Structure and Properties of the Vacuolar (H\(^{+}\)) -ATPases*

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Function of V-ATPases

The vacuolar (H\(^{+}\)) -ATPases (or V-ATPases) are a family of ATP-dependent proton pumps that are responsible for acidification of intracellular compartments in eukaryotic cells (1–3). Acidification of vacuolar compartments plays an important role in a variety of cellular processes, particularly membrane traffic processes. In receptor-mediated endocytosis, acidification of endosomes serves as a signal that activates release of internalized ligands (such as low density lipoprotein and insulin) from their receptors. This uncoupling allows unoccupied receptors to recycle to the cell surface where they can be reutilized, whereas the ligands are targeted to lysosomes for degradation (1). Acidification of endosomes is also required for the formation of endosomal carrier vesicles (which are involved in moving ligands along the endocytic pathway (4)) and in activating fusion between the endosomal membrane and internalized envelope viruses (such as influenza virus) that is necessary for viral infection (5).

In intracellular targeting of newly synthesized lysosomal enzymes, vacuolar acidification plays a very similar role to that observed for endocytosis. Thus, lysosomal enzymes bound to the mannose 6-phosphate receptor are delivered to a late recycling compartment where the low pH causes dissociation, allowing receptors to recycle to the trans-Golgi and the enzymes to be targeted to lysosomes (6). Disruption of vacuolar acidification in yeast also causes a perturbation in vacuolar targeting (7), although the molecular basis of this effect is not yet clear. In lysosomes and other digestive organelles, like the central vacuole of yeast, the low pH is required to support the activity of acid hydrolases involved in degradation of macromolecules. In both central vacuoles and secretory vesicles, such as synaptic vesicles and chromaffin granules, the proton gradient or membrane potential generated by the V-ATPase is utilized to drive the coupled transport of small molecules into the vesicle lumen (8, 9).

In addition to their role in intracellular compartments, V-ATPases have also been shown to play an important role in the plasma membrane of various specialized cells. Thus V-ATPases in the apical membrane of renal intercalated cells function in renal acidification (10), whereas plasma membrane V-ATPases in macrophages and neutrophils assist in pH homeostasis (11). Osteoclasts (12) and tumor cells (13) are able to target V-ATPases to the plasma membrane where they create an acidic extracellular environment that is necessary for bone resorption or tumor metastasis, respectively. V-ATPases in the midgut of insects have been shown to establish a membrane potential across the apical membrane that drives K\(^{+}\) transport via an electrogenic H\(^{+}\)/K\(^{+}\) antiporter (14). Finally, using the specific V-ATPase inhibitor bafilomycin (15), V-ATPases have been implicated in apoptosis (16). It is thus clear that V-ATPases serve a wide variety of roles in eukaryotic cells.

Structure of V-ATPases

Our current model for the structure of the V-ATPases is shown in Fig. 1. The subunit composition of the V-ATPase, together with the yeast genes encoding the V-ATPase subunits, is shown in Table I. The V-ATPases are composed of two functional domains (1–3). The V\(_{0}\) domain is a 260-kDa integral complex that is responsible for proton translocation across the membrane. V\(_{0}\) contains five different subunits of molecular mass 100–17 kDa (subunits a, d, c, c\(^{\prime}\), and c\(^{\prime\prime}\)), with six copies of the c/c\(^{\prime}\) subunits and single copies of the other subunits (17).

The V-ATPases are structurally and evolutionarily related to the F-ATPases (or ATP synthases) of mitochondria, chloroplasts, and bacteria (18–20) that normally function to couple the synthesis of ATP to the movement of protons down their electrochemical gradient. This relationship is evident both in their overall structure (3) and in the sequence of the nucleotide binding and proteolipid subunits (subunit c) (2). Despite this similarity, however, the V- and F-ATPases differ in many important respects, particularly in their function and regulation. An important goal is to determine the structural basis for these differences between the V- and F-ATPases.

Comparison of electron microscopic images of the V-ATPase from Neospora and the F-ATPase of mitochondria revealed that although both contained a globular head attached to the membrane by a central stalk, the V-ATPase had additional projections emanating from the base of the stalk (21). More recent images of the V-ATPase from Clostridium fervidus have revealed the presence of a second or peripheral stalk (22), similar to that reported for the F-ATPase of Escherichia coli (23). Negatively stained images of the bovine coated vesicle V-ATPase reveal an even more extensive secondary stalk. This peripheral stalk is crucial for the current rotary model of ATP-driven proton transport by both the F- and V-ATPases (20, 24, 54), discussed in detail below.

Structure and Function of V\(_{1}\) Subunits

The nucleotide binding sites of the V-ATPase are located on two subunits: the 70-kDa A and 60-kDa B subunits. Several lines of evidence suggest that the catalytic nucleotide binding sites are located on the A subunit. First, ATP-protectable modification of the A subunit by such reagents as N-ethylmaleimide, 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD-Cl), and 2-azido-ATP correlates well with loss of activity (3, 25). Second, the A subunit contains all of the catalytic residues in the A subunit (18). From the x-ray crystal structure of F\(_{1}\) (26, 27), although no high resolution structural data are yet available for the V-ATPases, mutation of conserved residues in these sequences has been identified from the x-ray crystal structure of F\(_{1}\) (26, 27). One property that distinguishes the V- and the F-ATPases is the sensitivity of the V-ATPases to sulfhydryl reagents, such as N-ethylmaleimide (1–3). Cys-254 of the bovine V-ATPase A subunit has been identified as the residue responsible for the sensitivity of the V-ATPases to sulfhydryl reagents (31). Cys-254 is located in the C-terminal domain of the A subunit, and disulfide bond formation is thought to

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1. S. Wilkens and M. Forgac, unpublished observations.
inhibit activity by preventing the conformational separation of domains associated with nucleotide release, as visualized in the crystal structure of F1 (26). The possible role of disulfide bond formation in regulation of V-ATPase activity is discussed below.

By contrast with the nucleotide binding sites on the A subunit, the nucleotide binding sites on the B subunit appear to be “non-catalytic.” The evidence for this is as follows. First, the B subunit does not possess the glycine-rich loop consensus sequence shown to be essential for ATP hydrolysis by the F- and V-ATPases (2). Second, mutational analysis of residues postulated to be at the nucleotide binding sites on the B subunit by BzATP does lead to loss of activity (34). The function of the noncatalytic sites on the V-ATPases is uncertain, but one recent observation is suggestive. If Arg-483 of the A subunit (which is thought to be contributed by the A subunit to the noncatalytic site) is mutated to glutamine or glutamic acid, a slow activation following addition of MgATP is observed (30), suggesting that the noncatalytic sites may need to be occupied to achieve maximal activity.

Two approaches have been taken toward understanding the role of other V1 subunits (subunits C–H) in activity or assembly of the V-ATPase complex. First, deletion of the genes encoding these subunits in yeast has been shown to lead to a loss of V-ATPase activity (1, 9), typically because the V1 domain is no longer able to assemble onto the integral V0 domain (35). One exception to this is subunit H, which is required for activity, but not assembly, of the V-ATPase complex (36). In addition, in vitro reassembly studies have suggested a role for many of the V1 subunits in supporting Ca2⁺-ATPase activity, or more recently Mg2⁺-ATPase activity, by the V1 domain (37). Although the V1 domain (unlike F1) does not typically hydrolyze MgATP (38), subunit G appears to confer this property on V1 (37).

To identify contacts between subunits in the V-ATPase complex, several approaches have been taken. First, a direct interaction between subunits C and E has been demonstrated by coimmunoprecipitation (38). In yeast, the absence of particular V1 subunits leads to the formation of a variety of V1 subcomplexes, including those containing subunits D + F and subunits E + G (39), suggesting that these subunits also contain contact regions. Cross-linking studies of the bovine V-ATPase revealed contacts between subunits C, D, and E and a protein originally interpreted to be subunit c of V0 (40). Because subunits F and G are very similar in molecular weight to subunit c, however, these cross-linked products may have instead reflected D/F and E/G contacts.

An important question is the subunit composition of the central and peripheral stalks observed in the electron microscopic images of the V-ATPase (see above). In the F-ATPase, the central stalk is composed of the highly α-helical γ and ε subunits, whereas the peripheral stalk is made up of the δ subunit and the soluble domain of subunit b (41). The high α-helical content of subunits D and E suggests that one of these subunits may function as the V-ATPase homolog of the γ subunit, thus forming part of the central stalk (42, 43). The subunit composition of the peripheral stalk for the V-ATPase is unknown, but subunits C, G, and H of V1 and subunits d and the large soluble domain of subunit a in V0 are all potential candidates.

**Structure and Function of V0 Subunits**

The V0 domain contains five different types of subunits, three of which (c, c′, and c″) are highly hydrophobic proteins termed proteolipids. The proteolipid subunits are homologous both to each other (44) and to the c subunit of the F-ATPases (19). The F-ATPase c subunit is composed of two transmembrane helices containing a single buried carboxyl group in TM2 that is essential for proton transport (19). The V-ATPase subunits c and c′ are composed of four transmembrane helices containing a single essential carboxyl group in TM4 (44). Both c and c′ of the V-ATPases were derived by gene duplication and fusion from the F-ATPase c subunit (45). Because there are 12 copies of subunit c in F0 (19) and 6 copies of subunits c/c′ in V0 (17), these subunits contribute the same number of transmembrane helices (twenty-four) to their respective domains. The number of buried carboxyl groups contributed by the c subunits in V0, however, is only half that in F0, and this has been suggested to account for the difference in H⁺/ATP stoichiometry observed for the V- and F-ATPases (46). For both V0 and F0, reaction of a single c subunit with DCCD2 is sufficient to completely block proton transport (3, 19), indicating that all the buried carboxyl groups must be functional for proton transport to occur.

By contrast with subunits c and c′, subunit c″ is a 23-kDa protein that contains five transmembrane helices, with the essential carboxyl group in the third transmembrane segment (44). Why the V-ATPases require three different proteolipid subunits (in contrast to one for the F-ATPases) is unclear, but mutagenesis studies indicate that there must be at least one copy of each proteolipid subunit in every V-ATPase complex (44). Electron micrographs of F0, which has the structure a,b2c2F2, indicate that the c subunits form a ring, with the a and b subunits to one side and the soluble domain of subunit b projecting from the membrane (47). Although comparable studies have not been carried out on V0, the V-ATPase subunit c has been observed to form rings that are morphologically identical to gap junctions (48). In addition, the V-ATPase c subunit has been shown to associate with the transforming oncprotein E5 of bovine Papillomavirus (49). These findings suggest the possibility that subunit c of the V-ATPases may serve multiple roles in the cell.

The 100-kDa subunit (subunit a) of the V-ATPase is a transmembrane glycoprotein possessing an N-terminal hydrophilic domain and a C-terminal hydrophobic domain containing multiple putative transmembrane helices (50). In yeast, the 100-kDa subunit is encoded by two genes, VPH1 and STV1 (51, 52). Vph1p localizes to the central vacuole whereas Stv1p localizes to some other intracellular membrane, possibly Golgi or endosomes (52), suggesting that the 100-kDa subunit may target the V-ATPase to different compartments in the cell.

Although no sequence homology exists between them, mutagenesis studies suggest that, like the a subunit of the F-ATPases (53, 54), the V-ATPase a subunit contains several buried charged residues that participate in proton translocation (55, 56). Although not absolutely required for proton transport for either the V- (56) or F-ATPases (57), these residues appear to be in a position to influence proton movement, as reflected in the pH dependence of activity (56). Thus the a subunit may form water-filled channels that allow protons to gain access to the buried carboxyl group of subunit c and to exit this site to the opposite aqueous compartment, as has been suggested for the F-ATPase a subunit (54). Evidence has also been obtained suggesting that the 100-kDa subunit possesses the binding site for the highly specific V-ATPase inhibitor bafilomycin (58).

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2 The abbreviation used is: DCCD, dicyclohexylcarbodiimide.
Minireview: Structure and Properties of the V-ATPases

TABLE I

| Domain | Subunit | Bovine $M_f$ | Yeast $M_f$ | Yeast gene$^a$ | Subunit function |
|--------|---------|-------------|------------|---------------|----------------|
| $V_1$  | A       | 73          | 69         | VMA1          | Catalytic site, regulation (?) |
|        | B       | 58          | 57         | VMA2          | Noncatalytic site, targeting (?) |
|        | D       | 34          | 22         | VMA5          | Activity, assembly |
|        | E       | 33          | 27         | VMA8          | Activity, assembly |
|        | F       | 14          | 14         | VMA4          | Activity, assembly |
|        | G       | 15          | 13         | VMA7          | Activity, assembly |
|        | H       | 50          | 54         | VMA10         | Activity, assembly |
| $V_0$  | a       | 100         | 95         | VMA13         | Activity (not assembly) |
|        | d       | 38          | 36         | VPH1/STV1     | H$^+$ transport, assembly, targeting |
|        | c       | 17          | 17         | VMA6          | Activity, assembly |
|        | c'      | 17          | 17         | VMA3          | H$^+$ translocation, DCCD site |
|        | c''     | 19          | 23         | VMA11         | H$^+$ translocation, DCCD site (?) |

$^a$ In yeast, disruption of vma genes encoding the V-ATPase subunits leads to loss of V-ATPase activity and a conditional lethal phenotype in which cells are able to grow at acidic pH but not at neutral pH or in the presence of elevated extracellular Ca$^{2+}$ (1, 9).

In addition to the proteolipid subunits and subunit a, the $V_0$ domain also contains a single copy of subunit d, a hydrophilic protein with no putative transmembrane helices (45), which nevertheless remains tightly bound to $V_0$ upon dissociation of $V_1$ (40). No function has yet been assigned to subunit d.

Mechanism of V-ATPases

Although the mechanism by which the V-ATPases carry out ATP-dependent proton translocation has not been established, it is thought to be similar to the rotary mechanism recently proposed for the F-ATPases (20, 24, 54). In the rotary mechanism, hydrolysis of ATP forces rotation of the central $\gamma$ subunit within the $\alpha_5\beta_2$ hexamer. Rotation of $\gamma$ within $F_1$ has been demonstrated by several methods (20, 24), most recently by fluorescence microscopy (59). The $\gamma$ subunit is believed to be attached to the ring of $c$ subunits in $F_0$ (41) so that rotation of $\gamma$ forces rotation of the ring of $c$ subunits relative to subunit a. Subunit a is held stationary relative to the $\alpha_5\beta_2$ hexamer by a peripheral stalk and forms two hexamers (displaced relative to each other) that allow protons to reach and leave the buried carboxyl groups on subunit c (54). As the ring of $c$ subunits rotates, protons are picked up by the c subunit carboxyls from one hexamer, rotate through the bilayer, and are then released to the opposite side of the membrane through the second hexameter. This mechanism thereby converts the rotary motion of $F_1$ into a linear gradient of protons across the membrane.

Assembly and Targeting of V-ATPases

Studies of the yeast V-ATPase indicate that although the $V_0$ domain is able to assemble and target to the central vacuole in the absence of the $V_1$ subunits, the absence of any of the $V_0$ subunits prevents both attachment of $V_1$ and assembly and targeting of $V_0$ (35). This suggests that the targeting information for the V-ATPase complex in yeast resides in the $V_0$ domain, which must be fully assembled to reach the vacuolar membrane. For the $V_1$ domain, deletion of any of the $V_0$ subunits prevents assembly of $V_1$ and attachment of $V_1$ to $V_0$, although various $V_1$ subcomplexes are observed under these conditions (39). As noted above, the exception in this case is subunit H, which is required for activity but not assembly of the V-ATPase complex (36). Assembly of the V-ATPase has also been studied in mammalian cells, where it appears that attachment of $V_1$ and $V_0$ can occur in the endoplasmic reticulum (3).

In yeast, a number of gene products, including Vma12p, Vma21p, and Vma22p, appear to function in assembly of the V-ATPase but do not form part of the final complex (1). All three of these proteins reside in the endoplasmic reticulum where they appear to be involved in the early stages of assembly of $V_0$ (60). These proteins are interesting in that they appear to function only in assembly of the V-ATPase complex, not in the assembly of other membrane proteins.

Several subunits have been suggested to contain information necessary for intracellular targeting of the V-ATPases. As indicated above, the two isoforms of the 100-kDa subunit (Vph1p and Stv1p) are targeted to different intracellular compartments in yeast (52). In mammalian tissues, two isoforms of subunit B have been identified (10). The brain isoform of B is expressed in all cell types whereas the kidney isofrom is expressed at high levels in renal intercalated cells, which are capable of targeting V-ATPases to the plasma membrane (10). Whether the B subunit actually contains targeting information necessary to direct the V-ATPase to the plasma membrane or whether the two isoforms of B simply recognize the appropriately targeted 100-kDa subunits remains to be determined.

Regulation of Vacular Acidification

It is clear from studies employing a variety of probes that cells are able to maintain different intracellular compartments at different pH values and that this property is essential for the functions performed by the V-ATPases (1). Thus, for example, endosomes must become progressively more acidic following receptor-mediated endocytosis to activate ligand-receptor dissociation and receptor recycling from the correct compartment. A central question in this field is how cells regulate vacuolar acidification, and evidence has been obtained in support of several possible mechanisms.

The first mechanism to be discussed involves reversible dissociation and reassembly of the V-ATPase complex. In yeast, glucose deprivation has been shown to cause a rapid dissociation of the V-ATPase complex into free $V_1$ and $V_0$ domains (61). This dissociation is rapidly reversible and independent of new protein synthesis, indicating that the dissociated domains are reutilized during reassembly. Dissociation of $V_1$ and $V_0$ domains is also observed in insects during molting (62), and free $V_1$ and $V_0$ domains have been identified in mammalian cells (3), suggesting that a similar mechanism may be operating in these cases. The factors controlling the assembly state of the V-ATPase remain to be elucidated.

A second mechanism proposed to regulate V-ATPase activity in vivo involves reversible disulfide bond formation between cysteine residues at the catalytic site of the V-ATPase. As described above, an inhibitory disulfide bond can be formed between Cys-254 and Cys-261 in vivo, which is uncertain, but the bovine V-ATPase has also been observed (64) and has been suggested to be responsible for the sensitivity of the V-ATPases to nitrate. The mechanism of disulfide bond formation in vivo is uncertain, but the bovine V-ATPase has been shown to be sensitive to the nitric oxide-generating agent nitrosoglutathione (65). Interestingly, a mutation in the cysteine biosynthetic pathway in yeast leads to defective vacuolar acidification, and this defect can be corrected by the C261V mutation (66), suggesting that disulfide bond formation can cause inhibition of V-ATPase activity in vivo.

Another mechanism that has been proposed to regulate vacular acidification is a change in the coupling efficiency of the V-ATPase (45). A variety of treatments causes a change in the tightness of coupling of proton transport and ATP hydrolysis, including high concentrations of ATP and mild proteolysis (3), suggesting that the V-ATPase is poised to exist in a partially coupled state. In addition, it has been suggested that an increase in coupling efficiency is one
factor contributing to the ability of the lemon fruit V-ATPase to achieve a vacuolar pH of 2 (67). As with the dissociation mechanism discussed above, the cellular signals controlling coupling efficiency are currently unknown.

A number of low molecular weight activator and inhibitor proteins that directly modulate V-ATPase activity have been identified (10), suggesting that these may help to control vacuolar acidification in some tissues. In addition, changes in pump density have been shown to control proton transport across the plasma membrane of renal intercalated cells (10), although the role of changes in pump density in controlling vacuolar pH has not been established. Finally, because V-ATPases are electrogenic, a parallel ion efflux must accompany proton transport for significant acidification to occur. Under in vivo conditions, the most important ion conductance involved in dissipation of the membrane potential generated during proton transport appears to be chloride influx. This chloride flux is mediated by a family of intracellular chloride channels whose activity is controlled by protein kinase A-dependent phosphorylation (3). Thus regulation of chloride channel activity likely represents an additional mechanism of controlling vacuolar pH in cells.

Conclusions

The V-ATPases are a novel family of ATP-dependent proton pumps that serve a variety of important functions in the cell, particularly in various membrane traffic pathways. They are composed of a soluble domain responsible for ATP hydrolysis and an integral domain that carries out proton transport and are structurally related to the ATP synthases. Because of their diversity of functions, it appears likely that a number of mechanisms are employed in controlling their activity in vivo.

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