Research Letter

Features of V-ATPases that distinguish them from F-ATPases

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Abstract The general structure of F- and V-ATPases is quite similar and they may share a common mechanism of action that involves mechanochemical energy transduction. Both holoenzymes are composed of catalytic sectors, F_1 and V_1 respectively, and membrane sectors, F_o and V_o respectively. Although we assume that a similar mechanism underlies ATP-dependent proton pumping by F- and V-ATPases in eukaryotic cells, the latter cannot catalyze pmf-driven ATP synthesis. The loss of this ability is probably due to a proton slip that is a consequence of alterations in its membrane sector. The major events include gene duplication of the proteolipids and the presence of three distinct proteolipids in each complex.

Key words: V-ATPase; F-ATPase; Proteolipid; Slip mechanism

1. Introduction

V-ATPase and F-ATPase are evolutionarily and functionally related [1–3]. Both are complexes of more than 10 subunits assembled into two distinct sectors: A catalytic sector involved in energy conversion and a membrane sector that functions in proton transport across the membrane. In eukaryotic cells both enzymes exist, but the F-ATPase is confined into semiautonomous organelles (mitochondria and chloroplasts), whereas V-ATPase is present in most membranes connected with the secretory pathway. Although the activity of both enzymes is coupled to proton translocation through the membrane in which the enzyme is located, their location designates a different function to both complexes: F-ATPase is mainly an ATP synthase, while the V-ATPase only has an H^+-ATPase function.

It was demonstrated that several types of mammalian cells can survive without functional F-ATPase, whereas V-ATPase is vital even in lower organisms [4–6]. To date, yeast cells are the only eukaryotic ones capable of living without V-ATPase [7,8]. Null V-ATPase mutants exhibit a low-pH-dependent phenotype, thus yeast became instrumental in the V-ATPase research. Yeast turned out to be an excellent model system to identify essential V-ATPase subunits. With the exception of *VPH1* and *STV1* that encode homologous proteins [9,10], all other genes encoding V-ATPase subunits are present as a single copy in the yeast genome [3]. Disruption of each of the genes encoding essential V-ATPase subunits (except for Vph1p and Stv1p, the analogues of the α subunit of F-ATPase) resulted in an identical phenotype that is unable to grow at a pH higher than 7, and sensitive to low and high calcium concentrations in the medium [7].

Definite functions have been assigned to all but four of the 13 subunits that comprise the V-ATPase, the functional assignments being based primarily on homology to subunits in the F-ATPase (V-A to F-β, V-B to F-α, V-G to F-b, and V-c to F-c). It is assumed that the function of the homologous subunits is similar in F- and V-ATPases. However, the β subunit of F-ATPase is a typical F_o subunit, whereas the G subunit of V-ATPase was reported to be associated both with V_1 and V_o sectors [11–14]. The structure is most clearly conserved between the V_1-A and F_1-β and between the V_1-B and F_1-α subunits. Other subunits of the catalytic sector are less conserved. The extreme is subunit D which is only analogous to the γ subunit of F1-ATPase, and the appearance of additional subunits that are absent in the latter [15]. The same is true for the membrane sector. In the yeast V-ATPase, the membrane sector includes subunit c (proteolipid) which has four transmembrane helices, and two additional proteolipids c′ and c″, as well as Vph1 and Stv1, two analogs of a subunit of F1-ATPase [16–18]. Why is one proteolipid sufficient for F-ATPases, whereas three of them are required for V-ATPases? The likely mechanistic constraint that may have led to the evolution of this deviation is the change in the coupling efficiency of V-ATPases that is referred to as the 'slip' mechanism [1,19].

2. Materials and methods

2.1. Strains, media, and reagents

The wild-type (WT) that was used is *Saccharomyces cerevisiae* W303 (*MATa/α trp1 ade2 his3 leu2 ura3*). The other strains used in this work are: *vma3Δ (MATa ade2 trp1 ura3 his3 VMA3::LEU2)*; *vma11Δ (MATa ade2 trp1 ura3 his3 VMA11::ura3)*; *vma16Δ (MATa ade2 trp1 his3 leu2 VMA16::URA3)*; *vph1Δ/stv1Δ (MATa ade2 trp1 ura3 his3 VPH1::LEU2 STV1::URA3)*. The cells were grown in a YPD medium containing 1% yeast extract, 2% bactopeptone, and 2% dextrose or galactose. The medium was buffered by 50 mM Mes and 50 mM Mops, and the pH was adjusted by NaOH [7,20]. Agar plates were prepared by the addition of 2% agar to the YPD buffer medium at the given pH. Yeast transformation was performed as previously described [21,22], and the transformed cells were grown on minimal plates containing a 0.67% yeast nitrogen base, 2% dextrose, 2% agar and the appropriate nutritional requirements.

2.2. Preparation of yeast vacuoles

For preparation of vacuoles, cells were grown in YPD medium

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adjusted to pH 5.5 by HCl and harvested at a cell density of about 0.8 OD units at 600 nm. Vacuolar membranes were prepared according to Uchida et al. [23], except that the 8% Ficoll gradient purification step was omitted, the homogenization buffer contained no magnesium and the vacuoles were washed only once with the EDTA buffer. ATP-dependent proton uptake activity was assayed by following the absorbance changes of acridine orange at 490–540 nm as previously described [24]. The 1-ml reaction mixture contained 20 mM Mops–Tris (pH 7), 15 mM KCl, 135 mM NaCl and 15 μM acridine orange. Isolated yeast vacuoles containing 10 to 30 μg were added to the reaction mixture followed by 10 μl of 0.1 M MgATP. The reaction was terminated by the addition of 1 μl of 1 mM carbonyl cyanide *p*-(trifluoromethoxy)phenyl-hydrazone (FCCP).

2.3. Staining of vacuoles in yeast cells with LysoSensor Green DND-189

All strains were grown at 30°C to an OD_{600} between 0.7 and 1. For LysoSensor Green staining (Molecular Probes Inc.), cells were harvested and washed with uptake buffer (YPD containing 100 mM HEPES, pH 7.6). The cell pellets were resuspended at 20 OD_{600}/ml in uptake buffer and dye was added to a final concentration of 15–30 μM from a stock solution of 1 mM in DMSO. Cells were then incubated for 30 min at 30°C and washed once with the same buffer. Cell pellets were resuspended in fresh YPD pH 7.6 at 10–15 OD_{600}/ml, placed on standard slides and photographed within 1 h after staining. The samples were viewed with a LSM510 confocal laser scanning microscope (Zeiss) equipped with an argon 458-nm laser, and a C-Apochromat 63× water immersion objective. A LP505 filter was used for LysoSensor Green DND-189 fluorescence. The images were recorded, merged and processed using the Zeiss LSM Image browser.

2.4. Construction of chimeras

Random chimeras of *VMA3* and *VMA11* were constructed using the method of R. Reed by which chimeras of two homologous genes are created in situ in transformed *Escherichia coli* cells with linear plasmid [25]. A series of chimeras were obtained with a construct in which *VMA3* was followed by *VMA11*. Both genes' reading frames (Met to stop codon – 0.5-kb fragments) were amplified by PCR on WT yeast genomic DNA using appropriate primers carrying unique restriction sites at their ends. The sense primer of *VMA3* carried the *Sac*I site and its antisense carried an *Eco*RI site. For *VMA11*, the sense primer carried an *Eco*RI site and the antisense carried a *Xho*I site. In this way, both reading frames were ligated tail-to-head, with a unique *Eco*RI site between them, into pYES2 (Invitrogen) plasmid predigested with *Sac*I and *Xho*I. The *Eco*RI-linearized plasmid was used for transformation of competent *E. coli* cells. From the transformed colonies plasmid DNA was prepared, and the ones containing an insert of about 500 bp were chosen for further analysis. *Hin*dIII, a unique site of *VMA3*, and *Bst*XI, a unique site for *VMA11*, were used for sequential digestions of the plasmid minipreps to ascertain the approximate location of the chimera's junction. Each chimera was checked for complementation to both *vma3Δ* and *vma11Δ* on YP-galactose plates buffered at pH 7.5. The chimeras were sequenced to ascertain restriction analysis and verify their in-frame configuration. Using this method we have generated a series of chimeras in regions of sequence conservation between *VMA3* and *VMA11*.

3. Results and discussion

3.1. Dissociation of the catalytic sector under physiological conditions

Very few enzymes are inactivated by cold treatment, F-ATPase holoenzyme being no exception to the rule. The discovery by Racker and his colleagues of cold sensitivity of the catalytic sector (F_1) of F-ATPase, was pivotal for the pioneering studies of the enzyme [26]. The isolated F_1 part of the complex dissociates to its subunits upon its incubation on ice and its ATPase activity is abolished. This cold inactivation can be protected by including ATP in the incubation medium or by binding it back onto the membrane sector. We were therefore surprised to discover that V-ATPase complex (V_1–V_o) of chromaffin granules was cold-sensitive [27]. More-

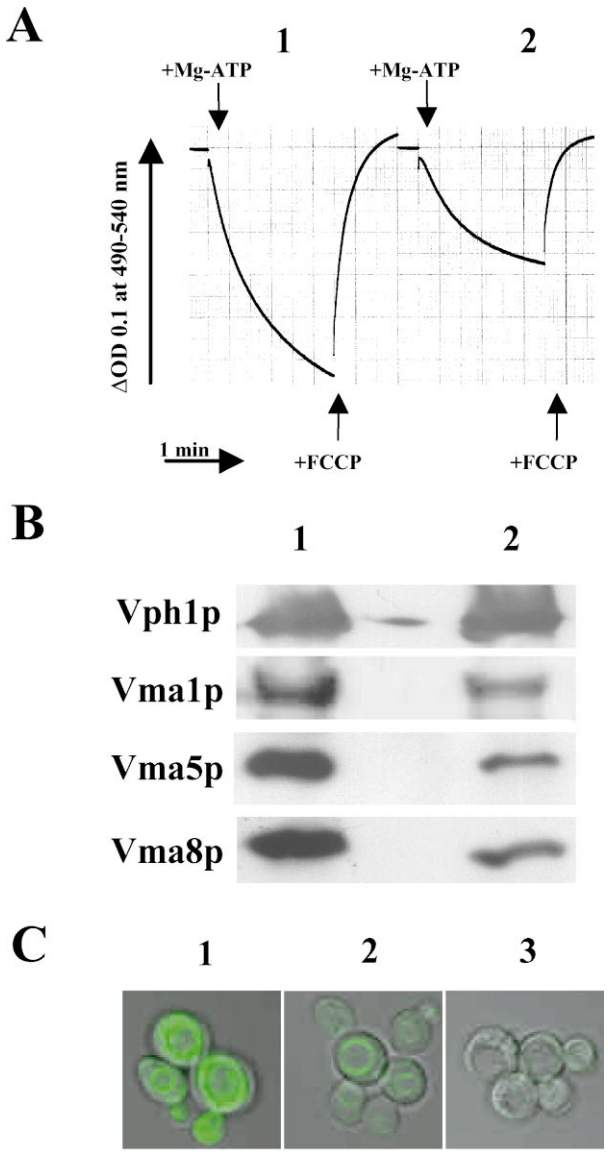

Fig. 1. Addition of 0.8 M NaCl in the growth medium results in reduced V-ATPase activity. A: Yeast vacuoles were isolated as described under Section 2 and adjusted to protein concentration of 3 mg/ml. Vacuoles containing 50 μg protein were assayed in each sample. ATP-dependent proton uptake was measured by absorption changes at 490–540 nm due to acridine orange accumulation. Where indicated, 10 μl 0.1 Mg-ATP or 1 μl 1 mM FCCP were added. (1) Cells grown on YPD. (2) Cells grown on YPD containing 0.8 M NaCl. B: Western blot of vacuolar membranes decorated with antibodies against V-ATPase subunits. The preparations of yeast vacuoles (10 μg protein) applied to 10% SDS–PAGE, and immunoblotted with the indicated antibodies. (1) Cells grown on YPD. (2) Cells grown on YPD containing 0.8 M NaCl. C: Yeast cells labeled with the pH indicator LysoSensor Green DND-189. Cells grown on YPD (pH 5.5) with or without addition of 0.8 M NaCl at 30°C were transferred to uptake buffer (YPD containing 100 mM HEPES buffered at pH 7.5) and stained with the dye (20 μM final concentration). Vacuolar acidification was monitored by accumulation of the fluorescent dye LysoSsensor Green DND-189 on the vacuolar membrane. Dye uptake was monitored by confocal laser scanning microscope (see Section 2). (1) Cells grown on YPD. (2) Cells grown on YPD containing 0.8 M NaCl. (3) The double mutant *stv1Δ*/*vph1Δ* was grown on YPD.

over, Mg-ATP is required for its cold inactivation and inclusion of EDTA in the medium protects against this sensitivity. In addition treatment by NEM that binds at the ATP-binding site of the enzyme had a protective effect as well. Similar effects were observed with yeast vacuoles and membranes containing V-ATPase from other sources, including Golgi and lysosomes [28,29]. Sedimentation of the membranes and analysis of the supernatants of those cold-inactivated preparations revealed that V1, the catalytic sector, dissociated from the membranes upon the cold treatment. This V1 has no ATPase activity, and when H^+ leakage of cold-treated vacuoles was checked, no such leakage was observed. This demonstrates that the membrane sector of V-ATPases, contrary to the F_o of F-ATPases, is by itself not capable of proton translocation [30]. If the V_o were to keep its proton conductivity intact after the V_1 dissociation, the organism would be doomed under cold and other stress conditions. The cold inactivation of V-ATPase is reversible. It was shown that the V_1–V_o reassembly is pH-dependent and it was proposed that the E188 of c″ is the pH sensor for the complex [31,32].

Dissociation of V_1 from the membrane sector was demonstrated in situ in plant and yeast cells following glucose deprivation and during the molting period of insects [33–35]. We recently observed that elevated NaCl concentrations in the growth medium of yeast cells have a similar effect as glucose deprivation. As shown in Fig. 1, the proton uptake activity of isolated vacuoles of cells grown overnight in YPD containing 0.8 M NaCl was diminished as compared to the control in YPD alone (Fig. 1A). The reduction of proton-pumping activity under those conditions reflects a lower amount of assembled holoenzyme in the vacuolar membrane (Fig. 1B). In parallel, intact cells were analyzed for vacuolar acidification in vivo by the pH-sensitive fluorescent dye LysoSensor Green (Molecular Probes). This is a hydrophobic indicator that is absorbed into membranes of acidic compartments in the cell. The salt-treated cells show much lower fluorescence intensity (Fig. 1C). The controls of this experiment are V-ATPase null mutant and WT cells grown in YPD and incubated in YPD containing 0.8 M NaCl prior to their labeling with the dye. The controls demonstrate that there is no dye accumulation in the vacuolar membrane without V-ATPase activity, and that NaCl by itself does not interfere with the dye penetration and its accumulation in the membranes in short time periods.

V-ATPase responds to physiological stresses not only by the above-mentioned dissociation, but also by modulation of its biosynthesis, degradation, assembly and change in distribution patterns [2,36]. The latter differentiates the V-ATPase from its related enzyme, F-ATPase. Moreover, the bioenergetics of F-ATPase does not permit a dissociation event, as the proton leakage of its membrane sector or the ATPase activity of its catalytic sector will be deleterious to the functionality of the organelles it functions in namely mitochondria and chloroplasts. This constraint requires coordinated assembly of the catalytic and membrane sectors so that at no time during the biogenesis process will either catalytic or membrane sectors of F-ATPase be in excessive amounts. In contrast, the lack of ATPase activity of V_1 of V-ATPase or proton conductivity of its V_o, allows their independent accumulation in the cytoplasm or membranes respectively. Indeed the biogenesis of V-ATPase takes advantage of this feature. An assembled complex of V_1 can be detected in the cytoplasm of eukaryotic cells, and we observe the presence of an excess V_o in almost every membrane containing V-ATPase [37–39].

Consequently it is possible to utilize the separate V_1 and V_o parts for different metabolic and cellular processes which enable the flexible modes of modulation necessary for an enzyme that is involved in a wide variety of interactions with other complexes. Very recently, a V_o participation in membrane fusion processes was demonstrated, as well as the formation of a complex between V_1 and the RAVE complex that was proposed to be involved in regulation of V-ATPase assembly [40,41].

3.2. The unique properties of the membrane sector (V_o) of V-ATPase

As mentioned above, both F and V-ATPases contain membrane sector which functions in proton translocation across their membranes [42,43]. It is likely that a similar mechanism of an ATP-dependent proton translocation operates in both enzymes; nevertheless the subunit composition and structure of their respective membrane sectors is remarkably different [3]. From all the subunits, only the proteolipids are homologous. However the V-ATPase proteolipid is double the size of its counterpart in F-ATPase [44]. Moreover, three different proteolipids comprise the central V_o core, whereas only one gene product comprises the parallel core in F-ATPase [18,45]. In the F-ATPase, subunit c (proteolipid) is an 8-kDa protein containing about 80 amino acids. It is highly hydrophobic and soluble in a chloroform/methanol solution. The *E. coli* proteolipid has two transmembrane helices with a hairpin turn facing the catalytic sector in the cytoplasm [46]. In the middle of the second helix there is a glutamyl or aspartyl residue that provides the binding site for *N*,*N*′-dicyclohexylcarbodiimide (DCCD). DCCD binding blocks proton conductance across the membrane and therefore inactivates the enzyme. In V-ATPases, subunit c (proteolipid) is also a highly hydrophobic protein that binds DCCD, but it contains about 160 amino acids and has a relative molecular mass of 16 kDa [16,44]. DCCD binding inactivates the proton pumping and ATPase activities of the enzyme [43]. The sequences revealed that the proteolipid might have evolved by gene duplication and fusion of an 8-kDa encoding ancestral gene homologous to that present in F-ATPases [1]. The sequence data also revealed four potential transmembrane helices with a single buried carboxyl group in helix IV that is thought to be the DCCD-binding site. The proteolipid is thought to be the principal subunit involved in proton translocation across the membrane. The yeast genome contains three genes encoding proteolipids of V-ATPase: *VMA3* (the principal proteolipid), *VMA11* and VMA16 [18]. The yeast V-ATPase contains several copies of subunit c and one copy of subunits c′ and c″ each essential for the activity of the enzyme [45]. Substitution of the active glutamyl residue (to Ala or Gly) in each of the proteolipids inactivated the V-ATPase but same substitution in subunits c′ and c″ produced a fully assembled enzyme that reached the vacuolar membrane [18,45]. Recently, the genes encoding subunits c′ and c″ were cloned from *Caenorhabditis elegans* [47]. Plant and mammalian expressed sequence tags (est) encoding subunit c″ are present in the GenBank and it is likely that subunits c′ and c″ will also be constituents of other V-ATPases.

Site-directed mutagenesis of the principle yeast V-ATPase proteolipid (Vma3p) revealed unusual sensitivity for changes

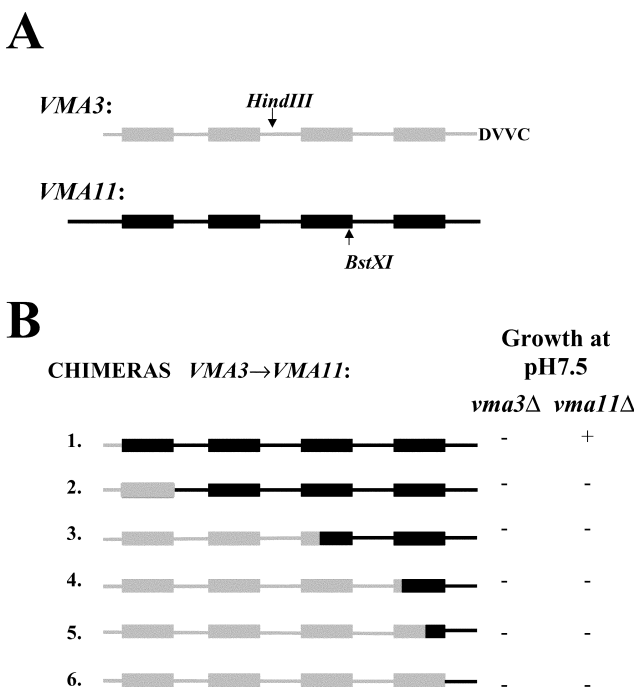

Fig. 2. Schematic presentation of *VMA3* and *VMA11* genes and their chimeras. A: The positions of restriction site of *Hin*dIII in *VMA3* (gray) and *Bst*XI in *VMA11* (black). The last four amino acids of *VMA3* are indicated. B: Six of the chimeras are schematically represented along with the complementation to both *vma3Δ* and *vma11Δ* on YP-galactose pH 7.5 plates.

in the amino acid composition [20]. Very subtle substitutions, especially near the glutamyl residue in transmembrane helix IV which may be involved in proton conductance, inactivated the enzyme [20]. Moreover, very subtle changes in different transmembrane helices could suppress the inactivation and render the enzyme fully active [24]. These observations suggest that the proteolipids are tightly packed in V_o and small alterations in this packing inactivate the enzyme. Recent studies with mutations introduced into the hydrophobic core of the chymotrypsin inhibitor and interactions of α-helices in the model system demonstrated that subtle changes in the size of the amino acid residues in the interface of two helices can cause significant destabilization [48]. Our studies of site-directed mutagenesis and suppressor mutations are in line with these studies as is the suggested model for intermolecular interaction between helices II and IV in an isolated proteolipid fraction from *Nephrops norvegicus* as well [49].

Since the three different proteolipids that comprise the c-ring in V-ATPases are quite homologous, we initiated a study with chimeric proteolipids. An in vivo method was used to generate a series of chimeric proteolipids between *VMA3* and *VMA11* (Fig. 2). Out of 200 plasmids obtained, 33 showed monomer-sized chimeras which were further checked by restriction analysis using a unique *Hin*dIII for *VMA3* and *Bst*XI for *VMA11* (see Section 2). All chimeras were subjected to complementation analysis to both *vma3Δ* and *vma11Δ*. Only one of the chimeras has regained the ability to grow on YP-galactose plates buffered to pH 7.5. Only chimera #1 was able to complement *vma11Δ* but not *vma3Δ*. Sequence analysis of this chimera showed that the 11 *N′* amino acids of *VMA11* were replaced by the 7 *N′* amino acids of *VMA3*, the rest of the sequence being that of *VMA11*. Sequencing of other chimeras revealed that even when all four transmembrane helices of *VMA3* were present and only the C′ was replaced by *VMA11*, the chimera did not complement *vma3Δ*. To investigate which residue is critical for activity we created a mutant of *VMA3* in which the last three amino acids VVC were deleted. This change rendered the proteolipid inactive, meaning it could not complement *vma3Δ*. We next replaced the last Cys to Ala, and this change alone also rendered the proteolipid completely inactive.

To summarize, the results support the notion that *VMA3* is very sensitive even to mild changes, although it does not rule out the possibility of creating chimeras between *VMA3* and *VMA11*, since in all the above chimeras the last Cys was missing. The fact that omitting the last Cys in the c subunit of yeast completely inactivated the V-ATPase suggests a key role of that Cys, either in enabling close interactions between adjacent proteolipids or neighboring phospholipids. This was quite unexpected since there is no proteolipid in any other known organism that has a cysteine before the stop codon [50]. Heterologous expression of *N. norvegicus* and lemon fruit proteolipids partially complemented the *vma3Δ* yeast mutant, so the requirement of the cysteine is an intrinsic property of the yeast protein.

3.3. Factors to be considered for the proton slip in V-ATPases

Why is one proteolipid sufficient for F-ATPases, whereas three of them are required for V-ATPases? The c-ring of F-ATPase is a very tight structure containing variable number of proteolipid molecules. Mitochondrial c-ring contains 10, some bacteria contain 11 and chloroplasts contain 14 molecules per complex [51–53]. These variable numbers already show that theoretical stoichiometry of ATP hydrolysis and proton pumping in various F-ATPases is not uniform and this stoichiometry is not an integer. The likely mechanistic constraint that may have led to the gene duplication and evolution of two other proteolipids in V-ATPase, led to the change in the coupling efficiency of V-ATPases that is referred to as the 'slip' mechanism [19,54]. The physiological expression of a mechanistic proton slip is manifested in limited production of proton motive force (pmf) produced by energy of ATP hydrolysis that cannot reach a thermodynamic equilibrium. A likely explanation of this phenomenon is depicted in Fig. 3, which proposes drastic changes in the stoichiometry of

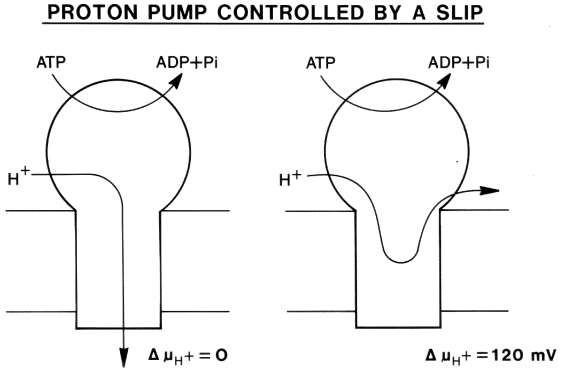

Fig. 3. pmf-induced proton slippage in V-ATPase. At pmf ($\Delta\mu_H+$) of 0 mV, the ATPase activity results in net proton uptake of about two protons per ATP. The buildup of pmf results in an increase proton slippage such that at about 120 mV there is no net proton uptake. The pmf in which this equilibrium is reached is dependent on the quality of the membrane.

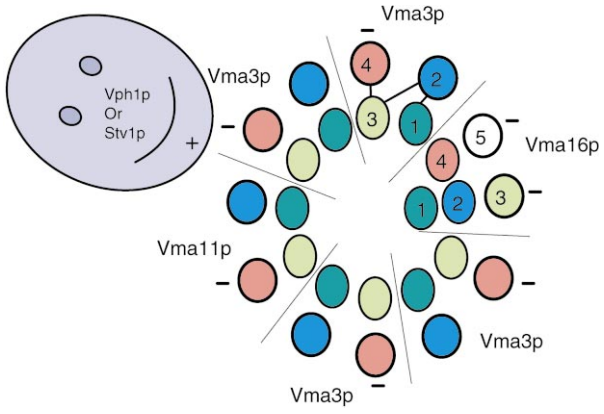

Fig. 4. Possible organization of the proteolipids in the membrane sector of V-ATPase. The various transmembrane segments are numbered and colored accordingly. The negatively charged residues within the transmembrane segments are marked and the possible location of Vph1p or Stv1p is indicated.

ATP hydrolysis to proton pumping, when the pmf reaches a value of about 120 mV. This value is about half way to the maximal pmf value generated at a thermodynamic equilibrium (about 250 mV were recorded in insect midgut and calculated for lemon fruit). Fig. 4 shows a proposed model for the structure of the c-ring in various V-ATPases. It is based on the assumptions that similar mechanisms underlie ATP-dependent proton pumping by F and V-ATPases even though the latter has three different proteolipids in its V_o sector. It is apparent that the c-ring of V-ATPase is an asymmetric structure and the distribution of the negative charges facing the phospholipids is not homogeneous. Moreover, the distance between two negative charges is double the distance in the c-ring of F-ATPase. We propose that this distribution of negative charges is the main culprit in the generation of proton slip during the catalytic activity of the enzyme. The long distance between those negative charges that bind protons in a hydrophobic environment provides a higher probability for the protons to slip back into the cytoplasmic face of the membrane especially when positive potential is building up in the lumen of the organelle. The lipid environment around the c-ring may influence the extent of proton slippage. The analysis of phospholipid composition of lemon fruit and lemon seedling membranes demonstrated that a different phospholipid composition affected the degree of coupling of ATP-dependent proton pumping [55]. However we expect that subtle changes in the amino acid sequences of one or more of the three proteolipids present in V_o will also be a determining factor.

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