Structure of the Vacuolar ATPase from *Neurospora crassa* as Determined by Electron Microscopy*

William J. Dschida and Barry J. Bowman†

From the Department of Biology, Sinsheimer Laboratories, University of California, Santa Cruz, California 95064

We have examined the structure of the vacuolar ATPase of *Neurospora crassa* using negatively stained preparations of vacuolar membranes and of detergent-solubilized and gradient-purified ATPase complexes. We also examined the peripheral sector (V₁) of the enzyme after it had been removed and purified. Using different stains, vacuolar membranes displayed ball-and-stalk structures similar to those of the intact mitochondrial ATPase. However, the vacuolar ATPase was clearly different from the mitochondrial ATPase in both size and structural features. The vacuolar enzyme had a much larger head domain with a distinct cleft down the middle of the complex. This domain was held above the membrane by a prominent stalk. Most intriguing was the presence of basal components. These structures appeared to project from the vacuolar membrane near the base of the stalks. Detergent-solubilized, gradient-purified ATPases displayed the same head, stalk, and basal features as those found with the intact enzyme on vacuolar membranes. The mitochondrial ATPase was significantly smaller, and no clefted head domains or basal components were observed.

When V₁ and F₁ particles were directly compared, a significant difference in size and shape between these two soluble ATPase sectors was apparent. V₁ retained all of the features seen in the globular head of the intact complex: V-shaped, triangular, and square forms around a stain-filled core.

The endomembrane system of eucaryotic cells contains several organelles: Golgi, lysosomes, vacuoles, secretory vesicles, and coated vesicles, which can be acidified by an ATP-driven proton pump called the vacuolar ATPase. These vacuoles, which are the fungal equivalent of mammalian lysosomes, have relatively high levels of ATPase activity and appear to be thickly studded with vacuolar ATPases. In an earlier study we found that negatively staining vacuolar membranes with phosphotungstate revealed ball-and-stalk structures which superficially resembled the F-type ATPase of mitochondrial membranes but appeared to differ from the mitochondrial enzyme in size and in structural details (12). Treatment of the vacuolar membranes with nitrate removed the peripheral sector of the vacuolar ATPase from the membranes and caused the disappearance of the ball-and-stalk structures. Thus, the structures seen in the electron micrographs appeared to be the vacuolar ATPases. Vacuolar ATPases have also been seen by negative staining of several higher plant tonoplasts (13-15), bovine chromaffin granules (16), and *Dictyostelium discoideum* acidosomes (17).

In this paper we have examined in detail the structure of the vacuolar ATPase of *N. crassa* as seen by electron microscopy of negatively stained membranes. We have made a direct comparison with mitochondrial membranes from the same cells. In addition, we have examined the structure of the peripheral sector of the vacuolar ATPase after it has been removed from the membrane. We wished to see if this part of the enzyme, designated V₁, is significantly different in size or structure from the F₁ sector of the mitochondrial ATPase. We also examined the structure of vacuolar ATPase that had been solubilized with detergent and purified on glycerol gradients.

**EXPERIMENTAL PROCEDURES**

Preparation of Vacular and Mitochondrial Membranes—Vacuoles and mitochondria were prepared as described previously (18, 19). The purified organelles were lysed and washed in 1 mM EGTA, pH 7.5, and centrifuged at 100,000 × g for 30 min. Vacuolar and mitochondrial membrane pellets were resuspended in 1 mM EGTA, pH 7.5, to a protein concentration of 2-3 mg/ml, frozen in liquid nitrogen, and stored at −70 °C. Both membrane preparations had specific ATPase activity.

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†To whom correspondence should be addressed. Tel.: 408-459-2245; Fax: 408-459-3139.

1 The abbreviations used are: EGTA, (ethylenebis(oxyethylenenitrito)l]tetracetic acid; PIPES, piperazine-N,N'-bis[2-ethanesulfonic acid].
activities of 2.0-4.0 μmol/min/mg of protein.

Purification of Vacuolar and Mitochondrial ATPases—The vacuolar membrane ATPase was purified essentially as described by Uchida et al. (20). Briefly, EGTA-washed vacuolar membranes were solubilized in detergent 3% digitonin, centrifuged at 180,000 g for 15 min. The supernatant was layered onto a linear 20-40% glycerol gradient and centrifuged at 200,000 × g for 5.5 h at 4 °C. The ATPase activity migrated halfway through the gradient, and these fractions were treated for electron microscopy as described below. The peripheral complex (V) of the V-ATPase was prepared as described previously (12), except for one minor change. Prior to the final MacConkey step, potassium nitrate was replaced with ATPase membranes were washed in 100 mM KNO₃ in the absence of ATP. The peripheral F₁ sector of the mitochondrial ATPase was prepared using a chloroform extraction method as described previously (21).

Other Methods—Gel electrophoresis was performed with samples dissolved in sodium dodecyl sulfate as described (22). Proteins were visualized by silver staining. Vacuolar and mitochondrial ATPase activities were assayed as described previously (18).

The molecular diameters of the soluble complexes were estimated by gel permeation chromatography on a 1 × 15-cm column of S-500 resin (Pharmacia LKB Biotechnology Inc.). Vacuolar and mitochondrial peripheral complexes were diluted 1:10 in vacuolar ATPase assay mix containing 5 mM Na₂ATP, 5 mM MgSO₄, 10 mM NH₄Cl, 10 mM PIPES (adjusted to pH 7.4) and concentrated to 50 μl (protein concentration of 1-2 mg/ml) in Centricon tubes (Amicon Corp.) and concentrated in Centricon tubes (Amicon Corp.) spun at 10,000 g for 5.5 h at 4 °C. The ATPase activity migrated halfway through the gradient, and these fractions were used to calibrate exact sizes. The ATPase was solubilized in detergent (1% digitonin) on a 1 × 15-cm column of S-300 gel permeation chromatography on a 1 × 15-cm column of S-300 resin (Pharmacia LKB Biotechnology Inc.). Vacuolar and mitochondrial peripheral complexes were diluted 1:10 in vacuolar ATPase assay mix containing 5 mM Na₂ATP, 5 mM MgSO₄, 10 mM NH₄Cl, 10 mM PIPES (adjusted to pH 7.4) and concentrated to 50 μl (protein concentration of 1-2 mg/ml) in Centricon tubes spun at 10,000 g for 5.5 h at 4 °C. The ATPase activity migrated halfway through the gradient, and these fractions were used to calibrate exact sizes.

Transmission Electron Microscopy—Vacuolar and mitochondrial membranes were diluted to a protein concentration of 0.5-1.0 mg/ml in 1 mM EGTA, pH 7.5. One μl of a suspension was applied to a Formvar-coated 200-mesh grid. After 1 min, the droplet was drawn off and allowed to semidry. One μl of a 1% solution of phosphotungstate, uranyl oxalate, or ammonium molybdate (all at pH 6.5) was applied over the grid, and immediately wicked off with filter paper. The remaining stain was quickly blown dry (within a few seconds) to minimize exposure of the samples to the heavy metal solution. Gradient- and column-purified ATPase fractions were diluted 1:10 in 1 mM EGTA, pH 7.5, and concentrated in Centricon tubes. The ATPase samples were subsequently applied to grids as described above. Specimens were examined and photographed on a Jeol 100B electron microscope at a primary magnification of 67,000 × with an accelerating voltage of 80 kV.

Image Processing and Analysis—Vacuolar and mitochondrial ATPases were digitally imaged and processed using the NIH Image 1.43 program on a Pulnix TM-545i CCD camera/Macintosh II computer. To avoid subjective selection of images, we measured every apparent ATPase in Figs. 2 and 4 and 65 particles in Fig. 5, A and B. Briefly, ATPase projections were magnified 75 X to a final magnification of 180,000 X. Images were digitally processed using filtering functions to reduce background noise and sharpen edges of stain-protein boundaries. Measurements of the computer-enhanced images were made using digital calipers and mean values and standard deviations for dimensions determined. A negatively stained catalase standard was used to calibrate exact sizes.

RESULTS

Analysis of Vacuolar and Mitochondrial Membranes—Vacuolar membranes were treated with three types of negative stains as described under “Experimental Procedures.” As shown in Fig. 1, the vacuolar ATPase was clearly visible as a ball-and-stalk structure when the membranes were stained with phosphotungstate (panel A), uranyl oxalate (panel C), or ammonium molybdate (panel D). The highest density and best resolution of these structures were observed when phosphotungstate was used. Occasionally, ATPases were seen in surface view (as in panel B), but in most cases, the ball-and-stalk structures were most prominent along the edge of the vesicles (panel A). For all subsequent experiments membranes were stained with phosphotungstate.

When mitochondria and vacuolar membranes were compared, both were thickly studded with ball-and-stalk structures; however, the density, size, and shape of the vacuolar ATPase (Fig. 2, A–C) were clearly different from those of the mitochondrial ATPase (panel D). The particles were essentially uninterrupted along the mitochondrial membrane with 10-12 ATPases/100 nm of linear membrane segment. By contrast the vacuolar ATPases appeared in small clusters along the membrane. In regions in which the vacuolar particles were of highest density, with no apparent gaps, there were 8-9 ATPases/100 nm of membrane segment. The vacuolar ATPase showed three characteristic features. First, the enzyme appeared to have a globular head with a prominent cleft or bifurcation visible in many of the particles (see structures labeled H in Fig. 2, A–C). In many views the stain penetrated to the center of the globular head, giving rise to a V-shaped or bilobed structure. In the case of the intact mitochondrial ATPase, penetration of stain into the head was rarely observed (see panel D). Second, the head domain of the enzyme was connected to the membrane by a thin stalk (see S in panels A–C). Third, the vacuolar membranes appeared to have additional basal components associated with the ATPase (see B in panels A–C). In occasional views, these basal components appeared as projections from the membrane originating near the stalk of the ATPase (see B in panel A). These projections measured approximately 7-10 nm long (as measured from the center of stalk-membrane junction) × 2-4 nm wide. With the mitochondrial membranes no apparent basal components were found in the region between the membrane and the globular heads (panel D).

Negatively stained vacuolar ATPases appeared to be significantly larger than the mitochondrial enzymes. We measured several dimensions of both enzymes as summarized in Fig. 3. The globular head of the vacuolar enzyme was 11.5 nm wide × 9.3 nm high. The mitochondrial head sector measured 9.6 nm wide × 8.4 nm high. The stalk of the vacuolar ATPase was 6.6 × 3.1 nm, both longer and thicker than the mitochondrial counterpart, which measured 4.7 × 2.6 nm.

Examination of Detergent-solubilized Vacuolar ATPase—We examined detergent-solubilized ATPases that had been partially purified on a glycerol gradient (see “Experimental Procedures”). This preparation contained both integral membrane and peripheral subunits of the ATPase (18). As shown in Fig. 4, structures were visible which closely resembled those seen on intact vacuolar membranes. The solubilized ATPase had a roughly spherical sector separated from an apparent membraneous domain by a thin stalk. The dimensions of these structures (n = 14) were very similar to those of the membrane-bound vacuolar ATPase. The head domain measured 12.0 ± 0.9 nm wide × 10.5 ± 1.3 nm high and the stalk, 6.4 ± 1.1 nm long × 3.5 ± 0.7 nm wide. The basal components seen on intact membranes (see B in Fig. 2, panel A) were also apparent on the solubilized enzyme (Fig. 4, far left panel). The putative membrane sector was roughly the same size as the head domain but did not appear to have a regular structure. In some cases the enzymes were clustered, resembling small strips of membrane (Fig. 4, right panels).

Examination of the Detached V₅, Sector and Comparison with the F₁, Sector from Mitochondria—We have shown previously that peripheral subunits of the vacuolar ATPase can be specifically released from the membrane (11). Incubation of membranes with 100 mM KNO₃ releases a complex of five subunits. When analyzed by size exclusion chromatography (see “Experimental Procedures”), the V₅ behaved like a particle of 12.5 nm in diameter with an apparent molecular mass of 480 kDa (data not shown). We examined negatively stained peripheral V₅ complexes in electron microscopy to see if they resembled the globular heads attached to the membranes (Fig.

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FIG. 1. Electron micrographs of vacuolar membranes treated with various negative stains. Vacuolar membranes were prepared and examined as described under "Experimental Procedures," using 1% phosphotungstate (panels A and B), 1% uranyl oxalate (panel C), or 1% ammonium molybdate (panel D), all at pH 6.5. All magnifications are 200,000 x. The bar in panel D represents 100 nm.
FIG. 2. Electron micrographs of intact vacuolar and mitochondrial membranes. Fields are of ATPases along vacuolar (A–C) and mitochondrial membranes (D) negatively stained with phosphotungstate. Some of the vacuolar ATPase particles have been marked to indicate well resolved head groups (H), stalks (S), and basal components (B). All magnifications 400,000 ×. Bar in panel D, 50 nm.

FIG. 4. Electron micrographs of detergent-solubilized vacuolar ATPases. Vacuolar ATPases were solubilized and purified as described under "Experimental Procedures." Purified enzyme was negatively stained with phosphotungstate. All magnifications 400,000 ×.

2, panel C). As shown in Fig. 5, panel A, relatively homogeneous fields of particles were present with an average diameter of 11.8 ± 0.8 nm (n = 65). For comparison, we also examined the F₁ sector of the mitochondrial ATPase (panel B). When analyzed on the same size exclusion column, the F₁-ATPase had a diameter of 11.5 nm with an apparent molecular mass of 380 kDa (data not shown). In the electron microscope the F₁-ATPase had a diameter of 9.2 ± 0.7 nm (n = 65), exhibiting a distinct ring structure with a hollow central region (panel B). F₁ particles have been shown to lie preferentially upright.
can be seen with good resolution. Furthermore, the $V_1$ sector of the ATPase is easily released from the membrane and can also be clearly resolved at high magnification after negative staining.

Analysis of the primary sequence of subunits of V-type and F-type ATPases, together with analysis of the subunit composition of the enzymes, strongly suggests that these two types of ATPases are derived from a common ancestor and have similar overall structures (4, 6–9). Therefore, it is somewhat surprising that mitochondrial and vacuolar ATPases can be readily distinguished from each other in electron micrographs. The sizes and shapes of the enzymes are clearly different. Some difference in size is expected because subunits of the vacuolar ATPases are larger than the corresponding subunits of the F-type enzymes. For N. crassa the predicted molecular mass of the $V_1$ sector is 466 kDa (67,57,48,39,16, 12), whereas the mitochondrial $F_1$ sector is approximately 382 kDa (21, 29, 30). These molecular mass differences are proportional to the difference in the diameters of the head groups seen on vacuolar and mitochondrial membranes, 11.5 nm versus 9.6 nm. However, if these were filled spheres, the $V_1$ would have 72% greater volume than the $F_1$, and the 22% difference in molecular masses does not account for this. Thus, in addition to having larger subunits, the vacuolar ATPase may also have more open space between some of these components.

In the electron micrographs the most characteristic feature of the vacuolar ATPase is a prominent cleft often seen in the $V_1$ sector. Presumably this is caused by a heavily stained cavity in the middle of the enzyme. This feature can also be seen in views of the detached $V_1$, suggesting that the major subunits are held together at the base of the head component in the region attached to the stalk (see model in Fig. 3). When stained with phosphotungstate, most intact mitochondrial ATPases lacked a prominent cleft. However, Gogol et al. (10, 25), using unstained $F_1$-ATPase examined in amorphous ice, observed a central cavity in this enzyme. Furthermore, a recent crystal structure of rat mitochondrial $F_1$-ATPase, at 3.6 Å resolution, provides evidence that the $\alpha$ subunits are joined at the base of the $F_1$, whereas the $\beta$ subunits straddle the $\alpha$ subunits but do not contact each other (31). The vacuolar ATPase homologue of the $F_1\beta$ subunit is the 67-kDa subunit. In N. crassa this polypeptide is 30% larger than the mitochondrial $\beta$ subunit. Thus, the V-type and F-type ATPases could have similar overall quaternary structures, but the V-type may look somewhat different because of larger subunits in a more open cluster in the $V_1$ sector.

The other distinct features of the vacuolar ATPase are the prominent stalk and the additional basal components. The globular head of the enzyme, which contains the ATP-binding subunits, appears to be held 6.6 nm above the surface of the membrane by a stalk that is approximately 3.1 nm wide. One of the most intriguing aspects of the F-type and V-type ATPases is the question of how changes in the ATP binding sites on $F_1$ and $V_1$ can be coupled to proton movement through a membrane sector which, on a molecular scale, is very far away.

For the vacuolar ATPase we do not know which subunits make up the stalk and basal components. $V_1$, detached from the membrane contains five different subunits but does not appear to have an extended stalk in the electron micrographs. Thus, the stalk may be composed, at least in part, of membrane-associated polypeptides. Membranes stripped of the $V_1$ still have small protrusions, but they are too small and variable to be easily identified. The basal projections seen on the vacuolar membrane (Fig. 2) have no counterpart in F-type ATPases.
FIG. 5. Electron micrographs of the V₁ sector of the vacuolar ATPase and the F₁ sector of the mitochondrial ATPase. Peripheral sectors of the two ATPases were isolated and negatively stained with phosphotungstate as described under "Experimental Procedures." Panels A, C, and D show the V₁ sector of the vacuolar ATPase. Panel B shows the F₁ sector of the mitochondrial ATPase. Some of the prominent V₁ complexes, observable in deeper stain, have been marked to indicate V-shaped (V), triangular (T), or square (S) forms. The magnification of panels A and B is 200,000 x. The magnification of panels C and D is 400,000 x. The bars in panels B and D represent 50 nm.
ATPases. In the future we plan to use antibodies raised against specific polypeptides to see if some of the distinctive components visible in the electron microscope can be specifically labeled.

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