Three-dimensional Structure of the Vacular ATPase Proton Channel by Electron Microscopy*

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Vacular ATPases are ATP hydrolysis-driven proton pumps found in the endomembrane system of eucaryotic cells where they are involved in pH regulation. We have determined the three-dimensional structure of the proton channel domain of the vacular ATPase from bovine brain clathrin-coated vesicles by electron microscopy at 21 Å resolution. The model shows an asymmetric protein ring with two small openings on the luminal side and one large opening on the cytoplasmic side. The central hole on the luminal side is covered by a globular protein, while the cytoplasmic opening is covered by two elongated proteins arranged in a collar-like fashion.

In eucaryotic cells, acidification and energization of organelles such as Golgi-derived vesicles, clathrin coated vesicles, synaptic vesicles, lysosomes, and the plant vacuole are accomplished by a vacular type ATPase (V-ATPase)1, and its proton pumping action plays a vital role in processes like protein trafficking, receptor-mediated endocytosis, neurotransmitter release, intracellular pH regulation, and waste management (1–3). The vacular ATPase contains a large cytoplasmic ATPase domain (V1) and a membrane-embedded proton channel, V0. The two parts are connected via a stalk structure that functions to transmit the energy released during ATP hydrolysis taking place on the V1 to drive proton translocation across the membrane-bound V0. The vacular ATPase contains a large cytoplasmic ATPase domain (V1) and a membrane-embedded proton channel, V0. The two parts are connected via a stalk structure that functions to transmit the energy released during ATP hydrolysis taking place on the V1 to drive proton translocation across the membrane-bound V0. The vacular ATPase is composed of 13 different polypeptides, with molecular masses ranging from 13 to 100 kDa. Eight of these, subunits A-H, form the cytoplasmic ATPase domain, while the membrane-embedded proton channel is made of the remaining five, subunits a, c, c’, c'' and d. A 14th subunit, Ac45, which is associated with the membrane-bound domain, is present in the mammalian enzyme in some tissues (4).

The V-ATPase is structurally and functionally related to the F-ATPase, and it is believed that both enzymes have evolved from a common ancestor (5). In case of the F-ATPase, a high resolution crystal structure exists for the cytoplasmic domain (6), and based on the degree of sequence identity between the catalytic subunits of the F- and V-ATPase, it is likely that the V1-ATPase catalytic core has a similar three-dimensional-fold. The functional element proton channel is less well understood mainly because there is no detailed structure available for the intact membrane domain of either V- or F-ATPase. It is generally assumed that the c subunits are arranged in a ring-like fashion with the a subunit bound at the outside of the ring, and there is evidence for such an arrangement from electron microscopic images of both enzymes (7, 8). Furthermore, it is believed that the proton channel is formed at the interface of the a and c subunits and that a rotation of the c subunit ring against the a subunit is an integral part of the mechanism of ATP hydrolysis-coupled proton translocation (9). The structure of the proton channel-forming c subunit of the F-ATPase from Escherichia coli has been determined by nuclear magnetic resonance (NMR) spectroscopy (10), and it is known from low resolution x-ray (11), atomic force microscopy, (12) and electron microscopy studies (13) that isolated F-ATPase proteolipids from different species are able to form symmetric oligomeric rings of between 20 and 28 transmembrane α helices. The same is true for isolated V-ATPase proteolipids in that they are able to form symmetric six-membered rings as seen in ordered gap junction sheets from Nephrops norvegicus, which are entirely formed by a protein identical to the V-ATPase c subunit (14).

The situation in the V-ATPase membrane domain is somewhat more complex in that its c subunit ring contains three different proteolipid isoforms, namely c, c’, and c”. This means that the proteolipid ring in the V0, in contrast to its F-ATPase counterpart, is most likely asymmetric depending on whether all three proteolipid isoforms are part of the ring and what their stoichiometry is. While the ring-like arrangement of the isolated proteolipids is now well established, there is essentially no structural information available with regard to the intact, subunit a-containing proton channel. Here we report the three-dimensional structure of the V0 domain determined at a resolution of 21 Å by angular reconstitution from electron microscopic images of a negatively stained specimen. The model is discussed in the context of the conventional proton pumping function of the V0 but also with respect to a recent report that the free V0 domain may play an important role in vacular membrane fusion (15).

EXPERIMENTAL PROCEDURES

Purification of the V0 Domain—The V0 domain of the vacular ATPase from bovine brain clathrin-coated vesicles has been purified in absence of added phospholipids as described (16).

Electron Microscopy—Purified V0 domain was diluted 10–20-fold with buffer containing 20 mM HEPES/NaOH, pH 7, 50 mM NaCl, 20 mM KCl, and 0.5 mM EGTA and applied to freshly glow-discharged carbon-coated copper grids. Protein was stained with 1% uranyl acetate. Grids were examined in a Philips CM300 transmission electron microscope operating at 100 kV in “low dose” mode. Electron micrographs were recorded at a magnification of ×47,000 with an underfocus of –0.38 μm on Kodak SO193 film.

Image Analysis—Electron micrographs were digitized on an Owonics ColorGetter drum scanner at a sampling rate of 18.75 μm corre-

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‡ The abbreviations used are: V-ATPase, vacuolar type ATPase; MSA, multivariate statistical analysis.
Three-dimensional Reconstruction—After several rounds of multireference alignment, 33 of the most characteristic projections were chosen as a starting set for the three-dimensional reconstruction (13 side- and 20 top-view projections). The images were normalized and centered with the “self-center” option, a circular mask with soft edges was applied, and the image size was reduced to 50 × 50 pixels. A first set of Euler angles was determined using the option “C1-start up” as implemented in IMAGIC 5. From these initial angles, a three-dimensional model was calculated. This first model was then back projected to produce 30 views of the molecule with projection angles uniformly distributed over the entire Euler sphere. The Euler angles of the 33 input projections were then refined against the set of back projections (anchor refinement). The anchor refinement was iterated with an increasing number of back projections and a decreasing angular increment (down to 1° steps) until no further improvement of the errors in the reprojections was obtained. The final anchor set was then used to introduce an extended set of 61 input projections. New rounds of anchor set refinement were performed leading to an improved anchor set that was then used to assign angles to 81 and later on to 97 projections (36 projections from the side view and 61 projections from the top view set of images). The final model was then forward projected to produce 50 projection images with Euler angles distributed uniformly over the Euler sphere. The 50 forward projections were used as references in a subsequent multireference alignment step with the data set of all 10,259 molecules at the same time. The aligned data set was sorted into 121 classes, and Euler angles to the 109 best class sums were assigned based on the final three-dimensional model. The procedure was iterated until in the end, 288 forward projections were used as references leading to a final number of 220 input projections from which the final reconstruction was calculated.

Resolution of the Reconstruction—The resolution of the final reconstruction was estimated by calculating the Fourier shell correlation between two reconstructions calculated from each 110 of the final 220 input projections. A cut-off of 0.5 correlation (18) and the 3σ criterion implemented in IMAGIC 5 (19) were chosen to estimate the resolution in the final model.

RESULTS AND DISCUSSION

Free V-ATPase membrane domain can be found in endosomal membranes including the membranes of bovine brain clathrin-coated vesicles where it exists in excess over the intact V-ATPase complex (16). In contrast to the F-ATPase proton channel, the free V₀ complex does not function as a passive proton pore (16, 20) and it can be purified as a stable complex containing subunits α, c, c', c'', d, and Ac45 (16). Fig. 1A shows SDS-polyacrylamide gel electrophoresis of clathrin-coated vesicles, isolated in absence of added phospholipids as described (16). The lanes contain ~2 μg of protein and the bands were visualized by silver staining. The right lane was allowed to develop for a longer time to visualize Ac45, which migrates as a diffuse band in polyacrylamide gels (see also Ref. 4). B, schematic structure representation of the subunits contained in the coated vesicle V₀. The orientation is such so that the globular domain of Ac45 is on the luminal side of the complex (bottom), while the N-terminal domain of subunit α and subunit d are facing the cytoplasm (top). Stoichiometry measurements indicate that the V₀ each contains one copy of subunits α, c, c', c'', d, and Ac45 and four to five copies of subunit c. For details see “Results and Discussion.”
domain used in this study. Glycoprotein Ac45 tends to run as a rather diffused band on polyacrylamide gels (4) and is best visualized in the gel by prolonged development of the silver stain (right lane in Fig. 1A). Fig. 1B gives a graphic representation of the subunits contained in the coated vesicle V0. The α subunit is a two-domain protein with a hydrophilic N-terminal and a membrane-bound C-terminal domain. Studies conducted with the yeast enzyme indicate that the C terminus contains nine transmembrane α helices while the N-terminal half forms a domain exposed to the cytoplasm (21). The c and c′ subunits contain four predicted membrane-spanning α helices, and it is assumed that the corresponding gene has evolved through a fusion of an ancestral two-transmembrane α helix proteolipid preserved in the F-ATPase (5). The larger c isoform displays about 30% sequence identity to the c isoform and contains five predicted transmembrane α helices. All three isoforms contain lipid-exposed glutamate residues (c contains two, but only one is essential; (22)) and these glutamates together with polar residues in the C-terminal domain of the α subunit are essential for proton pumping (22, 23), suggesting that all three isoforms are part of the proteolipid ring. Subunit d is not membrane-anchored and may be connected to the V0 by interaction with the α subunit cytoplasmic domain (24). Glycoprotein Ac45 is predicted to be oriented toward the luminal side of the complex anchored to the membrane by a single α helix in the C terminus of the polypeptide (4).

The stoichiometry of the V0 subunits has been determined by quantitative amino acid analysis for the coated vesicle V0 to α1cα2c′α′ and d1, respectively, assuming that the polypeptide migrating with an apparent molecular mass of 19,000 is subunit c (25, 26). Consistent with this assumption are studies in yeast that indicate that both the c′ and c isoforms are present in each one copy per complex (27). Ac45 was found to be present in varying amounts (26), possibly a result of the fact that clathrin-coated vesicles are derived from both the plasma membrane and the Golgi and that Ac45 might be present only in one of these membranes.

Fig. 2 summarizes electron microscopy and image analysis of the V0. Fig. 2A shows electron microscopy of the negatively stained V0 domain. In the images, the complex appears as a rather featureless globular object. Close inspection of the projections showed that the majority of the molecules had the appearance of a small disc with small globular densities on each side while others seemed to be more or less circular with no discernible features (see arrows in Fig. 2A). A data set of 10,259 V0 images was collected and subjected to single particle image analysis with the IMAGIC 5 package of programs (17). Fig. 2B shows two averages that were obtained by the bias-free “alignment by classification” protocol (28). Members of the class whose average is shown in image 2 of Fig. 2B are oriented on the carbon film to produce the so-called side-view projection in which the complex is seen parallel to the membrane bilayer. This projection of the V0 corresponds to the projection of the V0 in the intact V-ATPase (see Fig. 2C; taken from Ref. 8), allowing assignment of the cytoplasmic and luminal side of the V0 complex. The other projections are from complexes seen more or less normal to the bilayer (image 1 in Fig. 2B). The data set was then treated by several rounds of multireference alignment and MSA/classification to extract all the characteristic views of the complex, some of which are shown in Fig. 2D.

A visual inspection of the projections shown in Fig. 2D suggested that their variation in viewing directions might be sufficient for calculating a three-dimensional model of the V-ATPase membrane domain. A set of 33 projection images, including the ones shown in Fig. 2D, was used for starting up the angular reconstitution procedure as implemented in IMAGIC 5. The structure was refined with an increasing number of input projections leading to a model that was calculated from 97 class averages. The model was then forward-projected along 50 directions distributed uniformly over the Euler sphere, and the projections were used as references in a multireference alignment step leading to new input projections. The procedure was iterated until in the end 288 forward projections were used as references resulting in a total of 220 input projections. The 0.5 correlation criterion indicates a resolution of ~21 Å, whereas the 3σ criterion (●) shows that the model contains information down to 18 Å resolution.
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essentially isotropic information content in the structure. The resolution of the final model was estimated via the Fourier shell correlation computed between two reconstructions calculated from randomly selected subsets of the input images (Fig. 3B). At 0.5 correlation (18), the spatial frequency was 0.47 nm\(^{-1}\) corresponding to a resolution of 21 Å, while the 3σ criterion (19) indicated that the model contains information down to 18 Å resolution.

Fig. 4 summarizes the three-dimensional structure of the V-ATPase membrane domain. Overall, the structure can be described as an asymmetric protein ring with dimensions 11 × 14 × 6 nm (including detergent and lipid), which is capped by protein densities on both sides. One side (see arrow in image 1, Fig. 4A) is covered by a globular density with a diameter of ~4 nm. The other side is covered by two elongated proteins, ~7 × 3 and 6 × 3 nm, respectively, that are arranged as two half-circles (see arrows in image 2, Fig. 4A). One of these two densities is in contact with the asymmetric protein ring while the other is above the ring, connected to the first through a small bridge in the center. The orientation of the V0 in the membrane is such that the single globular density is directed toward the lumen of the vesicle (see Fig. 2C) suggesting that this density is Ac45 for which a luminal orientation had been predicted (4). The narrow connection between the luminal density and the proteolipid ring would be consistent with a single membrane anchoring α helix in the C terminus of the polypeptide (4). Additional evidence that supports this assignment comes from electron microscopy of the yeast V-ATPase complex, which does not contain a homologue to Ac45 and which does not show as such a luminal density.\(^2\) As mentioned above, Ac45 might be present in less than one copy per complex (between 0.3 and 0.7) as revealed by quantitative amino acid analysis performed with the holo enzyme (26). The ratio might be somewhat closer to one for the free V0 found in clathrin-coated vesicles as no class-sums of the side-view projection have been obtained in which the luminal density is missing. It can, of course, not be ruled out that some of the other projections do not have the luminal density bound; the clear presence of the luminal protein in the three-dimensional reconstruction, however, suggests that this is probably not the case to a significant degree. Assignment of Ac45 leaves the N-terminal domain of subunit a and subunit d for the proteins covering the cytoplasmic opening of the channel, again consistent with their predicted orientation.

Fig. 4B shows the V0 model with either the top half (image 1) or bottom half (image 2) removed to allow a view inside the channel. The bottom half, looking down toward the lumen, contains two openings roughly 2 nm in diameter, one of which is covered by the single globular protein (see arrowheads in Fig. 4B, image 1). The cytoplasmic opening, which is covered by the two elongated proteins (see arrows in Fig. 4A, image 2), has a diameter of ~6 nm (Fig. 4B, image 2). This would imply that the ring forming proteolipids is not arranged strictly perpendicular to the bilayer so they would form a cylinder but rather at an angle to one another to produce a funnel-shaped structure with a smaller luminal and a larger cytoplasmic opening. Channel openings of unequal size, ~2.5 and 3.5 nm in diameter, respectively, have been seen in atomic force microscopy images of the chloroplast ATP synthase subunit III (proteolipid) oligomer (12).

Fig. 4C shows contoured cross-sections of the three-dimensional model of the V0 at the positions indicated on the left in

\(^2\) S. Wilkens and M. Forgac, unpublished results.
Fig. 4A, image 1. Starting on the luminal side (bottom row, second image from the right) a peripheral density can be seen next to a ring-like structure (see arrow), which can be seen in the cross-sections going from the luminal to the cytoplasmic side. The density is ~4 x 3 nm and could therefore accommodate a bundle of nine transmembrane a helices, consistent with this density being the membrane-bound C-terminal a head of the a subunit. Although some densities within the proteolipid ring can be followed all the way through from the luminal to the cytoplasmic side (see arrowheads in Fig. 4C), the resolution of the model is not sufficient to define the number and arrangement of all the c subunit-like polypeptides in the proteolipid ring. An asymmetric protein ring comparable with the one shown here for the V-ATPase proton channel can be seen in electron microscopic images of the E. coli ATP synthase (29), again confirming that both enzymes share a similar overall architecture in their membrane domains.

As mentioned above, the luminal side of the V0 channel shows two openings. The one that is covered on the luminal side by the spherical density seems to lead into the central channel formed by the proteolipids, while the other opening might lead into a cavity formed at the interface of the C-terminal domain of the a subunit and the outside of the c subunit ring. Such a solvent-accessible half-channel had been postulated for the interface of the F-ATPase a and c subunits (30). Higher up in the structure, the two channel openings combine into one large cavity without a clear indication for a boundary between proteolipids and the membrane-bound part of subunit a. It is conceivable that the resolution of the model is not sufficient to show all the details of the structure inside the channel, but it is also possible that the ring-like arrangement of the proteolipids is somewhat perturbed by the interaction with the membrane-bound domain of the a subunit. Proton translocation through the V0 occurs via a protonation/deprotonation of lipid-exposed carboxyls in the C-terminal a helices of the c subunits (helix 3 of c''), most likely at the site of interaction with the a subunit. It has been shown by NMR spectroscopy that the E. coli F-ATPase proteolipid undergoes a large structural change when going from the protonated to the deprotonated state (31). The resulting structural symmetry in the proteolipid ring might allow an intercalation of part of the a subunit transmembrane a helices into the ring as suggested by the cross-sections shown in Fig. 4C.

An additional cellular function for the V-ATPase membrane domain besides proton translocation has recently been reported based on a study with the yeast system. The data show that the free V0 domain is involved in the late stage of membrane fusion and that this event involves a close contact between the cytoplasmic loops of the proteolipids of free V0 domains in the fusing vesicles (15). Calmodulin is then believed to interact with the proteolipids leading to a radial expansion of the proteolipid ring thus allowing the two bilayers to mix and fusion to occur. The structure here presented of the V0 shows that the cytoplasmic side of the proteolipid ring is covered by protein densities, most likely formed by the N-terminal domain of the a subunit and subunit d. This implies that in order for the c subunits of the opposing V0 domains to come in contact, a large structural change would have to occur. Such a conformational rearrangement might be induced by the interaction with calmodulin and/or other co-factors required for fusion. A large conformational change in the proteins covering the cytoplasmic domain of the V0 would also have to occur during binding of the V1 to enable the reported interaction of the N-terminal domain of a with the catalytic A subunits of the ATPase domain (32).

While the structure presented here gives a first picture of the subunit arrangement in the V-ATPase membrane domain, studies at much higher resolution will be needed to be able to more fully understand the molecular mechanism of proton translocation in this class of enzymes.

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