The coated vesicle (H\(^+\))-ATPase is composed of two domains, a peripheral Vo domain containing the 73 (A subunit)-, 58 (B subunit)-, 40-, 34-, and 33-kDa subunits and an integral Vo domain containing the 100-, 38-, 19-, and 17 (c subunit)-kDa subunits (Adachi, I., Puopolo, K., Marquez-Sterling, N., Arai, H., and Forgac, M. (1990) J. Biol. Chem. 265, 967-973). In the present manuscript we characterize the Vo domain with respect to its structural and activity properties. Glycerol density gradient separation of solubilized coated vesicle membrane proteins reveals the presence of an excess of Vo domains which migrate with a molecular weight of 250,000 and contain the Vo polypeptides in the same stoichiometry as in the intact V\(_{\text{V}}\) complex. Like the c subunit in V\(_{\text{V}}\), the c subunit of the free Vo domain is labeled by \([^{125}\text{I}]{\text{C}}\text{N},N'-\text{dicyclohexylcarbodiimide (DCCD)} and is extracted by chloroform:methanol. In addition, a monoclonal antibody specific for the 100-kDa subunit of the intact (H\(^+\))-ATPase recognizes the 100-kDa subunit of Vo. Tryptic cleavage of the Vo complex gives the same pattern of fragments for the 100- and 38-kDa subunits as in the intact complex, but with an increase in sensitivity, suggesting greater exposure of these subunits in free Vo. Proton conduction was measured in reconstituted vesicles containing the Vo domain and in native vesicles stripped of VI. No DCCD-inhibitable proton conduction was observed in either preparation, suggesting that unlike the corresponding Fo domain of F\(_{\text{F}}\)Vo, the free Vo domain is not an open proton channel.

EXPERIMENTAL PROCEDURES

Materials—Calf brains were obtained fresh from a local slaughterhouse. C\(_{12}\)E\(_9\), cholesterol, cholic acid, potassium iodide, DCCD, and ATP (grade II) were purchased from Sigma. Phosphatidylcholine and phosphatidylserine were obtained as chlortofm solutions from Avanti Polar Lipids, Inc. and stored at \(-20^\circ\text{C}\). Acridine orange was purchased from Eastman Kodak and 9-amino-3-chloro-2-methoxyacridine (ACMA) was purchased from Molecular Probes. p-Nitro blue tetrazolium chloride, 5-bromo-4-chloro-3-indolyl phosphate (toluidine salt), and affinity-purified goat anti-mouse IgG conjugated to alkaline phosphatase were obtained from Bio-Rad.

Preparation of Stripped Vesicles—Clastrin-coated vesicles were prepared from calf brain as described previously (28). Vesicles were stripped of their clathrin coat by dilution (40-fold) into 5 mM Tris (pH 8.5), 150 mM sucrose, and 0.5 mM EDTA followed by incubation for 1 h at 23 \(^\circ\text{C}\) and sedimentation for 1 h at 100,000 \(\times g\). Stripped vesicles displayed a 10-fold higher specific activity for the (H\(^+\))-ATPase relative to intact coated vesicles.

Glycerol Density Gradient Separation of V\(_{\text{V}}\) and V\(_{\text{V}}\) Complexes—Glycerol density gradient sedimentation was carried out by a modification of the procedure described previously (4). Stripped vesicles (1.0 mg of protein/ml) were solubilized with 1.0% C\(_{12}\)E\(_9\) containing 0.4 mg of phosphatidylcholine/ml and 0.2 mg of phosphatidylerine/ml in solubilization buffer (50 mM NaCl, 30 mM KCl, 20 mM HEPES (pH 7.0), 0.2 mM EDTA, 10% glycerol, 2 mM 2-mercaptoethanol) for 5 min at 23 \(^\circ\text{C}\) followed by 15 min at 4 \(^\circ\text{C}\). The insoluble material was removed by sedimentation at 150,000 \(\times g\) for 1 h in a Beckman SW 50.1 rotor, and 0.5 ml of the solubilized mixture was applied to an 11-ml linear glycerol gradient prepared in solubilization buffer containing 0.02% C\(_{12}\)E\(_9\), 8 \(\mu\)g of phosphatidylcholine/ml and spun for 16 h at 38,000 rpm in a Beckman SW-41 rotor. The resultant fractions were fractionated from the bottom using a peristaltic pump to give 19-20 fractions of 0.55-0.60 ml/fraction. The intact V\(_{\text{V}}\) complex, which has a molecular weight of 700,000-750,000 (17), typically peaks in fractions 4-6; whereas the dissociated Vo domain, containing properties (1), the vacuolar (H\(^+\))-ATPases also closely resemble the F\(_{\text{F}}\) class of (H\(^+\))-ATPases (13-16). This is true in terms of both the overall structure (17-19) and in the sequence homology observed between certain subunits (20-26). Thus the A and B subunits of the V-ATPases and the alpha and beta subunits of F, are all derived from a common ancestral nucleotide binding protein (20-25), whereas the c subunit of the V-ATPases appears to have arisen by a gene duplication and fusion of the gene encoding the corresponding c subunit of F\(_{\text{F}}\) (26).

Like the F-type ATPases, the V-ATPases are composed of a peripheral set of polypeptides (termed V\(_{\text{V}}\)) and an integral set of polypeptides (termed V\(_{\text{V}}\)) (19). We have demonstrated previously that these two domains could be dissociated and reassembled into a functional (H\(^+\))-ATPase (27). Because of the similarity between the V- and F-type ATPases, it was of interest to determine whether, as with the Fo domain, the Vo domain remained assembled as a discrete macromolecular complex in detergent. We also wished to compare the structural properties of the Vo subunits in the free Vo domain with those observed in the intact V\(_{\text{V}}\) complex. Finally, by analogy with Fo, we have investigated the ability of the Vo domain to act as a passive DCCD-inhibitable proton channel.

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Characterization of the Vo Domain of the Coated Vesicle (H\(^+\))-ATPase*

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* The abbreviations used are: (H\(^+\))-ATPase, proton-translocating adenosine triphosphatase; C\(_{12}\)E\(_9\), polyoxyethylene-9-lauryl ether; ACMA, 9-amino-3-chloro-2-methoxyacridine; DCCD, N,N'-dicyclohexylcarbodiimide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TLCK, 1-chloro-3-tosylamido-7-amino-2-heptanone; CCCP, carbonyl cyanide p-chlorophenylhydrazone.

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the 100, 38, 19, and 17 kDa subunits, typically peaks in fractions 10–12. Comparison with the sedimentation behavior of proteins of known molecular weight indicates the free V₀ complex has a molecular weight of approximately 250,000 (data not shown).

**Glycerol Density Gradient Sedimentation Analysis of the Peripheral V₁ Domain—** Dissociation of the peripheral V₁ domain was carried out essentially as described previously (27). Stripped vesicles (1.0 mg of protein/ml) were treated with 0.40 M KI and 5.0 mM ATP in buffer containing 20 mM HEPES (pH 7.0), 0.2 mM EGTA, and 2 mM 2-mercaptoethanol for 1 h at 4 °C and then sedimented for 1 h at 150,000 × g in a Beckman SW-50.1 rotor. The KI/ATP stripped membranes were then solubilized with C₁₂E₅ and the solubilized proteins separated by glycerol density gradient sedimentation as described above.

[¹⁴C]DDC labeling of the 17-kDa c Subunit of the V₁ Domain—[¹⁴C]DDC labeling of the 17-kDa c subunit of the V₁ domain was carried out as described previously for labeling of the c subunit of intact V₀V₁ complex (29). To 500 μl of the peak V₁ containing fraction from the glycerol density gradient (6.0 μg of protein) was added 50 μl [¹⁴C]DDC (specific activity 55 mCi/mmol), and the reaction was allowed to proceed for 30 min at 25 °C. The reaction was stopped by precipitation of the protein using 6% trichloroacetic acid, incubation for 30 min at 4 °C, sedimentation for 5 min at 10,000 × g, solubilization of the precipitated protein using Laemmli sample buffer and separation of the solubilized polypeptides on a 13.5% acrylamide gel as described below. Following electrophoresis, the gel was washed for 15 min in 10% acetic acid, 10% isopropyl alcohol, impregnated with 0.015% New England Biolabs Gel Stain and dried under vacuum, and exposed to Kodak XAR-5 film for 7 days at −70 °C using an intensifier screen.

**Chloroform/Methanol Extraction of the 17-kDa c Subunit of V₀—** Chloroform/methanol extraction was carried out essentially as described previously (28). 400 μl of the V₀ containing fraction (4.8 μg of protein) was placed in a 15-ml conical glass centrifuge tube to which was added 5 volumes of chloroform:methanol (2:1, v/v). The sample was vortexed vigorously and incubated on ice, with occasional vortexing, for 1 h. The sample was then spun for 5 min at 5,000 × g in a table top centrifuge, the upper aqueous phase and the interface (containing most of the denatured protein) were carefully removed, and the lower organic phase was transferred to a separate tube and dried under nitrogen. The extracted protein was then solubilized using 200 μl of Laemmli sample buffer and 30 or 60 μl were run on a 12.5% acrylamide gel as described below.

**Western Blot Analysis—** The presence of a 100-kDa polypeptide in the V₀ domain capable of reacting with a monoclonal antibody specific for the 100-kDa subunit of the intact V₀V₁ complex was demonstrated by Western blot analysis as follows. Membranes from which the clathrin had been stripped (1 mg of protein/ml) were solubilized with C₁₂E₅ and the solubilized proteins separated by glycerol density gradient centrifugation as described above with the exception that the solubilized proteins were solubilized with Laemmli sample buffer and applied to a 12% acrylamide gel. After electrophoresis, the proteins were transferred electrophoretically to nitrocellulose by blotting at 100 mA for 16 h at 4 °C using a Bio-Rad Trans Blot cell and a transfer buffer containing 25 mM Tris, pH 9.3, 192 mM glycine and 20% methanol.

After transfer, the blot was washed twice in TBS (20 mM Tris (pH 7.5), 0.5 mM NaCl) and then incubated for 30 min in TBS containing 1% gelatin. After blocking, the blot was washed twice in TBS (TBS containing 0.5% Tween 20) followed by incubation for 2 h in TBS containing 0.5% gelatin and a 1:50 dilution of the monoclonal antibody 3A6-D. The blot was then washed three times in TTBS and incubated for 1 h in TTBS containing 0.5% gelatin and a 1:1000 dilution of a goat anti-mouse IgG conjugated to alkaline phosphatase (Bio-Rad). After incubation with the secondary antibody, the blot was washed once in TTBS and twice in TBS and then incubated overnight in 10% acetic acid, 10% isopropanol, 0.2 mM EGTA, 10% glycerol, 0.5 mg of bovine serum albumin/ml, and 2 μM ACMA. To initiate proton flux, a membrane potential was generated by addition of 20 mM valinomycin. The resultant proton uptake was monitored by fluorescence quenching of ACMA using excitation and emission wavelengths of 410 and 490 nm, respectively. As a negative control, membranes from vesicles lacking protein were prepared and assayed in an identical manner. As a positive control, each vesicle preparation was tested for its ability to generate a membrane potential driven proton flux following addition of 1.0 μM of the proton ionophore CCCP.

**Stripped Vesicles (1.5 mg of protein/ml) that had been treated with KI and ATP as described above were loaded with potassium by permeabilization with 0.7% cholate, 0.4 μg of phosphatidylycerine/ml, 0.2 μg of phosphatidylserine/ml followed by dialysis against four changes of 200 volumes of 100 mM K₂SO₄, 20 mM HEPES (pH 7.0), 0.2 mM EGTA, 2 mM 2-mercaptoethanol, 10% glycerol, and the solubilized proteins separated by glycerol density gradient centrifugation.**

**RESULTS**

Fig. 1A shows the protein pattern obtained after solubilization of coated vesicle membrane proteins with C₁₂E₅ separation on 12–25% glycerol density gradients, and SDS-PAGE. As can be seen, the V₀ subunits of molecular masses 100, 38, 19, and 17 kDa migrate at two distinct positions on the density gradients. The heavier complex, which sediments with a molecular weight of approximately 700,000–750,000 (17), corresponds to the intact V₀V₁ complex and has the complete complement of nine subunits. The lighter complex (fractions 9–11), which by comparison with the V₀V₁ complex and other marker proteins, has a molecular weight of approximately 250,000, contains the V₀ subunits but not the V₁ subunits. There are also present a variety of contaminating proteins which do not peak with the V₀ subunits. We have demonstrated previously that the V₀ subunits are present in the V₀V₁ complex with a stoichiometry of (100,000), (38,000), (19,000), and (17,000) (17). As can be seen by comparing the relative staining intensities of the V₀ subunits in the two complexes, this same stoichiometry appears to apply to the free V₀ domain. If the vesicles are first treated with KI and ATP (Fig. 1B), the V₀ polypeptides (typically fractions 10–12) from either native membranes or membranes treated with KI/ATP were concentrated 2-fold using a Centricon 10 microconcentrator. To 1.0 ml of the concentrated V₀ was added 0.2 ml of 5% cholate, 4 mg of phosphatidylserine/ml in solubilization buffer followed by 0.34 ml 10% cholate, 4 mg of cholesterol/ml and 7 mg of phosphatidylinerine/ml in 150 mM NaCl, 2 mM 2-mercaptoethanol. The mixture was incubated for 5 min at 23 °C and then 10 min at 4 °C followed by dialysis for 2 days against five changes of 200 volumes of solubilization buffer using Spectrapor-2 dialysis tubing with a M₉ cut-off of 1,000–14,000. As previously described, reconstitution of the intact V₀V₁ complex under these conditions gave rise to reconstituted vesicles capable of ATP-dependent proton uptake as assayed by uptake of acridine orange (4).
subunits migrate entirely in the lighter complex. The slight shift in position of the V₀ complex in Fig. 1B relative to Fig. 1A corresponds to small variations between individual glycerol density gradient runs. These results suggest that the lighter complex corresponds to the free V₀ complex. B, stripped vesicles (1.0 mg of protein/ml) were treated with KI and ATP as described under "Experimental Procedures" and then solubilized with C₁₂E₉ and separated by glycerol density gradient sedimentation as described in A. The V₀ subunits appear only in the lighter complex which, on this gradient, peaks in fraction 11.

To test whether the 100-, 38-, 19-, and 17-kDa polypeptides migrating as a complex of 250,000 correspond to the authentic V₀ subunits, the following tests were performed. First, the ability of [¹⁴C]DCCD to label the 17-kDa c subunit was tested. As can be seen in Fig. 2A, the c subunit of V₀, like that in the intact V₁V₀ complex (29), was labeled by [¹⁴C]DCCD. In addition to labeling of the 17-kDa c subunit, a small amount of label was also observed at approximately 100 kDa. This may correspond to some labeling of the 100-kDa subunit, labeling of a different 100-kDa polypeptide, or to partial aggregation of the 17-kDa subunit, which is present in a stoichiometry of six copies per complex (17). In addition to labeling by [¹⁴C]DCCD, the 17-kDa polypeptide present in the V₀ containing fraction was extracted by chloroform:methanol (Fig. 2B), as demonstrated previously for the authentic c subunit (29). Thus in both its reactivity toward DCCD and its hydrophobicity, the c subunit of the V₀ complex was indistinguishable from the c subunit of V₁V₀.

As a further test of the relationship between these two complexes, Western blot analysis was performed using a monoclonal antibody specific for the 100-kDa subunit of the coated vesicle (H⁺)-ATPase. Fig. 3 shows that this monoclonal antibody recognized the 100-kDa subunit in both the intact V₁V₀ complex and in isolated V₀. Silver staining of a parallel SDS-PAGE gel run on the same gradient fractions indicated that, for this glycerol density gradient, the 38-, 19-, and 17-kDa polypeptides, like the 100-kDa immunoreactive band, peaked in fractions 6 and 12 (data not shown). This data provide strong evidence for the identity of the 100-kDa polypeptide in these two complexes.

To further characterize the V₀ domain, proteolysis was carried out on both the V₁V₀ and V₀ complexes in the detergent solubilized state. We have demonstrated previously that
FIG. 3. Western blot analysis of glycerol density gradient fractions of C17E6-solubilized stripped vesicle membrane proteins using the 100-kDa-reactive monoclonal antibody 3A-6D. Stripped vesicles (1.0 mg of protein/ml) were solubilized with C17E6 and the solubilized proteins separated by glycerol density gradient sedimentation as described in Fig. 1A. 60 μl of each fraction was applied to a 12% acrylamide gel and SDS-PAGE, and electrophoretic transfer to nitrocellulose and Western blotting using the monoclonal antibody 3A-6D and goat anti-mouse IgG conjugated to alkaline phosphatase were carried out as described under "Experimental Procedures." Silver staining of a second polyacrylamide gel run on the same gradient fractions indicated that the 38-, 19-, and 17-kDa polypeptides, like the 100-kDa immunoreactive band, peaked in fractions 8 and 12.

FIG. 4. Trypsin digestion of detergent-solubilized V1V0 and V0 complexes. Peak gradient fractions containing either intact V1V0 complex (lanes A–E) (1.8 μg of protein) or the free V0 domain (lanes F–J) (1.5 μg of protein) were treated with 0 (lanes A and F), 0.001 (lanes B and G), 0.01 (lanes C and H), 0.10 (lanes D and I), or 1.0 (lanes E and J) μg trypsin for 4 h at 23°C. Trypsin digestion was stopped by addition of 5 mM TLCK, and the samples were run on a 12% acrylamide gel followed by silver staining as described under "Experimental Procedures." The positions of the V0 subunits and the 80-kDa fragment of the 100-kDa subunit are indicated at the right of the figure.

trypsin treatment of the intact (H+)-ATPase generates an 80-kDa fragment of the 100-kDa subunit and cleaves the 38-kDa polypeptide approximately 1–2 kDa from the amino terminus (18). Moreover, both of these cleavage sites were shown to be present on the cytoplasmic side of the membrane (18). As can be seen in Fig. 4, the same proteolytic cleavage pattern is obtained for the 100- and 38-kDa polypeptides in the free V0 domain. The fact that these cuts happen at somewhat lower trypsin concentrations for V0 than for V1V0 suggests that there is greater accessibility of the protease to these polypeptides in the free V0 complex. That the same pattern is observed for both detergent-solubilized and reconstituted V0 (data not shown) indicates that, like the intact V1V0 complex, the free V0 domain reconstitutes primarily with the cytoplasmic face exposed.

To test whether the V0 domain could function as a passive proton channel, the following protocol was employed. The detergent-solubilized V0 domain from either untreated vesicles or vesicles which had been treated with KI and ATP was isolated by density gradient sedimentation as shown in Fig. 1 and then reconstituted into phospholipid vesicles by cholate dialysis as described previously (4). The vesicles were reconstituted in the presence of 150 mM KCl and then diluted into a potassium free buffer in order to establish a large potassium gradient across the membrane. The vesicles were incubated in the presence of the fluorescence dye ACMA, and the assay was initiated by addition of valinomycin. Because of the substantial potassium gradient present across the vesicle membrane, addition of valinomycin generated a negative interior membrane potential which acted as a driving force for proton uptake, which was monitored by fluorescence quenching of ACMA. Vesicles lacking protein acted as the negative control, whereas addition of the proton ionophore CCCP acted as the positive control. As can be seen in Fig. 5A, no greater passive proton transport was observed in vesicles containing V0 than was observed in vesicles lacking protein, despite the substantial fluorescence quenching observed on addition of CCCP. Moreover, treatment of reconstituted V0 with DCCD had no effect on this low background level of proton leakage (data not shown).

Because it is possible that the V0 domain may have become inactivated during the course of isolation and reconstitution, we also tested to see whether the V0 domain remaining in the native membrane after dissociation of V1 with KI and ATP could conduct protons. Native vesicles were loaded with potassium by permeabilization with cholate plus phospholipid followed by removal of the detergent by dialysis. We have observed that cholate is ineffective at solubilization of either the V0 domain or the intact V1V0 under these conditions (data not shown). As shown in Fig. 5B, the V0 domain in the native membrane is also incapable of DCCD-inhibitable proton translocation, despite the fact that, as we have demonstrated previously (27), this V0 is competent to reassemble with the V1 domain to give an active (H+)-ATPase complex.

DISCUSSION

The V0 domain of the coated vesicle (H+)-ATPase is composed of four subunits of molecular weight 100,000, 38,000, 19,000, and 17,000 (19). They are operationally defined as integral subunits by the observation that they remain attached to the membrane after dissociation of the peripheral V1 subunits by chaotropic agents such as KI and KNO3 (19).

In the present manuscript we provide the first demonstration that these integral subunits remain assembled as a complex following detergent solubilization. This complex has an approximate molecular weight of 250,000 and, by comparison with the intact V1V0 complex (17), has a subunit stoichiometry of one copy each of the 100-, 38-, and 19-kDa subunits and six copies of the 17-kDa subunit. The ability of the V0 subunits to remain assembled as a complex in detergent solution has also recently been reported for the vacuolar (H+)-ATPase from Neurospora (34).

As can be seen from Fig. 1, coated vesicles appear to contain an excess of V0 domains over what is required to form functional V1V0 complexes. It was therefore important to compare the properties of the polypeptides present in these "excess" V0 domains with the "authentic" V0 subunits to determine whether they were the same. According to the following criteria, the V0 polypeptides present in these two populations of V0 are identical: 1) [14C]DCCD labeling and chloroform:methanol extraction of the 17-kDa c subunit; 2) reaction of the 100-kDa polypeptide with a specific monoclonal antibody by Western blot; 3) trypsic cleavage pattern of both the 100- and 38-kDa subunits. With respect to proteolysis, it is interesting to note that although the same trypsic fragments of the 100- and 38-kDa subunits are generated, proteolysis appears to occur at lower trypsin concentrations for the free V0 domain relative to the intact V1V0 complex, suggesting that removal of the V1 domain has increased the accessibility
of the 100- and 38-kDa subunits to proteolytic cleavage.

Why there is an excess of $V_o$ domains in coated vesicles remains uncertain. It does not seem to be due to loss of the corresponding $V_i$ domains during dissociation of the clathrin coat or detergent solubilization and density gradient sedimentation of the $(H^+)$-ATPase, since Western blot analysis of fractions obtained throughout this procedure using the monoclonal antibody 3.2-F1 (specific for the peripheral $A$ subunit (30)) does not indicate any significant loss of $V_i$ during these steps (data not shown). It is possible that some loss of $V_i$ domains occurs during isolation of the clathrin-coated vesicles from bovine brain. Alternatively, there may exist a pool of unassembled $V_o$ domains in the cell. Consistent with this idea is the observation that in MDBK cells we are able to detect a pool of unassembled $V_i$ domains. The possible implications of these findings for regulation of vacuolar acidification are discussed below.

It has been demonstrated by a number of laboratories (35-37) that the $F_o$ domain of the $F_oF_1$ $(H^+)$-ATPases can act as a passive DCCD-inhibitable proton channel. Because of the similarity between the vacuolar and $F_oF_1$ classes of $(H^+)$-ATPase, both in overall structure (17-19) and in sequence homology (20-26), it was of interest to determine whether the $V_o$ domain, like $F_o$, could conduct protons. We investigated this question using both the isolated, reconstituted $V_o$ domain and native membrane vesicles from which the $V_i$ domains had been removed by treatment with $K_I$ and ATP (19). Proton movement was driven using a $K^+$/valinomycin-induced membrane potential and measured using uptake of the fluorescence dye ACMA. As can be seen in Fig. 5, in neither case did the $V_o$ domain exhibit DCCD-inhibitable proton translocation.

We have shown previously that following dissociation of the peripheral subunits with $K_I$ and ATP, the $V_o$ domain remaining in the membrane is competent to assemble with $V_i$ to form a functional $(H^+)$-ATPase (27). Thus treatment with $K_I$ and ATP has not rendered the $V_o$ domain nonfunctional. These results suggest that, unlike $F_o$, the free $V_o$ domain is not an open proton channel.

This absence of proton conduction by $V_o$ is interesting in a number of respects. First, it has been reported previously that the 17-kDa c subunit of the coated vesicle $(H^+)$-ATPase, when extracted with toluene and reconstituted into phospholipid vesicles, was itself competent to form a DCCD-inhibitable proton channel (38). Although surprising in light of the lack of channel activity of the isolated homologous c subunit of $F_o$ (31), if correct this result would suggest that one or more of the remaining $V_i$ subunits is supressing the channel activity of the c subunit. A second question concerns why if the fully assembled $F_o$ domain conducts protons, the corresponding $V_o$ domain does not. One possible answer is that the cell employs assembly of $V_iV_o$ as a mechanism of controlling vacuolar acidification. According to this model, acidification would be activated in a particular compartment by attachment of $V_i$ to free $V_o$ domains preexisting in the corresponding membrane. It would be important under these circumstances that free $V_o$ not conduct protons since otherwise, in any membrane containing multiple $V_o$ domains, it would be impossible to establish any significant $pH$ gradient until all of the available $V_o$ sites had been occupied. Whether free $V_o$ domains exist in the cell and what factors might control assembly of $V_i$ and $V_o$ in vivo remain important but unanswered questions.

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* M. Myers and M. Forgac, manuscript in preparation.
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