Cysteine-mediated Cross-linking Indicates That Subunit C of the V-ATPase Is in Close Proximity to Subunits E and G of the V₁ Domain and Subunit a of the V₀ Domain*

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The vacuolar (H⁺)-ATPases (V-ATPases) are multisubunit complexes responsible for ATP-dependent proton transport across both intracellular and plasma membranes. The V-ATPases are composed of a peripheral domain (V₁) that hydrolyzes ATP and an integral domain (V₀) that conducts protons. Dissociation of V₁ and V₀ is an important mechanism of controlling V-ATPase activity in vivo. The crystal structure of subunit C of the V-ATPase reveals two globular domains connected by a flexible linker (Drory, O., Frolow, F., and Nelson, N. (2004) EMBO Rep. 5, 1–5). Subunit C is unique in being released from both V₁ and V₀ upon in vivo dissociation. To localize subunit C within the V-ATPase complex, uniquely cysteine residues were introduced into 25 structurally defined sites within the yeast C subunit and used as sites of attachment of the photoactivated sulfhydryl reagent 4-(N-maleimido)benzophenone (MBP). Analysis of photocross-linked products by Western blot reveals that subunit E (part of V₁) is in close proximity to both the head domain (residues 166–263) and foot domain (residues 1–151 and 287–392) of subunit C. By contrast, subunit G (also part of V₁) shows cross-linking to only the head domain whereas subunit a (part of V₀) shows cross-linking to only the foot domain. The localization of subunit C to the interface of the V₁ and V₀ domains is consistent with a role for this subunit in controlling assembly of the V-ATPase complex.

The vacuolar (H⁺)-ATPases (V-ATPases) are a family of ATP-dependent proton pumps responsible for acidification of intracellular compartments (such as endosomes, lysosomes, and secretory vesicles) as well as proton transport across the plasma membrane (1–11). V-ATPases present in intracellular compartments function in such processes as receptor-mediated endocytosis, intracellular targeting, protein processing and degradation, coupled transport of small molecules and ions, and the entry of certain viruses and toxins from endocytic compartments into cells (1, 2). Plasma membrane V-ATPases are important for urinary acidification by renal intercalated cells, bone resorption by osteoclasts, K⁺ secretion by insect gill cells, sperm maturation in the male reproductive tract, and the invasiveness of certain tumor cells (1, 7–11).

The V-ATPases are composed of a peripheral V₁ domain responsible for ATP hydrolysis and an integral V₀ domain that carries out proton translocation (1, 2). V₁ is a 600–650-kDa complex with the subunit composition A₂B₂C₁D₁E₁F₁G₂H₁–2 (1, 2). The nucleotide binding sites are located at the interface of the A and B subunits (which form an alternating hexamer), with the catalytic sites located mainly on the A subunit and “non-catalytic” sites located mainly on the B subunit (12–14). The V₀ domain is a 250-kDa complex with the subunit composition α₁h₁ε₁c₁c′₁ c′₁ (1, 15). The three proteolipid subunits (c, c′, and c′) each contain a buried glutamic acid residue that is essential for proton transport (16, 17) and together they form a hexameric ring. The V-ATPases thus resemble the F-ATPases (or ATP synthases), which are involved in ATP synthesis (18–20). High resolution crystal structures have been obtained for both F₁ (21, 22) and F₀ bound to a proteolipid ring (23).

The F-ATPases operate by a rotary mechanism (20). ATP hydrolysis in F₁ causes rotation of a central stalk composed of the γ and ε subunits (23–26), which in turn causes rotation of the ring of proteolipid c subunits (27–29). A peripheral stalk (composed of the α subunit and the soluble regions of subunit b, Ref. 30) functions as a stator, holding subunit a fixed relative to the αβ₁ head during ATP-driven rotation of the proteolipid ring. It is rotation of the ring of proteolipid c subunits relative to subunit a that is believed to drive proton transport (19, 31, 32). Comparison of amino acid sequences of the V- and F-ATPases reveals clear sequence homology both between the nucleotide binding subunits and between the proteolipid subunits of the two classes (33, 34, 16). Because there is virtually no sequence similarity for the remaining subunits, it has not been possible to deduce the function or localization of the remaining V-ATPase subunits from their primary sequence.

Electron microscopy studies indicate that like the F-ATPases (35), the V-ATPases also contain multiple stalks connecting the peripheral and integral domains (36–38). To determine the composition of the central and peripheral stalks of the V-ATPase, we have previously employed cysteine mutagenesis and photochemical cross-linking using MBP to determine the arrangement of subunits relative to subunit B (39, 40). These studies suggest that subunit D is located in the central stalk whereas subunits E and G form part of the peripheral stalk connecting V₁ and V₀. These assignments have been confirmed by rotation experiments demonstrating that subunits D and F are present in the central rotor (41) whereas subunit G is located in the peripheral stator (42).

In the present study, we have used a similar photochemical cross-linking approach to localize subunit C within the
V-ATPase complex. This subunit is of particular interest because of its putative role in regulating dissociation of the V-ATPase complex in vivo (43). Dissociation of V₁ and V₀ represents an important mechanism of controlling V-ATPase activity in cells (44, 45) and occurs with release of subunit C from both the V₁ and V₀ domains. Subunit C has therefore been suggested to function in triggering dissociation of the V-ATPase in vivo (43). The availability of a recently published high resolution crystal structure of subunit C (46) has allowed us to introduce unique cysteine residues into structurally defined sites within this protein. The results have identified specific contacts between subunit C and other subunits present in the peripheral stalk of the V-ATPase and are consistent with a role for subunit C in controlling the stability of the interactions between the V₁ and V₀ domains.

EXPERIMENTAL PROCEDURES

Materials and Strains—Zymolyase 100T was purchased from Seika-gaku America, Inc. Concana~mycin A was obtained from Fluka Chemical Corp. 9-Amino-6-chloro-2-methoxyacridine (ACMA) was purchased from Sigma. SDs, nitrocellulose membranes (0.43 μm pore size), Tween 20, horseradish peroxidase-conjugated goat anti-
mouse IgG, and horseradish peroxidase-conjugated goat anti-rabbit IgG were from Bio-Rad. 4- (N-maleimidomethyl)benzophenone (MBP) and most common chemicals were obtained from Sigma. The yeast strain lacking subunit C (Vma5p) was constructed from YPH500 by replacing the VMA5 gene with the TRP1 gene (47). The strain was first selected on tryptophan-minus plates and the growth phenotype was then assessed on YEPD plates buffered with 50 mM KH₂PO₄ or 50 mM succinic acid to either pH 7.5 or pH 5.5.

Antibodies—The monoclonal antibodies 3F10 (against the HA antigen) with and without conjugation to horseradish peroxidase were purchased from Roche Applied Sciences. The monoclonal antibody 10D7 against the 100 kDa α subunit was from Molecular Probes. The poly-
clonal antibody against subunit E (Vma4p) was a gift from Dr. Daniel Klionsky (University of Michigan). The polyclonal antibody against subunit G (Vma10p) was a gift from Dr. Tom Stevens (University of Oregon).

Cloning of the VMA5 Gene Encoding Subunit C—The VMA5 gene was amplified from genomic DNA isolated from the yeast strain Oregon. The primers were designed using the Altered Sites II,

Cloning of Other V-ATPase Subunits—Subunit C resides at the interface of V₁ and V₀ (43). The availability of a recently published high resolution crystal structure of this protein (46). This structure supports an important mechanism of controlling V-ATPase

Isolation of Vacular Membrane Vesicles—Vacuolar membrane vesicles were isolated using a modification of the protocol described by Uchida et al. (50). Yeast cells were grown overnight at 30 °C to 5 × 10⁷ cells/mL in 1 liter of selective medium. Cells were pelleted, washed twice with water, and resuspended in 50 ml of 100 mM Tris-HCl, pH 9.4, containing 10 mM dithiothreitol. After incubation at 30 °C for 20 min, cells were pelleted again, washed once 25 ml of YEPD medium containing 0.7 mM sorbitol, 2 mM dithiothreitol, and 100 mM MES-Tris (pH 7.5), resuspended in 25 ml of YEPD medium containing 0.7 mM sorbitol, 2 mM dithiothreitol, 100 mM MES-Tris, pH 7.5, and 2 mg of Zymolase 100T, and incubated at 30 °C for 90 min with gentle shaking. The resulting spheroplasts were osmotically lysed, and the vacuoles were isolated by flotation on two consecutive Ficoll gradients and diluted in transport buffer (15 mM MES-Tris, pH 7.9, 4.8% glycerol).}

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was cloned from YPH500 genomic DNA and inserted into the pRS316 vector, which contains the URA3 selectable marker. An HA epitope tag was introduced at the N terminus of Vma5p to facilitate immunoprecipitation of the V-ATPase complex and to permit detection of subunit C and its cross-linked products by Western blot. Transformation of the vma5Δ strain with this epitope-tagged construct gave a wild-type growth phenotype (see below) and gave rise to a V-ATPase complex possessing wild-type assembly and activity properties (data not shown). Thus wild type in this study refers to cells transformed with this epitope-tagged construct of Vma5p.

To construct mutants of Vma5p containing single cysteine residues, it was first necessary to construct a Cys-less form of Vma5p in which the single endogenous cysteine residue at position 340 was replaced with serine. This was accomplished using site-directed mutagenesis (see “Experimental Procedures”). Using the Cys-less form of Vma5p as a starting point, 25 mutants containing single cysteine replacements were then constructed using the same mutagenesis protocol. Cysteine residues were introduced at exposed sites on each of the surfaces of the protein, as indicated from the x-ray structure of subunit C (Fig. 1). All mutants were selected on uracil-minus plates and tested for growth on YEPD plates buffered to either pH 7.5 or pH 5.5. Both the Cys-less Vma5p and the single cysteine-containing mutants were able to grow at both pH 7.5 or pH 5.5. Both the Cys-less Vma5p and the single cysteine-containing mutants were able to grow at both pH 7.5 and 5.5, indicating that they were able to complement the yeast strain expressing the Cys-less form of Vma5p. These membranes were then reacted with 1 mM MBP for 30 min in the dark followed by quenching of the reagent with dithiothreitol, washing by sedimentation and irradiation with a long wavelength UV lamp. The membranes were solubilized using C_{12}E_{6} and the V-ATPase complexes immunoprecipitated using an anti-HA antibody. The proteins were separated by SDS-PAGE and Western blotting was performed using the anti-HA antibody. As shown in Fig. 3A, no HA-reactive bands of molecular weight greater than that of the C subunit monomer were observed in the presence of MBP. These results indicate that any cross-linked products obtained with the single cysteine-containing mutants of Vma5p do not arise as a result of MBP reaction with sulfhydryls present in other subunits.

Vacuolar membranes were next isolated from the yeast strains expressing each of the single cysteine-containing mutants of subunit C and photocross-linking by MBP was evaluated as described above. Of the twenty five mutants tested, nine showed the generation of higher molecular weight cross-linked products when Western blotting was performed using the anti-HA antibody, indicating cross-linking of subunit C to other proteins in the complex. The remaining sixteen mutants showed no evidence of cross-linking, although this could be attributed to several possible causes. First, a given cysteine residue, although exposed on the surface of subunit C, may nevertheless be prevented from reaction with MBP by being located at the interface of two subunits. Second, although a cysteine may react with MBP, the photoactive group may not be sufficiently close or have the correct orientation to give rise to a cross-linked product. Thus, only a positive cross-linking result is interpretable.

Of the nine mutants showing evidence of cross-linking, two gave HA-reactive species of molecular mass ~70 kDa, including G27C and H324C. Because this mass corresponds to the sum of the molecular masses of subunit C (43 kDa) and subunit E (27 kDa), Western blotting was performed using an antibody specific for subunit E. As shown in Fig. 3A, the 70-kDa bands recognized by the anti-HA antibody were also recognized by the anti-E subunit antibody for both G27C and H324C, indicating that MBP-mediated formation of an E/C heterodimer can occur at both of these positions. Importantly, probing the blot of the Cys-less mutant using the anti-E subunit antibody revealed no...
such 70 kDa species (Fig. 3A). When blots of the other single cysteine-containing mutants of subunit C were probed with the anti-E subunit antibody, two others showed cross-linked products that were recognized by both the anti-HA and anti-E subunit antibodies: N216C and A220C (Fig. 3B). The apparent molecular mass of the cross-linked products formed for these mutants (90 kDa) was higher than that expected for an E/C heterodimer. We have previously observed such aberrant migration of cross-linked products upon MBP-mediated photocross-linking to subunit B (40). This aberrant migration may be caused by the formation of cross-linked products in which the polypeptides are joined near the middle of one or both sequences, giving rise to a non-linear species. Because subunit E does not contain endogenous cysteine residues, this aberrant migration cannot be because of the presence of other subunits that have become cross-linked as a result of MBP modification of subunit E in the initial reaction.

Of the four cysteine mutants showing cross-linking to subunit E, the A220C mutant showed a second cross-linked product recognized by the anti-HA antibody of molecular mass 60 kDa (Fig. 3B). Because this is close to the sum of the masses of subunits C and G (43 kDa + 13 kDa = 56 kDa), the blots were probed with an antibody against subunit G. As can be seen in Fig. 4, this species was recognized by both the anti-HA and anti-G subunit antibodies, suggesting that it corresponds to a C/G heterodimer.

Finally, for two subunit C mutants (S57C, S69C), a cross-linked product of 180 kDa was observed (Fig. 5). This product was recognized by both anti-HA and anti-a subunit antibodies, although the molecular mass was considerably larger than that of the other cross-linked products observed. This suggests that the cross-linked product of 180 kDa is a complex formed between subunits C and G, which has a molecular mass of 130 kDa, and a third subunit, likely subunit E.
predicted for a C/a heterodimer (43 kDa + 100 kDa = 143 kDa). This aberrant migration may be due either to the unusual migration behavior of the heterodimer or to the presence of other subunits that have been cross-linked as a result of MBP reaction with cysteine residues in subunit a. This latter explanation is made unlikely by the absence of higher molecular weight species recognized by the anti-a subunit antibody in the strain expressing the Cys-less mutant of subunit C (Fig. 5).

DISCUSSION

Subunit C is unique among the V-ATPase subunits in being released from both the V₁ and V₀ domains upon dissociation.
of the complex in vivo (44). For reversible dissociation to be used as a mechanism of regulating V-ATPase activity in vivo, it is necessary that ATPase activity of the free V1 domain and passive proton conduction by free V0 be silenced following dissociation, and these predictions have been confirmed experimentally (57, 58). Interestingly, overproduction of subunit C in yeast has a more lethal phenotype than its disruption, leading to the suggestion that binding of subunit C to free V1 may activate a futile ATPase activity (59). Thus, release of subunit C may function both in controlling in vivo dissociation and in preventing potentially lethal ATP hydrolysis by the released V1 domain. Subunit C has also been identified as one of two potential binding sites for actin within the V-ATPase complex (60, 61) and has been shown to display actin bundling activity (62).

The recently published high resolution crystal structure of subunit C (46) reveals a protein possessing two globular domains, termed the “head” and “foot” domains, connected by pair of α-helical segments. The larger foot domain contains both the N terminus (residues 1–151) and the C terminus (287–392) of the protein, whereas the head domain is composed of residues 166–263 located in the central region of the primary sequence. To localize subunit C within the V-ATPase complex, we have made use of this high resolution structure to select specific surface residues for mutagenesis to cysteine. These cysteine residues were then used as sites of attachment of the photo-activated maleimide reagent MBP. Photocross-linking followed by analysis of cross-linked products by Western blot thus allows us to place other V-ATPase subunits with respect to specific sites on the surface of the C subunit. A similar approach using photoactivated cross-linking to subunit B has allowed the localization of subunits E and G to the peripheral stalk and subunit D to the central stalk connecting V1 and V0 (39, 40). In addition, localization of subunit H to the interface of V1 and V0 using this method has been confirmed by electron microscopy (38).

The results in the present study indicate that subunit C binds to the V-ATPase complex with the foot domain oriented toward the membrane, interacting with the a subunit of V0. Although the site within the a subunit that is in proximity to subunit C has not been determined, the cytoplasmic localization of the N-terminal hydrophilic domain of subunit a (63) makes this region a likely candidate. By contrast, the head domain of subunit C is in close proximity to subunit G. Subunit G is a 13-kDa protein present in two copies per complex (64) that appears to form a dimer (65), analogous to the coiled-coil structure observed for the b subunit in the peripheral stalk of F1F0 (66). In fact subunit G and subunit b show partial sequence homology along one helical face (67) and both are tolerant to short deletions (68, 69). Previous cross-linking results indicate that at least a portion of subunit G is present near the top of the V1 complex farthest from the membrane (40), but this subunit may extend some length along the outer surface of the V1 domain.

Such an extended disposition perpendicular to the mem-
brane is clearly indicated for subunit E. Thus not only does subunit E cross-link to residues in both the head and foot domains of subunit C (a distance of nearly 100 Å), but also to residues near the top and bottom of subunit B (a distance of over 70 Å) (39, 40). An interaction between subunits C and E is consistent with previous cross-linking results (64) as well as the formation of a C/E heterodimer on dissociation of the V₁ domain of the bovine-coated vesicle V-ATPase (70). Similarly, interaction between subunits E and G has been suggested from both cross-linking (64) and the formation of an E/G heterodimer upon disruption of normal V₁ assembly in yeast (71, 72).

Recently, the N-terminal region of subunit E has been shown to play an important role in interaction with both subunits C and G (73). Thus, deletion of the first 19 amino acids of subunit E significantly perturbs binding to subunit C whereas deletion of the next 19 amino acids causes loss of binding to subunit G. Point mutations in subunit E that perturb binding to subunit C do not prevent subunit C from assembling with the remainder of the V-ATPase complex, leading the authors to suggest that subunit C makes contact with V-ATPase subunits in addition to subunit E (73). Results from the current study confirm this prediction and identify subunits G and a as additional contacts for subunit C. Thus, subunit C interacts with three different subunits (E, G, and a) of the peripheral stalk (Fig. 6).

Interactions between subunit C and subunits of the peripheral stalk may explain the postulated stimulatory role of subunit C on ATPase activity of the free V₁ domain (59). ATP hydrolysis by free V₁ may be suppressed by interaction between the central and peripheral stalks, which would prevent ATP-dependent rotation of the central stalk. In fact, cross-linking between subunits of the central and peripheral stalk in dissociated V₁ has been observed (64). By binding to multiple sites on subunit E, subunit C may prevent the peripheral stalk from folding inwards toward the central stalk, thereby preventing the normal inhibition of ATP hydrolysis by V₁.

Dror et al. (46) report two distinct crystal structures for subunit C. The second structure (solved at 2.9 Å) differs from the first in a −12° movement of the head domain relative to the foot domain, indicating considerable flexibility in the alpha helical neck connecting these two domains. Assuming subunit C remains stably bound to subunit E via both the head and foot domains during the transition between these two conformations, this would suggest that subunit E may need a comparable level of structural flexibility. This is consistent with the predicted high α-helical content of subunit E (74) where it existed in an elongated conformation. If, however, there is more than one copy of subunit E (as has previously been suggested (53, 75)), it is possible that the two different conformations of subunit C actually bind to two different copies of subunit E. Alternatively, the head domain may bind to one copy of E and the foot domain may bind the other. Definitive information on the number of copies of subunit E per complex should help to resolve this question. It should be noted that the two conformations of subunit C also make it possible that the head domain binds to subunit E in one conformation and to subunit G in the other conformation.

Mutagenesis studies of Vma5p have identified three mutations (F260A, Y262A, and F385A) that decrease the stability of the V-ATPase complex but nevertheless significantly increase the maximal velocity for both proton transport and ATP hydrolysis (43). Two of these mutations (F260A and Y262A) are in the head domain near the surface that shows cross-linking to subunits E and G (Asn216 and Ala220) whereas the third (Phe385) is in close proximity to Ser69 in the foot domain that shows cross-linking to subunit a. The increased catalytic activity despite decreased stability observed upon mutation of the three aromatic residues is not readily interpretable in terms of subunit C simply playing a structural role in the peripheral stalk. Curtis et al. (43) have suggested that during the catalytic cycle the V-ATPase adopts a conformation that is less stable, and that the C subunit controls the ability of the complex to adopt such a conformation. By interacting with peripheral stalk subunits in both V₁ (E and G) and V₀(a), subunit C is well positioned to play such a modulatory role.

Control of interactions between V₁ and V₀ is also crucial in regulating the assembly state of the V-ATPase in vivo, which in yeast is modulated by glucose levels in the media (44). If, as suggested above, subunit C is able to induce the complex to adopt a less stable conformation, glucose removal may trigger dissociation through changes brought about by subunit C. By interacting with key subunits of the peripheral stalk (including subunits E, G, and a), subunit C may be able to disrupt interactions between these subunits that would otherwise lead to a stably assembled peripheral stalk. In this regard subunit C plays a role that is unique to the V-ATPases. As previously pointed out (43), the peripheral stalk of the V-ATPases must be both sufficiently stable to survive the torque generated during rotary catalysis and sufficiently labile to lead to dissociation in the absence of glucose. Because there is no evidence for functional dissociation of the F-ATPase in vivo, it is not surprising that no homolog to subunit C exists for these enzymes.

In summary, we have identified novel interactions between specific domains of subunit C and both subunit G in the pe-
Subunit C Resides at the Interface of V₁ and V₀

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