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Minireview

Proton translocation driven by ATP hydrolysis in V-ATPases

Shoko Kawasaki-Nishi, Tsuyoshi Nishi, Michael Forgac*

Department of Physiology, Tufts University School of Medicine, 136 Harrison Ave., Boston, MA 02111, USA

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Abstract The vacuolar H^+-ATPases (or V-ATPases) are a family of ATP-dependent proton pumps responsible for acidification of intracellular compartments and, in certain cases, proton transport across the plasma membrane of eukaryotic cells. They are multisubunit complexes composed of a peripheral domain (V_1) responsible for ATP hydrolysis and an integral domain (V_0) responsible for proton translocation. Based upon their structural similarity to the F_1F_0 ATP synthases, the V-ATPases are thought to operate by a rotary mechanism in which ATP hydrolysis in V_1 drives rotation of a ring of proteolipid subunits in V_0. This review is focused on the current structural knowledge of the V-ATPases as it relates to the mechanism of ATP-driven proton translocation.

Key words: V-ATPase; Vacuolar acidification; Proton transport; ATP hydrolysis; Mechanism

1. Introduction

The vacuolar H^+-ATPases (or V-ATPases) are a family of ATP-dependent proton pumps responsible for acidification of intracellular compartments in eukaryotic cells [1–8]. Vacuolar acidification is essential for a variety of cellular processes [1,2], including ligand–receptor dissociation and receptor recycling following receptor-mediated endocytosis, intracellular targeting of newly synthesized lysosomal enzymes, protein processing and degradation, and coupled transport of small molecules, such as neurotransmitters. Acidification of endosomal compartments is also necessary for budding of transport vesicles [9] and infection by certain envelope viruses, such as influenza virus [10]. V-ATPases have also been identified in the plasma membrane of certain cells where they function in such processes as renal acidification [11], pH homeostasis [12], bone resorption [13] and coupled K^+ transport [14]. They have also been implicated in a variety of disease processes, including renal tubular acidosis [15], osteopetrosis [16] and tumor metastasis [17]. This review will focus on what is currently known concerning the structure of the V-ATPases and the mechanism by which they carry out ATP-dependent proton transport.

*Corresponding author. Fax: (1)-617-636 0445.
E-mail address: michael.forgac@tufts.edu (M. Forgac).

Abbreviations: V-ATPases, vacuolar proton-translocating adenosine triphosphatases; F-ATPases, F_1F_0 ATP synthases

2. Structure of the V-ATPases and their relationship to the F-ATPases

The V-ATPases are composed of two domains [1–8]. The peripheral V_1 domain is a 640 kDa complex responsible for ATP hydrolysis. It contains eight different subunits (subunits A–H) of molecular mass 70–13 kDa in a stoichiometry of $A_3B_3CDEFG_2H_{1-2}$ [18,19]. The integral V_0 domain is a 260 kDa complex responsible for proton translocation and is composed of five subunits (a, b, c, c′, c″) of molecular mass 100–17 kDa in a stoichiometry of adc′c″c_4 [18,20]. A model for the arrangement of subunits in the V-ATPase complex is shown in Fig. 1 and a list of the V-ATPase subunits, including the subunit name, molecular mass, corresponding gene in yeast and proposed function, is shown in Table 1.

The V-ATPases are structurally related to the F-ATPases (or F_1F_0 ATP synthases) that normally function in ATP synthesis in mitochondria, chloroplasts and bacteria [21–24]. This relationship between the V- and F-ATPases is evident both in the overall structure of the complexes and in the sequence of certain subunits. Electron microscopy has revealed a similar overall shape for the two proteins, with the peripheral domain of each complex connected to the corresponding integral domain by both central and peripheral stalks [25–27]. These stalks are believed to play an important role in the mechanism by which these enzymes catalyze ATP-dependent proton transport (see below).

On the other hand, there are also significant structural differences between the F- and V-ATPases revealed by electron microscopy. For example, images of the V-ATPase reveal a complex array of projections emanating from the V_1 domain [26], and a cuff-like cytoplasmic region on the V_0 domain [28] (Fig. 2). Some studies suggest that V_1 and V_0 may be connected by as many as three stalks [29]. Functionally, although the V-ATPases have been shown to be reversible [30], they normally operate, unlike the F-ATPases, in the direction of ATP-driven proton transport. In terms of primary sequence, three subunits show clear sequence homology indicating a common evolutionary origin for the V- and F-ATPases. These include the two nucleotide binding subunits (A and B), which are approximately 20–25% identical to the α and β subunits of the F-ATPases [31,32], and the proteolipid subunits (c, c′ and c″), which are homologous to the c subunit of F_0 [33]. Because of the relationship between the V- and F-ATPases, it is useful to briefly review what is known about the structure of the F-ATPases.

Like the V-ATPases, the F-ATPases are composed of a peripheral and integral domain. For the F-ATPase from *Es-*

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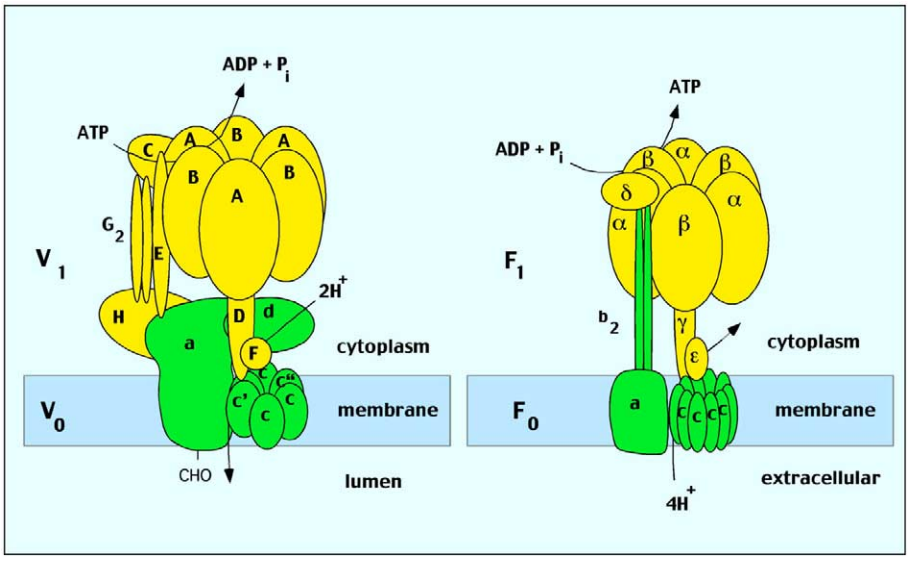

Fig. 1. Structural model of the V-ATPases and comparison with the F-ATPases. The V-ATPases are composed of a peripheral V_1 domain responsible for ATP hydrolysis and an integral V_0 domain responsible for proton translocation. The corresponding domains for the F-ATPases are F_1 and F_0. The nucleotide binding sites are located on the A and B subunits of V_1, with the catalytic sites located primarily on the A subunits. Proton translocation is postulated to occur at the interface of the a subunit and the ring of proteolipid subunits (c, c′ and c″). The V_1 and V_0 domains are connected by both a central stalk (composed of the D and F subunits) and a peripheral stalk (composed of the C, E, G and H subunits). These stalks play a crucial role in the proposed rotary mechanism of ATP-driven proton transport (see Fig. 6). Reprinted by permission from Nature Reviews Molecular Cell Biology [1], ©2002, Macmillan Magazines, Ltd.

cherichia coli, the peripheral F_1 domain has the structure $\alpha_3\beta_3\gamma\delta\varepsilon$ whereas the integral F_0 domain has the structure ab_2c_{10} [21–24]. High resolution structures of F_1 reveal a hexameric arrangement of alternating α and β subunits surrounding a central γ subunit which exists in a highly extended, coiled-coil conformation [34,35]. The nucleotide binding sites are located at the interface of the α and β subunits, with the catalytic nucleotide binding sites located principally on the β subunits and the 'non-catalytic' sites located on the α subunits [34,35]. The F_0 domain contains three integral subunits. The c subunit is a highly hydrophobic protein of 8 kDa composed of two transmembrane helices connected by a short cytoplasmic loop [23]. The X-ray structure of a partial F_1F_0 complex reveals a ring of 10 c subunits [36], in agreement with recent crosslinking studies [37]. The F_0 domain also contains one copy of subunit a, which contains five transmembrane helices [38,39], and two copies of subunit b, which contains a single transmembrane helix connected to a polar cytoplasmic loop [40]. Atomic force microscopy shows the a and b subunits to one side of the c subunit ring [41]. The F_1 and F_0 domains are connected by both a central and a peripheral stalk [27]. The central stalk is composed of the γ and ε subunits [36,42] whereas the peripheral stalk contains the δ subunit and the soluble domains of the b subunits [43,44]. With this background, we will next consider what is known concerning the structure, function and arrangement of the subunits in the V-ATPase complex.

3. Structure and function of the nucleotide binding subunits of V_1

The nucleotide binding sites of the V-ATPase are located on the 70 kDa A and 60 kDa B subunits [1–8]. The A and B subunits are encoded by the *VMA1* and *VMA2* genes in yeast, respectively [31,32,45–48]. The A subunit contains consensus sequences common to many nucleotide binding proteins (termed Walker A and B consensus sequences) and several lines of evidence suggest that the nucleotide binding sites on

Table 1
Subunit composition of the V-ATPase

Domain	Subunit	Molecular weight (kDa)	Yeast gene	F-ATPase homolog	Subunit function/location
V_1	A	70	*VMA1*	β	Catalytic site, regulation
	B	60	*VMA2*	α	Non-catalytic site, targeting(?)
	C	40	*VMA5*	?	Peripheral stalk
	D	34	*VMA8*	γ	Central stalk
	E	33	*VMA4*	?	Peripheral stalk
	F	14	*VMA7*	ε	Central stalk
	G	13	*VMA10*	b	Peripheral stalk
	H	50	*VMA13*	?	Peripheral stalk
V_0	a	100	*VPH1/STV1*	a	H^+ transport, targeting
	d	38	*VMA6*	?	Cytoplasmic side of V_0
	c	17	*VMA3*	c	H^+ transport, DCCD binding site
	c′	17	*VMA11*	c	H^+ transport
	c″	21	*VMA16*	c	H^+ transport

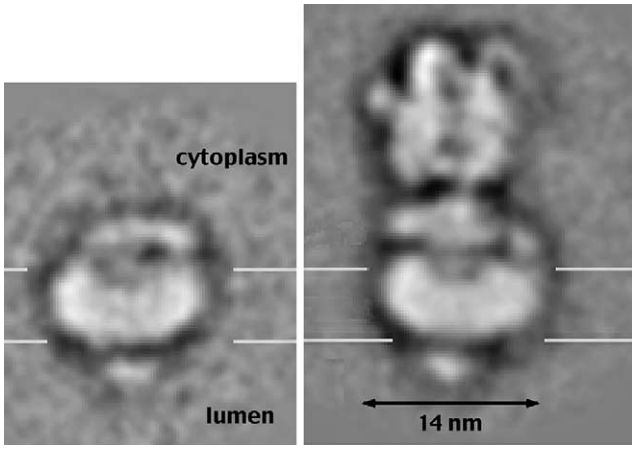

Fig. 2. Comparison of V_1V_0 and V_0 by electron microscopy. Negatively stained images of the isolated V_0 domain and the intact V_1V_0 purified from bovine brain clathrin-coated vesicles [28]. Reprinted with permission from the Journal of Biological Chemistry [28], ©2001, The American Society for Biochemistry and Molecular Biology and Nature Reviews Molecular Cell Biology [1], ©2002, Macmillan Magazines, Ltd.

the A subunit serve a catalytic function. First, modification of a conserved cysteine residue at this site by sulfhydryl reagents is prevented in the presence of ATP and leads to inhibition of activity [49]. The cysteine which is modified (Cys254 in the bovine A subunit sequence) is conserved in all available A subunit sequences and is located within the GXGKT Walker A motif [49]. Although this cysteine residue does not appear to play any direct role in catalysis, since mutation to serine gives a fully active enzyme [50], there is evidence that it participates in regulation of V-ATPase activity through reversible disulfide bond formation [51–54]. In addition, modification of the A subunit by 2-azido-ATP leads to irreversible inactivation of the V-ATPase [55]. Although the V-ATPase complex contains three copies of the A subunit [18], and hence three catalytic sites, modification of only one of these sites is sufficient to completely inhibit activity [55].

Molecular modeling studies based upon sequence homology between the nucleotide binding subunits of the V- and F-ATPases, the available X-ray coordinates of F_1 [34] and energy minimization have been used to generate the model of the catalytic nucleotide binding site on the A subunit shown in Fig. 3a [56]. Mutation of either Glu286 or Lys263 leads to complete loss of ATPase activity [50], suggesting an important role for these residues in ATP hydrolysis. The corresponding residues in the F_1 β subunit are also critical for catalysis [57,58], and the crystal structure of F_1 suggests that the lysine residue helps stabilize the negative charge on the phosphates of ATP whereas the glutamate residue serves to abstract a proton from the water molecule attacking the terminal phosphate during ATP hydrolysis [34,35]. Mutagenesis studies of the V-ATPase A subunit combined with photochemical labeling studies using 2-azido-ATP have also provided evidence for the presence of three aromatic residues at the catalytic nucleotide binding site (Phe452, Tyr532 and Phe538) [56,59]. These residues are postulated to stabilize the adenine ring of the bound nucleotide through π–π interactions.

Several lines of evidence suggest the B subunit also participates in nucleotide binding. First, the B subunit is homologous to the A subunit of V_1 and the α and β subunits of F_1 (approximately 20–25% identity at the amino acid level [32,48]), although it lacks the Walker A and B consensus sequences of these proteins. Second, the B subunit is modified by the photoactivatable nucleotide analog BzATP [60,61], and this modification occurs at a site distinct from the catalytic site [61]. Molecular modeling studies (described above) were used to derive the structure of the nucleotide binding site on the B subunit, shown in Fig. 3b [62]. Mutation of residues at this site, termed the 'non-catalytic' site, generally lead to only partial loss of activity [63]. To test the accuracy of this model, residues predicted to be at the non-catalytic site on the B subunit were mutated to cysteine and their reactivity towards the sulfhydryl reagent biotin maleimide in the presence and absence of BzATP was tested [62]. While a number of the mutant proteins were not reactive towards biotin maleimide (presumably because the corresponding cysteine residue was

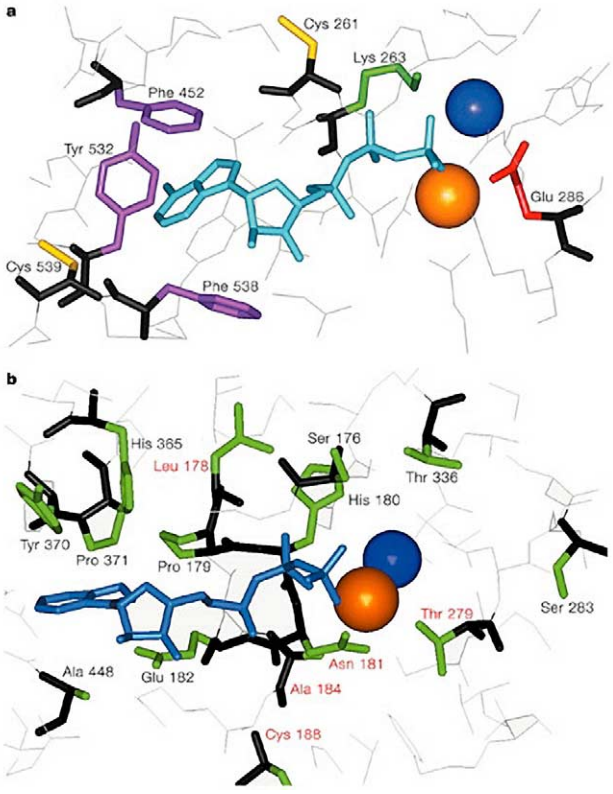

Fig. 3. Structural models of the catalytic and non-catalytic nucleotide binding sites on the V-ATPase. Models are based upon sequence homology between the nucleotide binding subunits of the V- and F-ATPases, the available X-ray coordinates of F_1 [34] and energy minimization [56,62]. Bound ATP is shown in blue. Blue spheres are water molecules and orange spheres are Mg^{2+} ions. a: The catalytic sites reside primarily on the A subunits. Lys263 is postulated to stabilize the negative charges on bound ATP while Glu286 is thought to directly participate in ATP hydrolysis [50] (see text). The aromatic residues (shown in purple) are postulated to assist in ATP binding through interaction with the adenine ring [56,59]. b: The non-catalytic sites reside primarily on the B subunits. The residues shown in red have been shown to be protectable from modification by sulfhydryl reagents in the presence of a nucleotide analog when mutated to cysteine [62]. The non-catalytic sites on the V-ATPases have been proposed to function in regulation of activity [56]. Reprinted with permission from the Journal of Biological Chemistry [56,62], ©1998 and 2000, The American Society for Biochemistry and Molecular Biology and Nature Reviews Molecular Cell Biology [1], ©2002, Macmillan Magazines, Ltd.

buried within the structure), all of those that were, including S152C, L178C, N181C, A184C, T279C and the wild type protein (containing a cysteine residue at position 188), showed biotin maleimide labeling that was blocked in the presence of BzATP [62]. These results support the model for the non-catalytic nucleotide binding site shown in Fig. 3b.

As with the F-ATPase [34,35], the nucleotide binding sites on the V-ATPase appear to be at the interface of the A and B subunits, with the catalytic sites located primarily on A and the non-catalytic sites located primarily on B. In support of this interfacial location, mutations of B subunit residues predicted to contribute to the catalytic sites (including R381 and Y352) lead to complete loss of activity [63]. Also, mutations of A subunit residues predicted to contribute to the non-catalytic sites lead to time-dependent changes in activity that occur upon exposure of the enzyme to ATP [56]. These latter results suggest that the non-catalytic sites on the V-ATPase may play a regulatory role in controlling activity [56].

4. Structure, function and arrangement of other V_1 subunits

Subunit C is encoded by the *VMA5* gene in yeast and has a molecular mass of approximately 40 kDa [64]. Subunit C is particularly interesting in that, during reversible dissociation of the yeast V-ATPase complex in response to glucose depletion (an important mechanism of regulating V-ATPase activity in vivo [65]), subunit C is released from both the V_1 and V_0 domains, suggesting that it may play a role in controlling this process. Mutations in a number of aromatic residues in subunit C appear to cause a destabilization of the V-ATPase complex but substantially increase the rate of both ATPase activity and proton transport of the enzyme that is assembled [66]. Both crosslinking studies [19] and co-immunoprecipitation [67] indicate that subunit C and E are in contact in the V-ATPase complex.

Subunit D is encoded by the *VMA8* gene in yeast and has a molecular mass of about 34 kDa [68]. Mutations in subunit D have been identified that lead to partial uncoupling of proton transport and ATPase activity [69]. This is similar to mutations in the γ subunit of F_1, which also lead to an uncoupled phenotype [70]. A synergistic effect of mutations clustering in two regions of the molecule near the N- and C-termini suggests the possibility that the D subunit may loop back upon itself such that these two regions interact. These observations, combined with the high predicted α-helical content, have led to the suggestion that subunit D may function as the γ subunit homolog in the V-ATPases [69]. This suggestion has been substantiated by crosslinking studies in which unique cysteine residues introduced into the B subunit were used as sites of attachment of the photoactivated crosslinking reagent maleimido benzophenone [71]. Only cysteine residues introduced into sites predicted to be oriented towards the center of the A_3B_3 hexamer resulted in crosslinking of subunits B and D [71]. These results suggest that subunit D, like the γ subunit of F_1, makes up part of the central stalk connecting the V_1 and V_0 domains.

Subunit E is encoded by the *VMA4* gene in yeast and has a molecular mass of 33 kDa in bovine and 27 kDa in yeast [64]. Temperature-sensitive mutants of subunit E in yeast have been identified that result in defective assembly of the V-ATPase complex [72]. Co-immunoprecipitation indicates that subunits E and C are able to form a complex [67] whereas crosslinking data indicate that subunit E makes contact with subunits C, G and H in V_1 as well as subunit a in V_0 [19]. In yeast strains lacking a number of different V_1 subunits, a subcomplex containing subunits E and G can be isolated [73,74], providing further support for contact between subunits E and G. Based upon its high predicted α-helical content [75] and protection from protease digestion in isolated V_1 [76], subunit E has been proposed to function as the γ subunit homolog in the V-ATPases [75,76]. This appears unlikely, however, since cysteine-mediated crosslinking of subunits B and E occurs only at sites on subunit B predicted to be oriented towards the outer surface of the complex [71,77]. Based upon these results, subunit E, together with subunits C, G, H and the soluble domain of subunit a, has been proposed to function as a peripheral stalk connecting the V_1 and V_0 domains [19,71,77].

Subunit F is a 14 kDa protein encoded by the *VMA7* gene in yeast [78]. In strains lacking various V_1 subunits, a subcomplex containing subunits D and F can be isolated [73,74]. That these subunits are in contact in both the intact V-ATPase complex and the isolated V_1 domain is indicated by covalent crosslinking studies [19]. Thus, subunits D and F have been proposed to both contribute to the central stalk connecting the V_1 and V_0 domains [19,77].

Subunit G is a 13 kDa protein encoded by the *VMA10* gene in yeast [79]. As indicated above, evidence for interaction between subunits E and G has come from both crosslinking [19] and isolation of an EG subcomplex [73,74]. That subunit G occupies a peripheral location in the V-ATPase complex near the top of the V_1 domain is supported by photoactivated crosslinking of subunit G to subunit B at sites oriented towards the outer surface of the complex near the amino-terminus of subunit B [71]. The fact that subunit G is present in two copies per complex [19] and exhibits some amino acid sequence homology with the soluble domain of subunit b of F_0 along one face of a predicted α-helix [80] has led to the suggestion that, like subunit b, subunit G forms part of the peripheral stalk connecting the V_1 and V_0 domains [80]. In further support of this idea, short deletions of the region of subunit G predicted to adopt a helical conformation were shown not to disrupt assembly of the V-ATPase [81], similar to findings made on subunit b of the F_0 domain [82]. Interestingly, a number of point mutations in subunit G have been identified which lead to increased stability of the V-ATPase complex and partially block glucose-dependent dissociation of the V_1 and V_0 domains [81].

Subunit H is a 50 kDa protein encoded by the *VMA13* gene in yeast [83]. Subunit H is unique in that it is the only V-ATPase subunit that is not absolutely required for assembly of the V-ATPase complex, although enzymes assembled in its absence show reduced stability [83]. Subunit H is also the only V-ATPase subunit for which high resolution structural data exist [84]. This structure has revealed the presence of motifs also found in importins, proteins involved in targeting of other proteins to the nucleus [84]. Although the function of these importin domains is not known, it has been suggested that they might mediate the interaction of subunit H with other V-ATPase subunits or with other cellular proteins outside the V-ATPase complex [84]. In fact, subunit H has been shown to interact both with the AP-2 adapter complex that mediates receptor-mediated endocytosis from the plasma membrane [85] and with the Nef protein that is involved in endocytosis

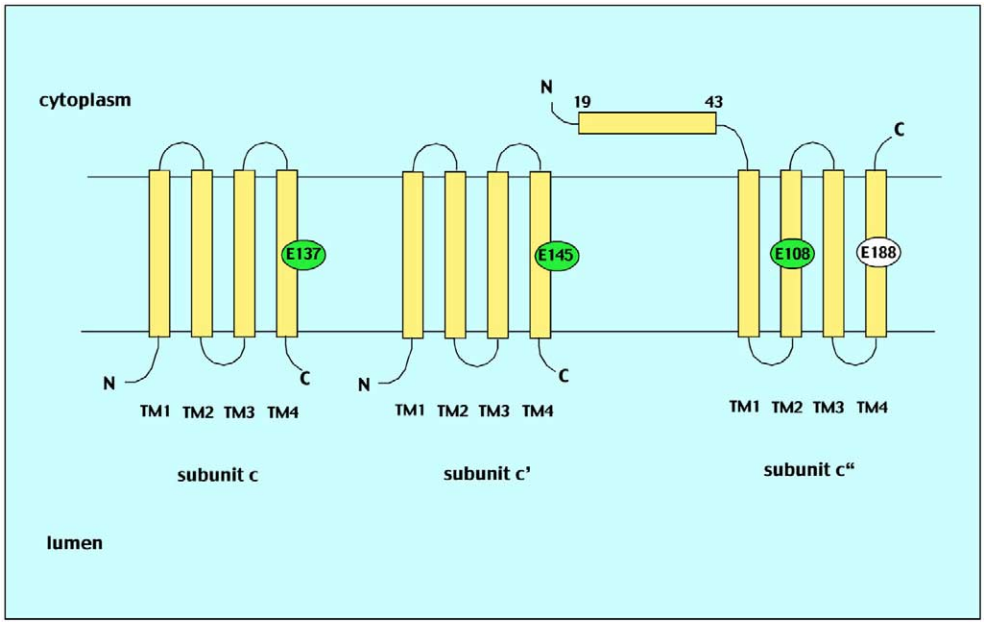

Fig. 4. Proposed topology of subunits c, c′ and c″ of the V-ATPase. Hydrophobicity analysis suggests both subunits c and c′ contain four transmembrane segments whereas subunit c″ is predicted to contain five transmembrane segments. Recent topological analysis, however, suggests that subunit c″ also contains four transmembrane segments, with the N-terminal hydrophobic region (originally TM1) dispensable for activity [91]. The C-terminus of subunit c, unlike subunit c″, resides on the luminal side of the membrane [92]. Subunits c and c′ each contain an essential buried acidic residue in TM4 [90,97] whereas the essential carboxyl group in subunit c″ (E108) is present in TM2 [90]. The acidic residue present in TM4 of subunit c″ (E188) is not essential for activity [90].

of the HIV receptor CD4 [86]. Subunit H has been shown to inhibit ATPase activity by the V_1 domain [87], thus keeping this soluble domain from participating in non-productive hydrolysis of ATP [67]. In addition, overexpression of subunit H has been shown to lead to functional uncoupling of ATP hydrolysis and proton transport [87]. Crosslinking studies indicate that subunit H is in proximity to subunit E [19] whereas co-immunoprecipitation has demonstrated interaction between subunit H and the amino-terminal hydrophilic domain of subunit a [88]. Subunit H is thus likely to form part of the peripheral stalk connecting the V_1 and V_0 domains.

5. Structure and function of V_0 subunits

The V_0 domain contains three different proteolipid subunits [33,89,90]: subunit c (product of the *VMA3* gene), subunit c′ (product of the *VMA11* gene) and subunit c″ (product of the *VMA16* gene). Both subunits c and c′ are 17 kDa polypeptides containing four putative transmembrane helices [33,90] whereas subunit c″ is a 23 kDa protein originally postulated to contain five putative transmembrane helices [90]. Recent results using cysteine mutagenesis and labeling by membrane-permeant and -impermeant maleimides, however, indicate that subunit c″ contains only four transmembrane segments, with both the amino- and carboxy-termini on the cytoplasmic side of the membrane (Fig. 4) [91]. Moreover, the hydrophobic segment originally suggested to correspond to TM1 has been shown to be dispensable for function [91]. Interestingly, the C-termini of subunits c and c″ appear to reside on opposite sides of the membrane [92]. All three proteolipid subunits are highly hydrophobic and are homologous to each other and to subunit c of the F-ATPases [33,90]. It appears as though the V-ATPase proteolipid genes arose through gene duplication and fusion of the F-ATPase c subunit gene, since the two halves of subunits c and c′ are homologous to each other. TM2 and TM3 of subunit c″ are similarly homologous to TM4 and TM5 of the same subunit [90]. Nuclear magnetic resonance analysis of the F-ATPase subunit c (an 8 kDa protein) reveals two transmembrane segments in a helical hairpin structure [93].

Quantitative amino acid analysis indicates the presence in V_0 of five or six copies of subunits c/c′ and a single copy of subunit c″ [18]. In addition, co-immunoprecipitation of epitope-tagged proteins demonstrates that both subunits c′ and c″ are present in single copies whereas subunit c is present in two or more copies [20]. There are also data suggesting the presence of two copies of subunit c″ per V_0 complex [94], but this may be due to the expression of Vma16p at high levels. Combining the former results, the likely proteolipid stoichiometry of the V_0 domain is $c_{4-5}c'_1c''_1$, suggesting that the proteolipids contribute a total of 25–29 transmembrane helices to the integral domain. Because the proteolipid ring in the F-ATPases contains only 10 c subunits (which contribute 20 transmembrane helices [36,37]), it appears that the F_0 c subunit ring is substantially smaller than that of V_0, although there are some reports of F_0 domains containing a larger number of c subunits [95,96].

Each of the V-ATPase proteolipid subunits contains a single buried glutamate residue that is essential for proton transport [90,97]. In subunits c and c′, this acidic residue is present in TM4 while in subunit c″ it is present in TM3 [90,97]. Mutagenesis of any of these glutamate residues completely inhibits proton transport [90]. In addition, the buried glutamate residues of subunit c (and possibly subunit c′) are the sites of covalent modification by the inhibitor dicyclohexylcarbodiimide (DCCD) [98]. Interestingly, reaction of a single copy of subunit c per complex with DCCD is sufficient to completely inhibit proton transport [98]. This suggests that the

enzyme is sequentially sampling each of the proteolipid subunits during catalysis. The F-ATPase c subunit also contains an essential buried acidic residue present in TM2 [23]. Why the V-ATPases require three different proteolipid subunits whereas the F-ATPases function with just one is not known. One possible explanation is that the asymmetry in V_0 may prevent this domain from acting as a passive proton channel in the absence of V_1 [99], a property commonly observed for F_0 [23]. Such passive proton conductance through V_0 would make it impossible to use reversible dissociation of the V_1 and V_0 domains as a mechanism of regulating V-ATPase activity in vivo [65], since the presence of free V_0 domains would make intracellular membranes highly permeant to protons.

Crosslinking studies and labeling by a membrane-permeant carboxyl reagent have been employed to investigate the arrangement of the c subunit helices in the V_0 domain [100,101]. These studies suggest that TM4 of subunit c containing the essential glutamate residue is oriented towards the hydrophobic phase of the bilayer, while TM1 lines the hole in the center of the proteolipid ring. This arrangement is similar to that proposed for the c subunit ring of F_0 (also based on crosslinking analysis [23]), although a rotation of TM2 of subunit c has been proposed to occur during catalysis such that the helical face containing the buried acidic residue becomes exposed [102,103].

Subunit c has also recently been shown to form at least part of the binding site for the highly specific V-ATPase inhibitors bafilomycin and concanamycin [104,105]. Thus, mutations conferring resistance to bafilomycin A1 were mapped to the c subunit gene in *Neurospora*, with two of these mutations located in TM4 on either side of the critical glutamate residue, leading to the suggestion that bafilomycin may interfere with rotation of this helix during proton transport [104]. In addition, subunit c was labeled with a photoreactive analog of concanamycin A [105].

In addition to the proteolipid subunits, the V_0 domain also contains two other subunits. Subunit d is a 38 kDa protein encoded by the *VMA6* gene in yeast [106]. Despite the fact that it contains no predicted transmembrane segments, it remains tightly bound to the V_0 domain upon dissociation of V_1 using chaotropic agents [107]. The function of subunit d is unknown, but it does appear from proteolysis and labeling studies [18,108] as well as alkaline extraction [106] to reside on the cytoplasmic side of the membrane.

The final subunit in V_0 is subunit a. The a subunit is a 100 kDa integral membrane protein composed of a hydrophilic amino-terminal domain and a hydrophobic carboxy-terminal domain containing multiple transmembrane segments [109]. It is the only subunit in yeast encoded by two genes: *VPH1* and *STV1* [110,111]. Vph1p targets the V-ATPase to the central vacuole whereas Stv1p targets the V-ATPase to the late Golgi compartment [111,112]. V-ATPase complexes containing Vph1p and Stv1p differ not only in intracellular localization but also in their degree of assembly with V_1, the tightness with

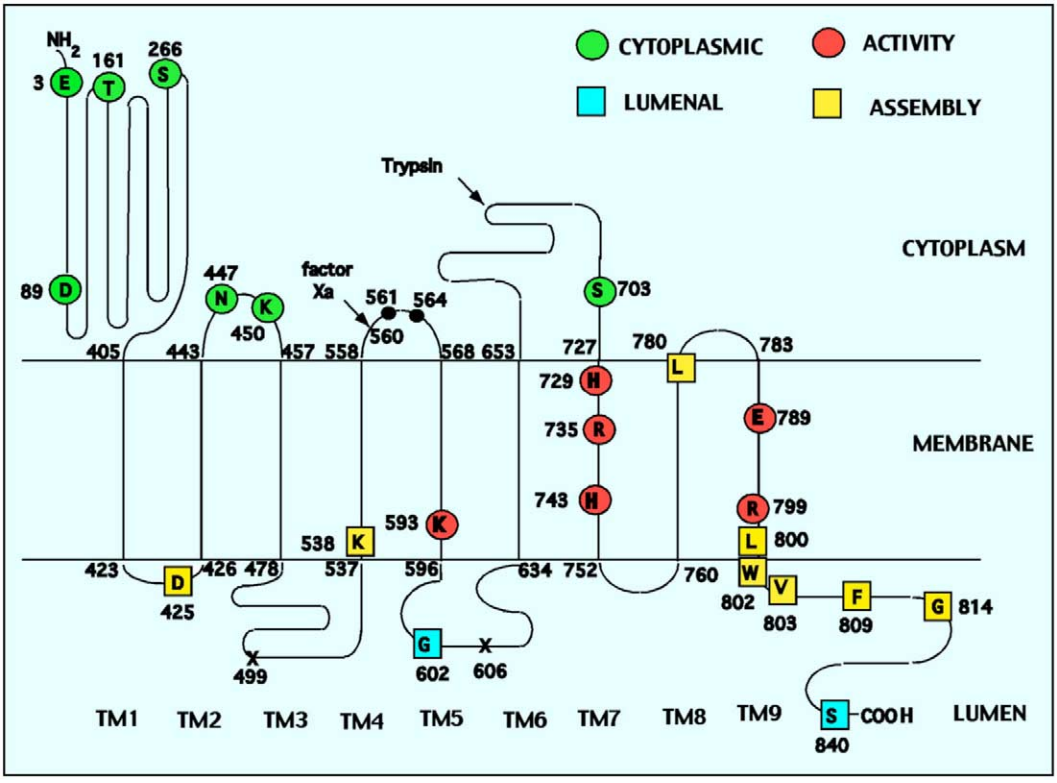

Fig. 5. Proposed topology and location of important residues in subunit a. Based upon studies employing cysteine mutagenesis and labeling by membrane-permeant and -impermeant maleimides [117], the a subunit is proposed to contain nine transmembrane segments, with the large hydrophilic amino-terminal domain located on the cytoplasmic side of the membrane and the carboxy-terminus located on the luminal side of the membrane. Residues shown in green were shown to have a cytoplasmic orientation whereas those shown in blue are oriented towards the lumen [117]. Mutations at residues shown in yellow affect assembly of the V-ATPase [118,119] whereas those shown in red affect activity [118–120]. Of the residues identified thus far, only Arg735 in TM7 is absolutely essential for proton transport [120]. Reprinted by permission from Nature Reviews Molecular Cell Biology [1], ©2002, Macmillan Magazines, Ltd.

which ATP hydrolysis is coupled to proton translocation and their ability to undergo reversible dissociation in vivo [113]. The signals controlling targeting and dissociation appear to be located in the hydrophilic amino-terminal domain whereas the signals controlling assembly and coupling efficiency are present in the carboxy-terminal domain [112]. In mammalian cells there are four isoforms of subunit a (a1–a4) [114–116]. These isoforms appear responsible not only for targeting the V-ATPase to different intracellular compartments, but also to the plasma membrane of cells such as osteoclasts and renal intercalated cells [16,115,116].

Topological studies of the a subunit using cysteine mutagenesis and labeling by membrane-permeant and -impermeant maleimides as well as protease cleavage have led to the proposed folding model shown in Fig. 5 [117]. The amino-terminal domain is located on the cytoplasmic side of the membrane and is separated from the C-terminus (which is located on the luminal side of the membrane) by nine putative transmembrane segments [117]. Mutagenesis studies have identified a number of buried charged residues present in TM7 and TM9 whose mutation affects proton transport, including E789 and H743 [118,119]. The only a subunit residue identified that is absolutely required for proton transport activity, however, is R735 in TM7 [120]. Even the conservative R735K mutation at this position leads to complete loss of proton translocation [120]. This situation is similar to that observed for subunit a of the F-ATPases, where R210 of the *E. coli* enzyme is absolutely required for ATP-driven proton transport whereas mutation of a number of other buried charged residues in subunit a also leads to varying degrees of inhibition [121–124]. As explained below, subunit a is believed to play an integral part in the mechanism by which protons traverse the membrane in both V_0 and F_0.

6. Mechanism of ATP-driven proton transport by the V-ATPases

Because of their structural similarity to the F-ATPases, the V-ATPases are thought to operate by a similar rotary mechanism. For the F-ATPases, ATP hydrolysis in the F_1 domain drives rotation of the central γ subunit which, together with the ε subunit, forms the central stalk connecting the F_1 and F_0 domains [36,42]. Rotation of both the γ and ε subunits of F_1 has been demonstrated using a variety of methods [125–127]. Because the γ and ε subunits are attached to the ring of c subunits [36,128], rotation of the central stalk also drives rotation of the ring of c subunits relative to subunit a. Rotation of the c subunit ring has also been demonstrated experimentally [129,130]. Subunit a is held fixed relative to the $\alpha_3\beta_3$ hexamer of F_1 by a peripheral stalk (or stator) composed of the δ subunit of F_1 and the soluble domains of the b subunit of F_0 [43,44]. Movement of the c subunit ring relative to subunit a has been proposed to lead to active proton translocation across the membrane as follows [131,132]. Subunit a is proposed to form two hemi-channels that allow access of protons from the cytoplasmic and extracellular sides of the membrane to the buried carboxyl groups on the ring of c subunits. Charged residues in subunit a that are not essential but whose mutation affects proton transport are proposed to line these hemi-channels [133,134]. Subunit a is also proposed to stabilize the carboxyl groups of those c subunits in contact with subunit a in a deprotonated state, possibly through their interaction with the critical arginine residue (R210 in *E. coli* [102,133,134]). During rotation of the c subunit ring, the buried carboxyl groups must pick up a proton from one hemi-channel (that leading to the cytoplasmic surface) in order to rotate into contact with the lipid bilayer. By contrast, when these protonated carboxyl groups rotate through the membrane to the other hemi-channel (in contact with the extracellular space), they are induced to release their proton through interaction with the a subunit arginine residue. Thus the rotary motion induced by ATP hydrolysis leads to vectorial transport of protons across the membrane.

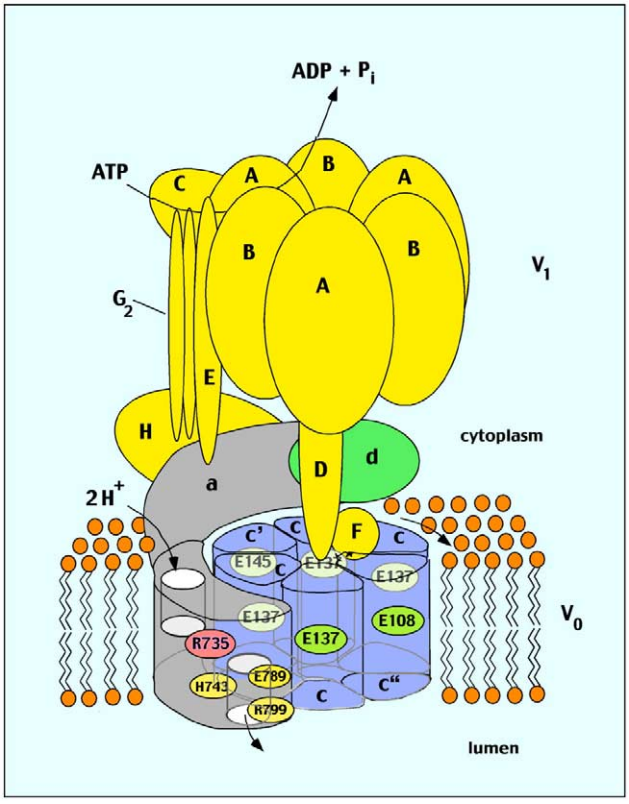

Fig. 6. Proposed rotary mechanism of ATP-driven proton translocation by the V-ATPases. ATP hydrolysis in the V_1 domain is proposed to drive rotation of the central stalk (composed of the D and F subunits), which in turn drives rotation of the ring of proteolipid subunits (c, c′ and c″) relative to subunit a in V_0. Subunit a is held fixed relative to the A_3B_3 head of V_1 by the peripheral stalk (or stator) composed of subunits C, E, G and H. Subunit a is shown forming two hemi-channels in communication with either the cytoplasmic or luminal side of the membrane. As each of the protonated carboxyl groups on the proteolipid subunits encounters the luminal channel, it is induced to lose a proton trough interaction with Arg735 of subunit a. By contrast, these acidic residues must pick up a proton from the cytoplasmic channel before re-entering the hydrophobic phase of the bilayer. This model is based upon that originally proposed for the F-ATPases [131,132]. Reprinted by permission from Nature Reviews Molecular Cell Biology [1], ©2002, Macmillan Magazines, Ltd.

Extending this rotary mechanism to the V-ATPases (Fig. 6), ATP hydrolysis in V_1 is proposed to drive rotation of the central D and F subunits which in turn drives rotation of the ring of proteolipid subunits. The fact that there are only approximately half as many buried carboxyl groups in the proteolipid ring of V_0 as there are in F_0 has been proposed as an explanation for the lower H^+/ATP stoichiometry of the V-ATPases (two) compared to the F-ATPases (three or four)

[135]. Subunit a is held fixed relative to the A_3B_3 hexamer by a peripheral stalk composed of subunits C, E, G, H and the soluble domain of subunit a [19,71,77,88]. The complexity of this peripheral stalk may be accounted for by the fact that it must serve not only as a stator during rotary catalysis but may also control reversible dissociation of the complex during regulation of V-ATPase activity in vivo [65,66]. Subunit a residues such as E789 and H743 are proposed to line the access channels leading to the c subunit carboxyl groups, whereas Arg735 is proposed to function in release of protons from the proteolipid subunits into the luminal access channel [120]. Bafilomycin may prevent rotation of the proteolipid ring by binding at the interface of the a and c subunits [104]. Consistent with such a rotary mechanism are the observations that proton transport and ATP hydrolysis are completely inhibited upon reaction of a single c subunit with DCCD [98] or a single A subunit with 2-azido-ATP [55]. Nevertheless, proof that the V-ATPases also operate via a rotary mechanism awaits direct experimental demonstration.

7. Conclusions

The V-ATPases have evolved to function as ATP-driven proton pumps in both intracellular membranes and the plasma membrane. Their structural similarity to the F-ATPases suggests that they operate via a similar rotary mechanism. The greater complexity of the V-ATPases, however, likely reflects the diversity of functions they perform and the need to regulate their activity in a variety of different cellular environments.

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