The structure of the vacuolar ATPase from bovine brain clathrin-coated vesicles has been determined by electron microscopy of negatively stained, detergent-solubilized enzyme molecules. Preparations of both lipid-containing and delipidated enzyme have been analyzed. The complex is organized in two major domains, a V₁ and V₀, with overall dimensions of 28 × 14 × 14 nm. The V₁ is a more or less spherical molecule with a central cavity. The V₀ has the shape of a flattened sphere or doughnut with a radius of about 100 Å. The V₁ and V₀ are joined by a 60-Å long and 40-Å wide central stalk, consisting of several individual protein densities. Two kinds of smaller densities are visible at the top periphery of the V₁, and one of these seems to extend all the way down to the stalk domain in some averages. Images of both the lipid-containing and the delipidated complex show a 30–50-kDa protein density on the lumenal side of the complex, opposite the central stalk, centered in the ring of c subunits. A large trans-membrane mass, probably the C-terminal domain of the 100-kDa subunit α, is seen at the periphery of the c subunit ring in some projections. This large mass has both a lumenal and a cytosolic domain, and it is the cytosolic domain that interacts with the central stalk. Two to three additional protein densities can be seen in the V₁-V₀ interface, all connected to the central stalk. Overall, the structure of the V-ATPase is similar to the structure of the related F₁F₀-ATP synthase, confirming their common origin.

A vacuolar ATPase (or V-ATPase) is found in the membrane of subcellular compartments of eucaryotic cells, where it functions to acidify the interior and at the same time energize the membranes of organelles such as clathrin-coated vesicles, endosomes, lysosomes, chromaffin granules, and Golgi-derived vesicles (1, 2). ATP hydrolysis-driven acidification of these organelles plays an important role in processes like receptor-mediated endocytosis, neurotransmitter release, protein trafficking, pH maintenance, and storage of metabolites. Early electron microscopic images (3–5) show the V-ATPase complex organized in two parts, a membrane extrinsic V₁ and a membrane-embedded V₀, named after the related F₁F₀-type ATP synthase. As in F₁F₀, ATP hydrolysis on the membrane extrinsic domain is coupled to proton translocation across the membrane bilayer, but unlike F₁F₀, the vacuolar enzyme cannot utilize the potential energy of a proton gradient to synthesize ATP. The V₁V₀-ATPase is composed of at least 12 different subunits. The V₁ contains subunits A–H with molecular weights of 73,000, 58,000, 40,000, 34,000, 33,000, 14,000, 10,000, and 50,000–57,000, and the V₀ contains subunits a, c, c’, and d having molecular weights of 100,000, 17,000, 19,000 and 38,000, respectively. The subunit stoichiometry of the complex is A₃B₃CDEF₃G₃H₂, giving a calculated molecular weight of approximately 840,000 (assuming one copy of subunits F, G, and H each), in good agreement with the value of 750,000 obtained from sedimentation experiments (6).

The vacuolar ATPase is similar in its structure to the well characterized F₁F₀-ATP synthase, and it is believed that both proteins evolved from a common ancestor (7, 8). Significant sequence similarities for the two ATPases exist for the nucleotide binding domains of the large subunits and for the proteolipids. Based on this structural similarity, it is assumed that both proteins also have a similar mechanism; however, little is known about the details of this process in the vacuolar enzyme. Biochemical studies conducted with the yeast and coated vesicle enzyme seem to confirm this idea; however, it is unclear at this point as to how far this similarity goes, considering the lack of significant sequence homology for any of the single copy “accessory” subunits of the two proteins. Whereas a high resolution structural model exists for the largest part of the F-type ATPase, there is essentially no detailed structural information available at all for the vacuolar ATPase at this point.

In the present study, we have used electron microscopy and single molecule image analysis to initiate a structural analysis of the V-ATPase from clathrin-coated vesicles. Based on the images and the known subunit stoichiometry of the enzyme, a refined model of the subunit arrangement of the vacuolar ATPase complex and its functional implications are discussed.

**EXPERIMENTAL PROCEDURES**

**Materials**—The vacuolar ATPase from clathrin-coated vesicles has been isolated in the presence or absence of added phospholipid as described (9). Both lipid-containing and delipidated preparations are fully active in ATP hydrolysis assays, and proton pumping activity can be restored by reconstituting the enzyme into phospholipid vesicles. The subunit composition of the preparations used for electron microscopy was determined by polyacrylamide gel electrophoresis in the presence of SDS.

**Electron Microscopy**—Protein was prepared on freshly glow-discharged carbon-coated copper grids and stained with 1% uranyl acetate. Images of the negatively stained V-ATPase complex were recorded on a Philips CM300 transmission electron microscope operating at 100 kV under “low dose” conditions with an electron optical magnification of ×47,000. Images were recorded on Kodak SO163 plates or a Gatan 1024 × 1024 CCD camera at a defocus value of −0.576 μm.

**Image Analysis**—Electron micrographs were scanned on an Optronics Color Getter Plus drum scanner with a sampling rate of 18.75 μm corresponding to a step size of 4 Å on the specimen level. All subsequent image analysis steps were performed within the IMAGIC 5 software package (10). Single molecules were selected interactively and extracted as 100 × 100 pixel images. Initial data sets were collected from 1024 × 1024 pixel CCD frames (lipid containing and delipidated enzyme). Two large data sets (from two delipidated V-ATPase prepara-
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**RESULTS AND DISCUSSION**

Overall Structural Features—Fig. 1 shows typical electron micrographs of the negatively stained coated vesicle V-ATPase, purified in the presence (Fig. 1A) and absence (Fig. 1B) of added phospholipids. As can be seen from Fig. 1, the protein is highly monodisperse in both preparations, a prerequisite for single particle image analysis. Most of the molecules are oriented on the carbon film in the "side view" position, which is a projection perpendicular to the long axis of the complex. In an initial step, small data sets of molecules in the side view orientation for each of the lipid-containing and lipid-free enzyme have been analyzed. Fig. 2 shows total averages calculated from the aligned data sets. The main difference between the two images is the appearance of the lower portion of the complex. This lower portion is ill defined in the image showing the complex in more detail, two data sets from two independent enzyme preparations (5,400 and 5,800 molecules, respectively) have been collected and subjected to objective alignment procedures followed by multivariate statistical analysis and classification to extract all the different projections of the V-ATPase. The 5,400 molecule data set was first mass centered and treated by alignment by classification, leading to a set of signal improved averages calculated from molecules having the same orientation “by chance.” The set of signal improved images was then treated by the double self-correlation function procedure resulting in one final average that was used as a reference in a first direct alignment step. By following this procedure, no raw image of a single enzyme molecule has to be used as initial reference for an alignment step, thus avoiding any form of bias toward potentially insignificant features of a noisy image. The direct alignment was iterated three times, and the aligned data set was treated by MSA/classification and sorted into classes of similar images. The quality of the aver-

![Fig. 1. Electron microscopy of the V-ATPase from bovine brain clathrin-coated vesicles. Enzyme was prepared in the presence (A) or absence of phospholipids (B) and stained with 1% uranyl acetate for electron microscopy. Electron optic magnification is × 47,000.](image1)

![Fig. 2. Total averages calculated from 890 lipid-containing molecules (A) and 980 of the lipid-free complexes (B).](image2)
aged classes of images (class sums) was further improved by using such class sums as references in a "multi-reference alignment" step. Analysis of the second data set was initiated by a direct alignment with the symmetrized sum of the first data set as a reference image, followed by MSA/classification and multi-reference alignment. The analysis of the two data sets resulted in virtually identical final averages (not shown). Class sums of the first, smaller data set had a slightly better resolution, but at the same time, about 1500 molecules of the data set did not align to produce signal improved averages and were therefore removed before the final MRA. The second, slightly larger data set was of better quality in terms of particle homogeneity, and no molecules had to be discarded from this data set.

Fig. 3 shows the most characteristic views of the V-ATPase obtained after the final MRA of the larger data set. All the averages show similar overall features as the total averages in Fig. 2, including the central cleft in the V₁, the doughnut-shaped V₀, and the 30–50-kDa lumenal density. In addition to these more or less invariant features, there are three areas of the molecule that look different in the projections depending on the orientation of the molecule along its long axis. These regions are the top periphery of the V₁, the stalk domain, and the periphery of the V₀.

**Structural Details of the V₁**—A 10–20-kDa elongated density can be seen attached at the top periphery of the V₁. Depending on the orientation of the complex on the carbon film used for electron microscopy, this elongated density can be seen either on the left (Fig. 3, images 1, 2, 4, 5, and 7, see black arrow in image 1) or on the right side (Fig. 3, images 3, 8, and 9, see black arrow in image 3). The appearance of the top part of the V₁ with respect to the rest of the molecule can be most straightforwardly explained on the basis that there are not one but three of the elongated densities and that in most averages two of these overlap in projection (see white arrow in image 4). If this were indeed the case, it would be reasonable to assume that the three copies of the 10–20-kDa density bind to either one of the large subunits, so the three copies would be attached alternatingly in the hexagonal arrangement of the A and B subunits. A 60° rotation around the long axis of the molecule would then make one of the three densities disappear on one side of the V₁ and a second one appear on the other. Images 5, 6, and 9 represent such a 60° rotation. In image 5, the elongated density is on the left, the same side as the strong peripheral stalk protein. An intermediate projection can be seen in image 6, in which the elongated density on the left is already hidden behind the V₁. At the same time, the strong peripheral stalk protein on the left is getting weaker in image 6 (see white arrow), whereas the two densities on the right, which are barely visible in image 5, are getting stronger (see black arrows). This trend is continued in image 9, in which the elongated density is now on the right and the two densities on the right of the central stalk are now clearly visible.

In addition to the 10–20-kDa elongated density, a second, "knob"-like density can be seen at the periphery of the V₁, always on the opposite side of the 10–20-kDa protein (see white arrowheads in images 1 and 3). The fact that this smaller density is always bound opposite the 10–20-kDa density indicates that there are three copies of this density as well. The possibility that there are not three but only one copy of the elongated and knob-like proteins and that images 1 and 3 or 7 and 8 are simply related by a 180° rotation around the long axis can be ruled out based on the accompanying variation of the projected densities in the stalk region described above.

The preferred orientation of the complex results in the so-
called “bilobed” view, in which each three of the large subunits overlap in projection leading to the central cleft or cavity seen in the total averages shown in Fig. 2. By classifying the large data set (5800 molecules) using only the image area corresponding to the V1 and sorting the data set into 36 classes, an average can be obtained which represents the so called “trilobed” view, in which each 2 large subunits are overlapping in projection (see Fig. 5, image 1). In this trilobed view, there are two of the elongated densities visible at the top of the V1 (see white arrowheads), consistent with above consideration. By looking at the left of the elongated densities in this image, it becomes obvious that the knob-like density described above and the elongated density belong together and that their different appearance in all the averages shown in Fig. 3 is a consequence of the predominantly bilobed orientation of the complex.

The trilobed projection of the bovine brain V-ATPase shown in Fig. 5, image 1, is very similar to averages obtained for the membrane-bound V-ATPase from Clostridium perfringens (12, 13). In these membrane-bound samples, the orientation of the complex is apparently less influenced by the interaction with the carbon film, leading to a higher abundance of this projection.

A possible interpretation as to what the elongated and knob-like densities can be obtained by comparison with the related F0F1-ATP synthase. There are about 90 amino acids near the N terminus of the V-ATPase A subunit for which there is no equivalent sequence in the bacterial F-ATPase β subunit (14). The insertion of this so-called “non-homologous region” into the mitochondrial F-ATPase β subunit occurs between conserved residues Pro-121 and Leu-133. According to the crystal structure of the mitochondrial F1-ATPase (15, 16), these residues form a loop at the outer periphery of the β subunit at the interface to an α subunit on a level just above the nucleotide-binding sites. Based on the sequence homology for the nucleotide-binding subunits of the V- and F-ATPase, it is believed that the V-ATPase A and B subunits have a similar three-dimensional fold as the β and α subunits and that they form a similar hexagonal arrangement as the αβγ complex.2

The position and size of the knob-like and elongated proteins at the periphery of the V1 would therefore suggest that these densities are at least in part formed by the non-homologous inserts near the A subunit N terminus. Also part of the elongated protein could be the F or G subunits of the V1 (both 10–15 kDa based on SDS-gel electrophoresis and sequence data); however, recent stochiometry measurements conducted with the coated vesicle enzyme indicate the presence of only a single copy of subunit F and two copies of G.3 A weak homology between the F-ATPase b subunit and chromaffin granule V-ATPase G subunit has been described (17). This leads to the proposal that subunit G might be part of the second stalk or stator in the V-ATPase (see below).

Structural Details of the V0—The subunit composition of the coated vesicle V0 is acβγc′d′. In most images shown in Fig. 3, the V0 seems to be a rather symmetric flattened sphere with a radius of approximately 100 Å. It is generally believed that the six ε subunits of the V0 form a ring-like structure in which the six 4-helix bundles of the proteolipids are arranged hexagonally around a central cavity. Such an arrangement of the proteolipids had been proposed based on electron microscopy and molecular modeling studies conducted with gap junction-like sheets from Nephros norvegicus, which are formed entirely by the V-ATPase c subunit (18). The diameter of the ε subunit hexamer according to the above study was 88 Å. The slightly larger size of the V0 described here might be due to bound detergent and residual lipid and the other membrane-bound subunits, α, c′, and d.

A large protein can be seen attached at the periphery of the V0 in some averages (Fig. 3, images 2, 7, 8, and 9), most prominently in image 7 (see black arrow). This density has both a small luminal and a small cytosolic domain. The cytosolic portion of this V0 subunit is connected to the central stalk via an elongated polypeptide (for a description of the stalk domain, see below). This large, peripheral V0 protein can only be the 100-kDa α subunit, since there is no other trans-membrane V0 subunit with that size. The vacuolar ATPase a subunit is a two-domain protein with a hydrophilic N-terminal half and a mostly hydrophobic C-terminal part. The C-terminal half contains 6–9 predicted trans-membrane helices, and it has been suggested that this portion of the a subunit is the homologue to the c subunit in F1F0 (19). The topology of the V0 a subunit has recently been determined for the yeast enzyme (20). The data suggest that there are nine trans-membrane α-helices in the C-terminal half of the α subunit and that the hydrophilic N-terminal portion is exposed to the cytosolic side of the membrane. Furthermore, approximately 10–15 kDa including the very C terminus are exposed to the luminal side of the membrane, consistent with the small density seen in image 7 (see small white arrowhead). A very similar arrangement of the a subunit of the related F0F1-ATP synthase had been proposed based on electron microscopic images of the F0 (21) and F1F0 (22). Not all the averages shown in Fig. 3 reveal the large V0 density as clearly as image 7, but the asymmetry of the V0 (see white arrowheads in images 2, 8, and 9) indicates its presence. In the averages that do not show any asymmetry of the V0 (see e.g. image 1 in Fig. 3), the large V0 subunit might be hidden in

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2 A preliminary analysis of electron microscopic images of the soluble V1 domain embedded in amorphous ice show a hexagonal arrangement of six large densities around a central cavity, in support of above assumption (S. Wilkens and M. Forgac, unpublished data).

3 T. Xu and Forgac, M., manuscript in preparation.
front or behind the projection of the c subunit ring. A second possibility is that in this class of molecules, the large transmembrane mass is dissociated from the complex as a result of the negative stain treatment. Only the analysis of ice-embedded enzyme molecules by cryoelectron microscopy, studies ongoing in our laboratory, will be able to avoid such potential break-down artifacts caused by the negative staining technique.

All averages in Fig. 3 show a 30–50-kDa protein density on the luminal side of the complex, centered in the ring of c subunits (see white arrow in image 2). A possible candidate for this density is the accessory polypeptide Ac45, originally described for the enzyme from chromaffin granules (23). The primary sequence of Ac45 predicts a C-terminal trans-membrane α-helix and potential N-glycosylation sites, suggesting a luminal orientation of the polypeptide. Although Ac45 has been found in the coated vesicle enzyme, it seems to be present in only about 50% of the enzyme molecules. It is therefore possible that part of this density is formed by the luminal portion of the V_{o} a subunit C terminus (see above). According to the above-mentioned model for the arrangement of the proteolipids, the central cavity is lined with polar residues from the first α-helix of the 4-helix bundle (24). Such a polar channel most certainly could not offer any insulation against the proton gradient, so it is obvious that the central cavity of the proteolipid hexamer has to be occluded in vivo. It is possible that the above described luminal density might have such a “sealing” function for the aqueous pore, but there must be other sealing mechanisms in the vacuolar enzyme since no homologue of Ac45 is present in the yeast V-ATPase. As can be seen in Fig. 4, all these subunits are present in the enzyme preparations analyzed by electron microscopy.

FIG. 5. Image 1, average of 97 trilobed projections. To identify this minor class in the large data set, only the image area corresponding to the V_{l} was used for the MSA calculation. Images 2–4, averages showing details of the stalk domain more clearly. The images have been obtained by sorting the data sets into 24 classes. The averages were calculated from 149, 152, and 114 images, respectively. Images 1 and 4 were calculated from the data set containing 5800 molecules, and the other two averages are from the data set containing 5400 images.
complex in vitro (27), and for the yeast system, an interaction between subunit pairs DF and EG has been described (28). Of the accessory proteins of the V0, only subunit H is dispensable for assembly of the complex (29). Removal of this subunit from the assembled complex leads to an inactivation without disrupting the V1/V0 interface (30), indicating a somewhat peripheral location for the subunit. In some averages, a large, ill-defined density can be seen on the side of the large transmembrane mass, interacting with the bottom of the V1 (see black arrowhead in image 9 of Fig. 3 and white arrowhead in image 4 of Fig. 5). The size and peripheral location would match the size of the 100-kDa H heterodimer, but also the 50-kDa N-terminal portion of the V0 subunit a might be part of this density. Except for subunit H, all the other accessory polypeptides are required for a structural and functional assembly of the catalytic sector with the membrane domain. Consequently, in current models, all of these subunits are therefore placed into the stalk domain in the interface between the V1 and V0/0p. However, at the current level of resolution in the two-dimensional projections, the few biochemical data do not help to unambiguously assign any of the stalk proteins. We are currently in the process of imaging V-ATPase complexes tagged with monoclonal antibodies against individual subunits in order to define the arrangement of these subunits in the coated vesicle enzyme.

Overall, the central stalk region of the V-ATPase is much more complex than the same region in the ATP synthase due to the larger variety of single copy accessory subunits in the vacuolar enzyme. ATP hydrolysis-driven proton pumping in the vacuolar ATPase is regulated by a reversible dissociation and association of catalytic sector and proton channel in vivo (31). This process has to be obviously controlled via the stalk proteins, which might be one reason for the more complicated structure of the V1-V0 interface.

The Second Stalk—It is generally believed that ATP hydrolysis-driven proton pumping in the related F1F0-ATP synthase involves a rotation of a domain formed by γ (Escherichia coli nomenclature) and possibly the subunit ring relative to the static remainder of the complex formed by αββαββ (32–36). In this model, the two b subunits bound to the a subunit in the F0, and the δ subunit in the F1 form a second stalk or stator keeping the two active domains of the complex in the correct spatial arrangement for energy coupling to occur. This second stalk or stator has only recently been observed by electron microscopy of negatively stained samples for both the V- and F-ATPase (12, 13, 27). Weak densities can be seen connecting the elongated proteins at the top of the V1 to the stalk region in some images (see small black arrows in images 2 and 5 of Fig. 3). In some averages (e.g. image 5 in Fig. 3), the second stalk seems to emerge from the strong density at the left periphery of the central stalk, whereas in image 2 of Fig. 3, it seems to be connected to the central stalk via a weak density all the way. This difference in the appearance of the stator could be caused by a slight rotation along the long axis of the molecule, a variation in the stain distribution in the stalk area or a negative stain-induced dissociation of the peripheral subunit, but it could also mean that there are two (or even three) “second” stalks in the V-ATPase.

As mentioned above, in the F-ATPase, the second stalk is formed by the δ and b subunits. In this model, the δ subunit binds at the top of the F1 molecule and interacts with the C-terminal portion of the two b subunits (38, 39). It is a possibility that in the V-ATPase, the above-mentioned non-homologous regions in the A subunit N termini could play the part of the δ subunit in the F-ATPase and that the two G subunits, possibly as a dimer, connect the A subunit inserts to the membrane domain. Although in the F1F0-ATP synthase, the second stalk clearly emerges from the membrane at the interface of the a and c subunits, the situation is different in the V-ATPase. In all the images showing a second stalk, there seems to be no clear connection of the stalk forming proteins into the membrane portion of the V0. A very similar looking picture, in which a weak density is running down on the side of the V1 to end in a subunit bound to the central stalk, not in the membrane, has been obtained for the Na+-transporting V-type ATPase from C. feroxius (12, 13). Whether this clear difference in the appearance of the second stalk in the images of the V- versus F-type ATPase is due to staining artifacts, or a genuine structural and possibly functional difference between the two related enzymes, requires further study.

Fig. 6 shows our current working model of the vacuolar ATPase based on the available biochemical data and the images presented here. In order to unambiguously define the subunit architecture in the V-ATPase, the two-dimensional projections of the protein will have to be combined to reconstruct a three-dimensional model of the complex. These studies are ongoing in our laboratory.

Note added in proof—After submission of this manuscript, a paper was published by Boekema et al. (Nature 401, 37–38) describing electron microscopic evidence of two “second stalks” in the V-ATPase from C. feroxius. In another paper, Rademaker et al. (FEBS Lett. 453, 383–386) provide evidence for a hexagonal arrangement of the A and B subunits in the V0 domain.

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