Subunit Interactions in the Clathrin-coated Vesicle Vacuolar (H\(^+\))-ATPase Complex*

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The vacuolar (H\(^+\))-ATPases (or V-ATPases) are structurally related to the F\(_{1}\)F\(_{0}\) ATP synthases of mitochondria, chloroplasts and bacteria, being composed of a peripheral (V\(_{1}\)) and an integral (V\(_{0}\)) domain. To further investigate the arrangement of subunits in the V-ATPase complex, covalent cross-linking has been carried out on the V-ATPase from clathrin-coated vesicles using three different cross-linking reagents. Cross-linked products were identified by molecular weight and by Western blot analysis using polyclonal antibodies raised against individual V-ATPase subunits. In the intact V\(_{1}\)V\(_{0}\) complex, evidence for cross-linking of subunits C and E, D and F, as well as E and G was observed. Subunits C and E as well as D and E could be cross-linked by 1-ethyl-3-(dimethylaminopropyl)carbodiimide, while subunits a and E could be cross-linked by 4-(N-maleimido)benzophenone. It was further demonstrated that it is possible to treat the V-ATPase with potassium iodide and MgATP in such a way that while subunits A, B, and H are nearly quantitatively removed, significant amounts of subunits C, D, E, and F remain attached to the membrane, suggesting that one or more of these latter subunits are in contact with the V\(_{0}\) domain. In addition, treatment of the V-ATPase with cysteine, which modifies Cys-254 of the catalytic A subunit, results in dissociation of subunit H, suggesting communication between the catalytic nucleotide binding site and subunit H. Finally, the stoichiometry of subunits F, G, and H were determined by quantitative amino acid analysis. Based on these and previous observations, a new structural model of the V-ATPase from clathrin-coated vesicles is proposed.

The vacuolar (H\(^+\))-ATPases (or V-ATPases)\(^1\) are a family of ATP-dependent proton pumps that carry out proton transport across both intracellular membranes and, in some cases, the plasma membrane (1–6). Acidification of intracellular compartments is important for such processes as protein degradation, intracellular protein targeting, and receptor-mediated endocytosis (1–6), while V-ATPases in the plasma membrane function in renal acidification, bone resorption, and tumor metastasis (7–9).

The V-ATPase is a heterooligomeric complex of molecular mass approximately 800 kDa composed of at least 13 different subunits arranged into two separate domains (1–6). The peripheral V\(_{1}\) domain has a molecular mass of about 570 kDa and contains eight different subunits of molecular mass 70 (A), 60 (B), 57 (H), 40 (C), 34 (D), 33 (E), 14 (F), and 16 (G) kDa. The integral V\(_{0}\) domain has a molecular mass of 260 kDa and contains five different subunits of molecular mass 100 (a), 38 (d), 19 (c\(_{2}\)), and 17 (c\(_{1}\)) kDa. Functional studies indicate that the V\(_{1}\) domain is responsible for ATP hydrolysis while the V\(_{0}\) domain carries out proton transport (1–6). We have previously demonstrated that each V-ATPase complex contains three copies each of the A and B subunits, six copies of the 17-kDa subunits (c\(_{1}\), c\(_{2}\)), and single copies of the C, D, E, a, d, and c\(_{1}\) subunits (10). The number of copies of subunits F, G, and H per complex has not been reliably determined.

The V-ATPases are known to be structurally and evolutionarily related to the ATP synthases (or F-ATPases) of mitochondria, chloroplasts and bacteria (11–13). Thus the nucleotide binding subunits of the V-ATPase (A and B) are homologous to the corresponding \(\beta\) and \(\alpha\) subunits of F\(_{1}\) (14, 15), while the proteolipid subunits of the two complexes are also homologous (16, 17). 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 (18, 19). F\(_{1}\) appears to be attached to the F\(_{0}\) domain via both a central stalk (believed to include both the \(\gamma\) and \(\epsilon\) subunits) and a peripheral stalk (composed of the \(\delta\) subunit and the soluble portions of subunit b) (20, 21). The F\(_{0}\) domain contains a ring of c subunits with the a and b subunits to one side (12, 23).

While the V-ATPase complex is thought to resemble the F-ATPases, electron micrographs have revealed significant differences (24, 25), and very little is known concerning the arrangement of subunits from the V-ATPase complex. In the current study, we have employed covalent cross-linking and Western blot analysis to identify subunit contacts within the V-ATPase. In addition, we have reevaluated the effects of treatment of the enzyme with cysteine or potassium iodide on dissociation of specific subunits from the complex. Finally, we have determined the stoichiometry of subunits F, G, and H by quantitative amino acid analysis. These studies have allowed us to construct a new model for the structure of the V-ATPases.

EXPERIMENTAL PROCEDURES

Materials—Calf brains were obtained fresh from a local slaughterhouse. Disuccinimidyl glutarate (DSG), 1-ethyl-3-(dimethylaminopropyl)carbodiimide hydrochloride (EDC), and N-hydroxysuccinimide (NHS) were from Pierce. SDS, niterocellulose membranes (0.2-µm pore size), Tween 20, and horseradish peroxidase-conjugated goat anti-rabbit IgG were from Bio-Rad. 1-Tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin, soybean trypsin inhibitor, 4-(N-maleimido)benzophenone (MBP), and most common chemicals were obtained from Sigma. The chemiluminescence substrate for horseradish peroxidase...
was from KPL Laboratories and prestained SDS-PAGE marker proteins were from Amersham Pharmacia Biotech.

**Antibody Production and Purification**—The bovine cDNAs encoding the full-length forms of subunits C, D, and E were cloned into the pET-21d (+) expression vector and expressed in BL-21 cells. Expression of the tagged proteins was induced by incubation in 1 mM isopropyl-1-thio-β-D-galactopyranoside, and disruption of cells was carried out using lysozyme and Triton X-100 as described previously (26). The recombinant proteins were recovered from inclusion bodies by sedimentation and solubilized with 8 M urea followed by dialysis against phosphate-buffered saline. The His-tagged proteins were then isolated by nickel-affinity chromatography and sent to Covance Inc. for production of polyclonal antibodies in rabbits. For subunits B, F, and H, the following synthetic peptides were prepared: subunit B, CPTSGPLAG-SREQAL; subunit C, CPSEKHYDAYDKSILRR; subunit H, CHSEK-FNRENPARLNKKN. The peptides were conjugated to keyhole limpet hemocyanin via the cysteine residues at the amino termini and were also sent to Covance, Inc. for production of polyclonal antibodies in rabbits. Antibodies were isolated from rabbit antiserum using protein A-Sepharose affinity chromatography.

**Preparation of Clathrin-coated Vesicles and Purification of the V-ATPase**—Clathrin-coated vesicles were prepared from calf brain as described previously (27). Vesicles were stripped of their clathrin coat using 0.5 M Tris (pH 7.0), 2 mM EDTA and the V-ATPase was solubilized with C₆E₆ and purified by glycerol density gradient centrifugation as described (27).

**Preparation of the V₁(Subunit C) Subcomplex**—Stripped vesicles (1.0 mg of protein/ml) were treated first with 0.2 M KI for 1 h at 4 °C, followed by sedimentation for 1 h at 100,000 × g and resuspension in 0.36 M KI and 2.5 mM MgATP, incubation for 1 h at 4 °C, and sedimentation. The supernatant was dialyzed overnight versus two changes of 100 volumes of solubilization buffer (50 mM NaCl, 30 mM RCI, 20 mM HEPES (pH 7.0), 0.2 mM EGTA, 10% glycerol) to allow reassembly of the V₁(subunit C) subcomplex (referred to as the V₁ subcomplex). The V₁ subcomplex was then isolated by sedimentation for 16 h at 175,000 × g on 15–30% glycerol density gradients as described previously (28).

**Covalent Cross-linking of the Purified V-ATPase and V₁ Subcomplex**—For DSG, the purified V-ATPase or V₁ subcomplex were concentrated to approximately 50 μg of protein/ml using a Centricon-30 microconcentrator and incubated with 0.2 mM DSG for 30 min at 23 °C. The reaction was stopped by addition of 50 mM ammonium acetate (pH 8.0). For EDC, the purified V-ATPase or V₁ subcomplex (50 μg of protein/ml) was incubated with 5 mM EDC together with 5 mM NHS for 20 min at 23 °C. The reaction was stopped by addition of 50 mM Tris (pH 8.0). For MBP, the reagent dissolved in dimethylformamide at 100 mM was added to the purified V-ATPase or V₁ subcomplex at a final concentration of 1 mM, followed by incubation in the dark for 30 min at 23 °C. The unreacted MBP was quenched by addition of 10 mM dithiothreitol. The samples were dialyzed against two changes of 200 volumes of solubilization buffer for a total of 4 h, followed by irradiation with a long wavelength ultraviolet lamp for 20 min at 4 °C.

**RESULTS**

**Covalent Cross-linking of the Coated Vesicle V-ATPase**—To facilitate identification of products following covalent cross-linking of the purified V-ATPase, polyclonal antibodies were prepared in rabbits against either full-length recombinant proteins expressed in *Escherichia coli* (subunits C, D, and E) or 15–19-amino acid synthetic peptides conjugated to keyhole limpet hemocyanin (subunits B, F, G, and H). Antibodies were purified by protein A-Sepharose chromatography and tested for specificity by Western blot analysis against the purified coated vesicle V-ATPase. As can be seen from Fig. 1, all antibodies showed specific recognition of the corresponding subunit by Western blot with the exception of the antisera raised against subunit G, which did not show significant reaction (data not shown). The antisera against subunit H recognized two bands of molecular mass 50–57 kDa, consistent with previous reports on the existence of two isoforms of subunit H in the coated vesicle enzyme (34).

The first cross-linking reagent employed was DSG, a bifunctional amino-reactive reagent containing a 7.7-Å linker arm. Cross-linking by DSG was tested in both the intact V₅V₀ complex and the free V₁ domain isolated by glycerol density gradient sedimentation following potassium iodide solubilization and centrifugation (28). As can be seen in Fig. 1, a cross-linked product of molecular mass 50 kDa showed cross-reaction with antibodies against both subunits D and F and was observed in both the intact V₅V₀ complex and the free V₁ domain. The migration of this product agrees well with the predicted molecular mass of a complex containing one copy each of subunits D and F (48 kDa). While a band at 50 kDa could also be observed...
in the V₁ lane (and more faintly in the V₁/V₀ lane) with antibodies against subunit E, experiments described below suggest that this band does not represent cross-linking of subunits E and F. However, a 46-kDa cross-linked product that reacted with the anti-E subunit antibody but did not react with the anti-F subunit antibody was also observed in both V₁/V₀ and V₁, and likely represents cross-linking of subunits E and G (predicted molecular mass 46 kDa).

In addition to the above complexes, cross-linking by DSG gave rise to a 90-kDa product in V₁ that reacted with antibodies against subunits H and E (predicted molecular mass 87–90 kDa) and a 75-kDa product in V₁/V₀ that was recognized by antibodies against subunits C and E (predicted molecular mass 73 kDa). Finally, a doublet around 70 kDa was observed in the V₁ lane with both the anti-H and anti-F antibodies (predicted molecular mass 68–71 kDa). It should be noted that treatment with DSG appears to cause some internal cross-linking of subunit E, such that a second (or even a third) band appears with slightly lower mobility. This may account for the additional band observed at 50 kDa with the anti-E subunit antibody.

To further test the identity of the observed cross-linked products, the V₁/V₀ complex was first treated with trypsin prior to cross-linking with DSG. Under these conditions, subunits D, F, and H were largely digested. As can be seen in Fig. 3, the product at 75 kDa recognized by the antibodies against subunits C and E as well as the 75-kDa cross-linked products were unaffected.

The second cross-linking reagent employed was EDC, which, following treatment with NHS, results in cross-linking of amino and carboxyl groups. As can be seen in Fig. 4, treatment of the V₁/V₀ complex with EDC/NHS gave rise to a pair of products around 75 kDa that reacted with both the anti-C and anti-E subunit antibodies (similar to that observed for DSG) and a new product of molecular mass 67 kDa that was recognized by both the anti-D and anti-E subunit antibodies. The predicted molecular mass of a complex between subunits D and E is 67 kDa.

The final cross-linking reagent tested was MBP, which is a photoactivatable maleimide that has a linker arm of 10 Å (Fig. 5). Reaction with MBP in the dark followed by irradiation with ultraviolet light gave rise to a 135-kDa product that was recognized by the antibody against subunit E. The size of this complex is consistent with cross-linking of subunit E and subunit a of the V₀ domain. We have recently shown that subunit a contains a large soluble domain at the amino terminus, which is exposed on the cytoplasmic side of the membrane and is therefore situated to interact with the V₁ domain (35).

**Dissociation of V₁ Subunits by Potassium Iodide and MgATP**—We have previously demonstrated that treatment of clathrin-coated vesicles with potassium iodide and MgATP results in the release of the V₁ subunits from the membrane (36, 37). Moreover, by Western blot analysis, the A subunit is released nearly quantitatively (38). An important question, how-
ever, is whether all of the V₁ subunits are equally well released from the membrane. To address this question, Western blot analysis was carried out on stripped coated vesicles either before or after treatment with potassium iodide and MgATP using the antibodies against subunits B, C, D, E, F, and H described above. As can be seen in Fig. 6, treatment with KI and MgATP results in nearly complete release of subunits B and H (as was previously observed for subunit A; Ref. 38), whereas significant amounts of subunits C, D, E, and F remain attached to the membrane. While it is likely that a larger fraction of subunits C, D, and E were removed at the lower protein concentration employed during the dissociation (1 mg of protein/ml) was higher than that used previously in the preparation of V₀ (28) in order to determine whether it is possible to observe a differential release of subunits A, B, and H versus subunits C, D, E, and F.

release of subunit H by treatment with Cystine—We have previously observed that treatment of the V-ATPase with cystine results in the release of the 50-kDa subunit of the AP2 adaptin complex that is associated with the enzyme and causes loss of V-ATPase activity (39). It has subsequently been reported that an active V-ATPase completely lacking the AP50 polypeptide could be isolated, but that removal of subunit H by urea treatment resulted in loss of ATPase activity (34). We therefore wished to determine whether cysteine treatment of the V-ATPase also led to loss of subunit H, which on SDS-PAGE has a molecular mass similar to AP50. Stripped vesicles were treated with 1 mM cystine at 4 °C as described previously and washed by sedimentation at 100,000 × g. The vesicles were resuspended in solubilization buffer and dialyzed overnight against the same buffer. Control vesicles were subjected to the same treatment except in the absence of KI and MgATP during the second incubation. The membranes were solubilized in Laemmli sample buffer, and 10 μg of protein was applied to each lane of a 15% acrylamide gel followed by SDS-PAGE according to the method of Laemmli (29). Following transfer to nitrocellulose, Western blotting using antibodies against the indicated subunits was carried out as described under “Experimental Procedures.” It should be noted that the protein concentration employed during the dissociation (1 mg of protein/ml) was higher than that used previously in the preparation of V₀ (28) in order to determine whether it is possible to observe a differential release of subunits A, B, and H versus subunits C, D, E, and F.

Dissection of V₁ subunits from stripped vesicles by treatment with potassium iodide and MgATP. Stripped vesicles (1 mg of protein/ml) that had been pretreated with 0.2 x KI for 1 h at 4 °C and washed by sedimentation at 100,000 × g were then treated with 0.36 x KI and 2.5 mM MgATP for 1 h at 4 °C, followed by sedimentation for 1 h at 100,000 × g. The vesicles were resuspended in solubilization buffer and dialyzed overnight against the same buffer. Control vesicles were subjected to the same treatment except in the absence of KI and MgATP during the second incubation. The membranes were solubilized in Laemmli sample buffer, and 10 μg of protein was applied to each lane of a 15% acrylamide gel followed by SDS-PAGE according to the method of Laemmli (29). Following transfer to nitrocellulose, Western blotting using antibodies against the indicated subunits was carried out as described under “Experimental Procedures.” It should be noted that the protein concentration employed during the dissociation (1 mg of protein/ml) was higher than that used previously in the preparation of V₀ (28) in order to determine whether it is possible to observe a differential release of subunits A, B, and H versus subunits C, D, E, and F.
V-ATPase was then solubilized with C12E9 and purified by density incubation under identical conditions but in the absence of cystine. The resuspension of vesicles in solubilization buffer. Control vesicles were the V-ATPase complex.

for 5 days at 4 °C followed by sedimentation for 1 h at 100,000 g. The data shown are for two independent trials. The blotting efficiency was determined as described under "Experimental Procedures" and used to calculate the total moles of amino acids in each band on the original gel. Moles of protein were calculated from corrected moles of amino acids based upon the number of amino acids in each polypeptide (which is known from the published amino acid sequences). The ratios shown in the last column are an average of the two independent determinations and are expressed relative to a value of 3.0 for subunit A (for subunit H and GP45) and 1.0 for subunit C (for subunits F and G).

V1 complex may distort relative to V1V0 to allow some subunit contacts to occur that do not normally occur in the intact complex. Because V-ATPase complexes lacking subunit H can be isolated both in vitro (39, 34) and in vivo in a yeast strain lacking the corresponding gene product (Vma13p) (44), it is likely that subunit H is located near the periphery of the complex. In fact, the VMA13 gene product is unique in being the only V-ATPase subunit whose absence does not disrupt assembly of the V-ATPase.

The final information obtained from the cross-linking studies presented concerns subunit E. Because EDC/NHS is a zero-length cross-linking reagent, the cross-linking of subunits D and E suggests that these subunits are, at least part of the time, in close contact with each other. A contact between subunit E and the 100-kDa subunit a of the V0 domain is also suggested by the appearance of a product of the appropriate size on cross-linking of the intact complex (but not the V1 domain) with MBP. While this assignment is again tentative pending the availability of an antibody that recognizes the a subunit by Western blot, it suggests that subunit E may play an important role in bridging the peripheral and integral domains of the protein.

We have suggested, based on mutagenesis studies (30, 45), that the 100-kDa subunit corresponds to the V-ATPase homolog of the F-ATPase a subunit, which functions not only in proton translocation (12, 46, 47) but as part of the stator that is held rigid relative to the 100-kDa hexamer (21). Most recently we have demonstrated that the large amino-terminal domain of the 100-kDa subunit is oriented toward the cytoplasmic side of the membrane (35), making it a likely candidate for forming part of the stator structure in the V-ATPase.

We had previously suggested, based on cross-linking by the reversible cross-linking reagent DTSSP and analysis by two-dimensional gel electrophoresis that subunits C, D, and E were in contact with subunit c of the V0 domain (36). Because of the similarity in molecular mass of subunits c, F, and G, the results in the current study suggest the possibility that the products observed previously actually represented complexes between subunits C, D, or E and subunits F or G. Nevertheless, the observation that treatment of stripped vesicles with potassium iodide and MgATP can result in nearly complete removal of subunits B and H (Fig. 6) as well as subunit A (38) while leaving behind substantial amounts of subunits C, D, E, and F suggests that one or more of these latter subunits likely form part of the bridge between the V1 and V0 domains.

We had previously reported that treatment of the V-ATPase with cystine results in dissociation of AP50 (the 50-kDa subunit of the AP2 adaptin complex) and loss of activity (39). AP50 was identified by NH2-terminal sequence analysis, and the

### TABLE I

| Subunit | Mass | Total nmol amino acids | Blotting efficiency | Correct nmol amino acids | Correct pmol protein | Average ratio |
|---------|------|------------------------|---------------------|--------------------------|----------------------|--------------|
| A       | 73   | 56.7                   | 58.0                | 0.64                     | 88.6                 | 90.6         |
| H       | 54   | 18.9                   | 10.0                | 0.59                     | 28.6                 | 16.9         |
| GP45    | 45   | 9.6                    | 5.3                 | 0.62                     | 15.5                 | 8.5          |
| C       | 40   | 2.25                   | 6.88                | 0.52                     | 4.33                 | 13.23        |
| F       | 14   | 0.87                   | 2.55                | 0.55                     | 1.58                 | 4.64         |
| G       | 16   | 2.13                   | 3.91                | 0.57                     | 3.74                 | 6.86         |

### TABLE II

Summary of subunit contacts identified by cross-linking of the intact V-ATPase and the V1 subcomplex

| Cross-linker | Product | Observed mass | Complex where identified |
|--------------|---------|---------------|--------------------------|
| DSG          | DF      | 50            | V1V0, V1                |
| DSG          | EG      | 46            | V1V0, V1                |
| DSG          | HE      | 90            | V1                      |
| DSG          | CE      | 75            | V1V0                    |
| DSG          | HF      | 70            | V1                      |
| EDC          | CE      | 75            | V1V0                    |
| EDC          | DE      | 67            | V1V0                    |
| MBP          | aE      | 135           | V1V0                    |

by DSG to both subunits E and F, although these products are observed only in the isolated V1 domain, suggesting that these contacts may be shielded in the intact V-ATPase. Alternatively, the V1 complex may distort relative to V1V0 to allow some subunit contacts to occur that do not normally occur in the
number of copies of the protein migrating at this position was measured using quantitative amino acid analysis (48). We have subsequently observed that only about 10–15% of the protein at this position actually corresponds to AP50, the remainder corresponding to the lower molecular weight form of subunit H, which is not detected by NH₂-terminal sequencing because it is blocked at the amino terminus. We therefore wished to determine whether treatment of the V-ATPase with cysteine also resulted in the loss of subunit H. As shown in Fig. 7, cysteine treatment caused nearly complete loss of the lower molecular weight form of subunit H with a partial reduction in the higher molecular weight form. Because this treatment results in nearly complete loss of activity, it appears that loss of either isoform of subunit H is sufficient to inactivate the enzyme. These two isoforms appear to be the product of alternative splicing of a single gene (34).

We have previously shown that cystine selectively modifies Cys-254 of the V-ATPase A subunit, leading to inactivation of the enzyme that can be reversed by treatment with diethiothreitol (33). It should be noted, however, that the activity of the V-ATPase depleted of subunit H by cysteine treatment cannot be restored by diethiothreitol (39) and this loss of activity is therefore not a direct result of the active site modification but rather loss of subunit H. Nevertheless, the results clearly indicate that there is intramolecular communication between the catalytic site of the enzyme (located on subunit A; Ref. 33) and the contact region between subunit H and the remainder of the complex. Such conformational cross-talk may play an important role in regulation of assembly or activity of the V-ATPases.

Our previous measurement of the subunit stoichiometry of the V-ATPase complex revealed that each V-ATPase molecule contains three copies of subunit A, three copies of subunit B, six copies of subunit C, and single copies of subunits C, D, E, a, d, and c⁻ (10). At the time, subunits F, G, and H had not been discovered and it was not known that the V-ATPase contained an additional proteolipid subunit of molecular mass 17 kDa (c⁻), as demonstrated first for the V-ATPase of yeast (17). In addition, it has been reported that the V-ATPase from chromaffin granules contains a 45-kDa integral membrane glycoprotein that has been cloned and sequenced (40). We have observed the presence of this polypeptide in the intact V-ATPase of clathrin-coated vesicles (49), as well as in the isolated V₀ domain, but there has been no report of a corresponding rma gene in yeast, despite the completion of the genome sequencing. We therefore wished to determine the copy number of the more recently discovered subunits of the V-ATPase and to determine whether the 45-kDa glycoprotein was in fact present in stoichiometric amounts in our preparation. Because different polypeptides display very different staining efficiencies, relative staining of proteins is a very unreliable method for determining subunit stoichiometry. Instead, we employed the method we had previously used involving electrophoretic separation and transfer to Immobilon followed by quantitative amino acid analysis (10).

As shown in Table I, subunit F and the lower molecular weight isoform of subunit H are present in single copies while subunit G is present in two copies per complex. Because Western blotting analysis suggests that the two isoforms of subunit H are present in equal amounts (Fig. 7), these results suggest that both isoforms of subunit H are present in one copy per complex. This result is consistent with the observation noted above that removal of just one of the subunit H isoforms is sufficient to lead to complete loss of activity, since if the two isoforms were present in different populations of V-ATPase, a less dramatic effect on activity would have been predicted. In addition, we find that the 45-kDa glycoprotein is present at a substoichiometric level, suggesting that it is only present in a subpopulation of the V-ATPases from coated vesicles. This is not entirely surprising, however, given that clathrin-coated vesicles are derived from both the plasma membrane and the Golgi, and that this 45-kDa polypeptide may be present in only one of these two populations.

Fig. 8 shows a revised structural model of the V-ATPase, incorporating both previous findings and the data presented in the current paper. Electron microscopic analysis of the V-ATPase from Clostridium fervidus (25) and from clathrin-coated vesicles shows that the peripheral stalk includes subunits C, E, G, and H, while subunits D and F are part of the central stalk. Both of these subunits show interaction with subunit E, which can also be cross-linked with the amino-terminal soluble domain of subunit a in the peripheral stalk. We speculate that the cross-link observed between subunits D and E in the intact complex may represent a point of very high helical content (50), we suggest that subunit D is the V-ATPase homolog to γ subunit in yeast, subunit F is the V-ATPase homolog to ε subunit, and subunit E is the V-ATPase homolog to δ subunit, which interacts with subunit C and H in the central stalk. By contrast, subunits C and H can be readily dissociated, suggesting that they form part of a peripheral structure. Both of these subunits show interaction with subunit E, which can also be cross-linked with subunit G in V₁ and subunit a of the V₀ domain. We therefore have placed subunits C, E, G, and H together with the amino-terminal soluble domain of subunit a in the peripheral stalk. We speculate that the cross-link observed between subunits D and E in the intact complex may represent a point of close contact between the peripheral and central stalks which may change during the course of catalysis. Such conformational changes in subunit contacts will be a key event during turnover of the enzyme. Such catalysis-dependent changes in subunit contacts have in fact been demonstrated for the F-ATPase complex using covalent cross-linking (22, 51). The picture presented in Fig. 8 thus represents a working model of the structure of the V-ATPases, which can serve as a basis for further experimental analysis.

FIG. 8 Structural model of the coated vesicle V-ATPase. Subunits in the peripheral V₁ domain are shown in white, while subunits in the integral V₀ domain are shown shaded. The central stalk is postulated to include subunits D (the likely V-ATPase γ subunit homolog) and F, while the peripheral stalk includes subunits C, E, G, and H. The model shown includes contacts identified in the current paper between subunits C and E, subunits D and F, subunits E and G, subunits H and E, and subunits a and E. The model also reflects the stoichiometry of two copies of subunit G, one copy of each isoform of subunit H, and one copy of subunit F per V-ATPase complex.

2 S. Wilkens and M. Forgac, submitted for publication.
REFERENCES

1. Forgac, M. (1999) *J. Biol. Chem.* 274, 12951–12954
2. Stevens, T. H., and Forgac, M. (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94, 1319, 12951–12954
3. Margolles-Clark, E., Tenney, K., Bowman, E. J., and Bowman, B. J. (1999) *J. Biol. Chem.* 274, 13888–13891
4. Kane, P. M. (1999) *Biochim. Biophys. Acta* 14836–14841
5. Anraku, Y., Umemoto, N., Hirata, R., and Ohya, Y. (1992) *J. Biol. Chem.* 267, 9184–9186
6. Adachi, I., Arai, H., Pimental, R., and Forgac, M. (1999) *J. Biol. Chem.* 274, 14655–14661
7. Adachi, I., Arai, H., Pimental, R., and Forgac, M. (1999) *J. Biol. Chem.* 274, 16618–16623
8. Ho, M. N., Hirata, R., Umemoto, N., Ohya, Y., Takatsuaki, A., Stevens, T. H., and Anraku, Y. (1999) *J. Biol. Chem.* 274, 18286–18292
9. Leng, X. H., Manolson, B. S., and Forgac, M. (1998) *J. Biol. Chem.* 273, 6717–6723
10. Arai, H., Berne, M., Terres, G., Terres, H., Puopolo, K., and Forgac, M. (1987) *Biochemistry* 26, 6606–6612
11. Vik, S. B., and Antonio, B. J. (1994) *J. Biol. Chem.* 269, 14655–14661
12. Adachi, I., Arai, H., Pimental, R., and Forgac, M. (1999) *J. Biol. Chem.* 274, 16618–16623
13. Ho, M. N., Hirata, R., Umemoto, N., Ohya, Y., Takatsuaki, A., Stevens, T. H., and Anraku, Y. (1999) *J. Biol. Chem.* 274, 18286–18292
14. Leng, X. H., Manolson, B. S., and Forgac, M. (1998) *J. Biol. Chem.* 273, 6717–6723
15. Cain, B. D., and Simoni, R. D. (1988) *J. Biol. Chem.* 263, 10479–10485
16. Adachi, I., Arai, H., Pimental, R., and Forgac, M. (1999) *J. Biol. Chem.* 274, 16618–16623
17. Ho, M. N., Hirata, R., Umemoto, N., Ohya, Y., Takatsuaki, A., Stevens, T. H., and Anraku, Y. (1999) *J. Biol. Chem.* 274, 18286–18292
18. Leng, X. H., Manolson, B. S., and Forgac, M. (1998) *J. Biol. Chem.* 273, 6717–6723
19. Vik, S. B., and Antonio, B. J. (1994) *J. Biol. Chem.* 269, 14655–14661
20. Adachi, I., Arai, H., Pimental, R., and Forgac, M. (1999) *J. Biol. Chem.* 274, 16618–16623
21. Ho, M. N., Hirata, R., Umemoto, N., Ohya, Y., Takatsuaki, A., Stevens, T. H., and Anraku, Y. (1999) *J. Biol. Chem.* 274, 18286–18292
22. Leng, X. H., Manolson, B. S., and Forgac, M. (1998) *J. Biol. Chem.* 273, 6717–6723
23. Vik, S. B., and Antonio, B. J. (1994) *J. Biol. Chem.* 269, 10479–10485
24. Myers, M., and Forgac, M. (1993) *J. Biol. Chem.* 268, 9184–9186
25. Myers, M., and Forgac, M. (1993) *J. Biol. Chem.* 268, 9184–9186
26. Peng, S. B., Stone, D. K., and Xie, X. S. (1993) *J. Biol. Chem.* 268, 23519–23523
27. Arai, H., Berne, M., Terres, G., Terres, H., Puopolo, K., and Forgac, M. (1987) *Biochemistry* 26, 6606–6612
28. Puopolo, K., Szczekan, M., Magner, R., and Forgac, M. (1992) *J. Biol. Chem.* 267, 5171–5176
29. Laemml, U. K. (1970) *Nature* 227, 680–685
30. Leng, X. H., Manolson, M., Liu, Q., and Forgac, M. (1996) *J. Biol. Chem.* 271, 22487–22493
31. Schagger, H., and von Jagow, G. (1987) *Anal. Biochem.* 166, 368–379
32. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265–275
33. Schagger, H., and von Jagow, G. (1987) *Anal. Biochem.* 166, 368–379
34. Zhou, Z., Peng, S-B, Crider, B. P., Slauughter, C., Xie, X. S., and Stone, D. K. (1998) *J. Biol. Chem.* 273, 5878–5884
35. Leng, X. H., Nishi, T., and Forgac, M. (1999) *J. Biol. Chem.* 274, 14655–14661
36. Adachi, I., Puopolo, K., Marquez-Sterling, N., Arai, H., and Forgac, M. (1990) *J. Biol. Chem.* 265, 967–973
37. Puopolo, K., and Forgac, M. (1990) *J. Biol. Chem.* 265, 14836–14841
38. Arai, H., Pink, S., and Forgac, M. (1989) *Biochemistry* 28, 3075–3082
39. Liu, Q., Peng, Y., and Forgac, M. (1994) *J. Biol. Chem.* 269, 31592–31597
40. Supék, F., Supékova, L., Mandiyan, S., Pan, Y. C., Nelson, H., and Nelson, N. (1994) *J. Biol. Chem.* 269, 24102–24106
41. Doherty, R. D., and Kane, P. M. (1993) *J. Biol. Chem.* 268, 16845–16851
42. Tsukazaki, S., and Forgac, M. (1992) *Biochemistry* 31, 5817–5822
43. Tomaszek, J. J, Garrison, B. S., and Klionsky, D. J. (1997) *J. Biol. Chem.* 272, 407–414
44. Tomashek, J. J, Graham, L. A., Hutchins, M. U., Stevens, T. H., and Klionsky, D. J. (1997) *J. Biol. Chem.* 272, 31592–31597
45. Tomashek, J. J, Garrison, B. S., and Klionsky, D. J. (1997) *J. Biol. Chem.* 272, 16618–16623
46. Ho, M. N., Hirata, R., Umemoto, N., Ohya, Y., Takatsuaki, A., Stevens, T. H., and Anraku, Y. (1999) *J. Biol. Chem.* 274, 18286–18292
47. Leng, X. H., Manolson, M., and Forgac, M. (1998) *J. Biol. Chem.* 273, 6717–6723
48. Cain, B. D., and Simoni, R. D. (1988) *J. Biol. Chem.* 263, 10479–10485
49. Vik, S. B., and Antonio, B. J. (1994) *J. Biol. Chem.* 269, 10479–10485
50. Myers, M., and Forgac, M. (1993) *J. Biol. Chem.* 268, 9184–9186
51. Adachi, I., Arai, H., Pimental, R., and Forgac, M. (1999) *J. Biol. Chem.* 274, 16618–16623