of the complex, but closer to the middle of subunit B, did not show the formation of the 84-kDa B-E cross-linked product. Interestingly, a faint band cross-reactive with both the anti-subunit B and E antibodies could be observed at ~110 kDa for the D199C mutant (Fig. 4B). Although this band is higher in molecular mass than predicted for a B-E heterodimer, it is possible that it migrated aberrantly upon SDS-PAGE due to cross-linking of the protein near the middle of subunit B.

One possible explanation for the lack of cross-linking of the remaining cysteine residues is an inability to react with MBP, possibly because of restricted accessibility due to subunit-subunit contacts or to less surface exposure. In fact, the T270C mutant showed no cross-linking of subunit B to any other subunits, consistent with such an explanation. However, the D341C and K417C mutants both showed higher molecular masses (Fig. 4B). Although these bands are higher in molecular mass than predicted for a B-E heterodimer, it is possible that it migrated aberrantly upon SDS-PAGE due to cross-linking of the protein near the middle of subunit B.

Electron micrographs of the V-ATPase from clathrin-coated vesicles show a relatively thin peripheral stalk that runs from near the top of the V₁ complex down toward the V₀ domain, with some space visible between most of the central core and the peripheral stalk (39). In addition, electron micrographs of the V₀ domain suggest a cytoplasmic region (consisting of subunit d and the amino-terminal domain of subunit a) relatively close to the membrane (61). We have incorporated these observations, together with the results from the present study, into the revised structural model shown in Fig. 5.

Several lines of evidence indicate that subunit E is in contact with subunit G (product of the VMA10 gene). First, subunits E and G can be cross-linked in the bovine coated vesicle enzyme using disuccinimidyl glutarate (52). Second, an E-G complex can be isolated from yeast strains disrupted in other VMA genes, including VMA1, VMA2, VMA7, and VMA8 (62, 63).
Finally, subunit E is unstable in a *uma10Δ* strain in which subunit G is absent (62). The amino-terminal half of subunit G has some homology to the soluble domain of the F-ATPase β subunit, at least along one helical face (64). In addition, as with subunit β (65), short deletions in subunit G do not severely disrupt function (66). These results suggest that subunit G may function like subunit β in the V-ATPase complex. One difference between these two subunits is that, unlike subunit β, subunit G has no membrane anchor. It has been proposed that subunit γ, by virtue of having a significant cytoplasmic domain, may serve to anchor the peripheral stalk in the membrane (52, 69). Because subunit E also makes contact with subunit a (52), subunit E may function as a critical bridge in the formation of the peripheral stalk.

Two other subunits (C and H) are also proposed to reside in the peripheral stalk. Cross-linking studies indicate that subunit E is in close proximity to subunit C in the intact V-ATPase complex (52), and subunits E and C have been shown to form a subcomplex by co-immunoprecipitation (68). In *vivo* dissociation of the V-ATPase complex in response to glucose depletion results in the absence of subunit C from both the V₁ and V₀ domains (69). Because of the ease of release of subunit C from the V-ATPase complex (58, 62, 68), subunit E may represent the principal contact of this subunit with the remainder of the complex. It is possible that changes in the interaction between subunits E and C may trigger the dissociation of the V₁ and V₀ domains in *vivo*. With respect to subunit H, we have found that cystine modification of a conserved cysteine residue at the catalytic site on subunit A causes release of subunit H and consequent loss of activity (52). Similarly, V-ATPase complexes catalytic site on subunit A causes release of subunit H and cystine modification of a conserved cysteine residue at the peripheral stalk. Cross-linking studies indicate that subunits E and C may trigger the dissociation of the V₁ and V₀ complex (52), and subunits E and C have been shown to form a subcomplex by co-immunoprecipitation (68).

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