

REVIEW ARTICLE

The vacuolar H⁺-ATPase: a universal proton pump of eukaryotes

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The vacuolar H⁺-ATPase (V-ATPase) is a universal component of eukaryotic organisms. It is present in the membranes of many organelles, where its proton-pumping action creates the low intravacuolar pH found, for example, in lysosomes. In addition, there are a number of differentiated cell types that have V-ATPases on their surface that contribute to the physiological functions of these cells. The V-ATPase is a multi-subunit enzyme composed of a membrane sector and a cytosolic catalytic sector. It is related to the familiar F₀F₁ ATP synthase (F-ATPase), having the same basic architectural construction, and many of the subunits from

the two display identity with one another. All the core subunits of the V-ATPase have now been identified and much is known about the assembly, regulation and pharmacology of the enzyme. Recent genetic analysis has shown the V-ATPase to be a vital component of higher eukaryotes. At least one of the subunits, i.e. subunit c (ductin), may have multifunctional roles in membrane transport, providing a possible pathway of communication between cells. The structure of the membrane sector is known in some detail, and it is possible to begin to suggest how proton pumping is coupled to ATP hydrolysis.

1. INTRODUCTION**1.1. Overview**

Virtually all eukaryotic cells contain a network of membrane-bound organelles, such as the vacuoles of plants and fungi, lysosomes, endoplasmic reticulum, Golgi bodies, secretory granules, etc. Although these organelles differ widely in the precise intracellular functions that they perform, they all need to be energized in some way and, as for the cell membrane, this energization occurs through the activity of electrogenic pumps. In the early 1980s one such pump was discovered in various endomembranes: the vacuolar H⁺-ATPase, or V-ATPase [1]. This proton pump has since turned out to be a ubiquitous component of eukaryotic organisms and, although other pumps have been found in endomembranes, it appears that the V-ATPase is the major electrogenic pump of vacuolar membranes. In the last few years V-ATPases have also been found in the cell membranes of various organisms, in particular insects, where they appear to be vital for the specialized physiological roles of these cells. Indeed, the first electron-microscopy images of the V-ATPase have been in existence for 30 years, when stud-like projections were seen in the apical membranes of insect epithelial cells [2].

The V-ATPase belongs to a class of pumps that includes the familiar F₀F₁-ATP synthase (F-ATPase) found in eubacteria, mitochondria and chloroplasts. This class is distinguished from other membrane pumps, such as the P-type and ABC cassette family, by being composed of two multi-subunit sectors: a transmembrane sector and a detachable catalytic sector (Figure 1). They also have different mechanisms of ATP hydrolysis, not having a phospho-enzyme intermediate characteristic of the P-type. The V- and F-ATPases are thought to have a common ancestral origin [3,4] and most, possibly all, of the subunits of the two ATPases have been identified and the genes cloned from a variety of organisms. As might be expected, the subunits of the

V-ATPase have greater sequence similarity to the subunits of the F-ATPases from the archaeobacteria (sometimes called the A-ATPase) than those from the eubacteria [5].

The F-ATPase has received much recent attention, with the solving of the core structure of the catalytic sector, F₁ [6]. By contrast, there is as yet no structural solution of any of the subunits of the V-ATPase, although it is highly likely that it will have the same basic architectural plan. However, the arrangement of the major subunit (subunit c) of the membrane sector, V_o, is known. This is as a result of the unexpected finding that subunit c is the sole protein component of another membrane complex found in metazoans, the connexon channel of gap junction structures [7], which was first imaged 20 years ago [8,9].

1.2. Functional aspects

The V-ATPases pump protons from the cytoplasm to the lumen of the vacuole using the energy released by ATP hydrolysis. As such, the V-ATPase is part of the cellular machinery that regulates the cytosolic pH, and there are a number of reported instances demonstrating the importance of the V-ATPase in pH regulation [10–12]. As the V-ATPase does not use a counter-ion, its pumping activity is electrogenic, creating an electrical potential difference ($\Delta\Psi$) across the membrane. This, in turn, is used to drive the movement of other ions and solutes across the organelle membrane. Such a mechanism is used to accumulate neurotransmitters into synaptic vesicles and amino acids into the vacuole lumen [13]. The acid pH of vacuoles is also used for other functions [14]. For example, it leads to the activation of hydrolytic enzymes in lysosomes. Low pH is also required for the dissociation of receptor–ligand complexes [15].

The low pH also may also enable the opposite of dissociation to occur, and may be required for the association of complexes such as the KDEL receptor with proteins resident in the lumen of the endoplasmic reticulum that contain the KDEL sequence at

Abbreviations used: V-ATPase, vacuolar H⁺-ATPase; F-ATPase, F₀F₁ ATP synthase; V_o, membrane sector of V-ATPase; V₁, catalytic sector of V-ATPase; F_o, membrane sector of F-ATPase; F₁, catalytic sector of F-ATPase; DCCD, *N,N'*-dicyclohexylcarbodi-imide; NEM, *N*-ethylmaleimide; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole chloride; $\Delta\Psi$, electrical potential difference.

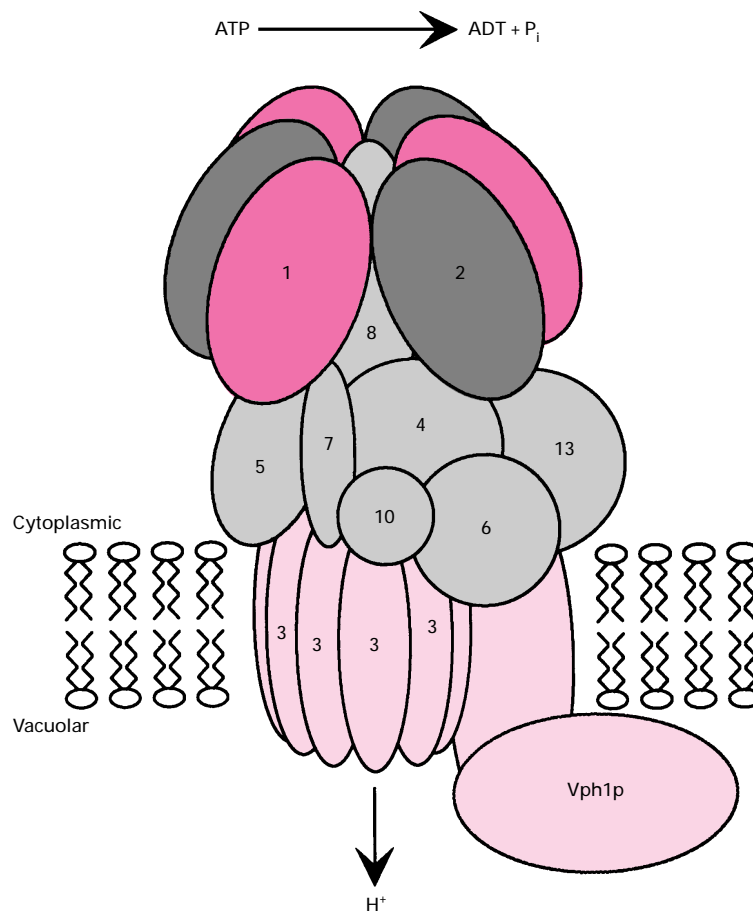


Figure 1 Structural model of the *Saccharomyces* V-ATPase

This naive model represents a synthesis of data relating primarily to the yeast enzyme and also inferred from studies on other systems (see sections 2–4). The functional core of V_1 contains three copies each of polypeptides Vma1p (1) and Vma2p (2) (shown in dark pink and dark grey respectively) and one each of Vma4p (4) and Vma8p (8) (shown in light grey). If Vma8p (perhaps in conjunction with Vma4p) is a homologue of the γ subunit of the F_1 -ATPase, it would by analogy be central to V_1 . Vma7p (7) has been proposed to be functionally analogous to the ϵ subunit of the F_1 -ATPase, and appears also to be part of a 'core' V_1 complex. The Vma5p subunit (5), although essential for coupling of V_1 to V_o , does not appear to be a crucial component in the assembly of V_1 , and the reconstituted enzyme is active even in the absence of this subunit. Conversely, Vma13p (13) is required for activity of the enzyme, but is not essential for assembly of V_1 with V_o , although it does appear to stabilize this association. Both Vma5p and Vma13p may be peripheral to the core V_1 complex. Cross-linking studies on the coated vesicle enzyme indicate that Vma4p (4), Vma5p (5) and Vma8p (8) are in contact with the 16 kDa proteolipid of V_o (3). V_o (subunits shown in light pink) comprises a core formed from a hexameric complex of the 16 kDa proteolipid Vma3p (3) which contains, at least in part, the pathway of proton translocation. Vma6p (6) is a soluble protein, but is tightly bound to V_o , and may also be involved in the control of proton flow. Vma10p (10) is required for the association of V_1 with V_o , but may be more tightly associated with V_o than with V_1 . Vma10p has (speculatively) been proposed to be a homologue of the b subunit of F_o , and may be present in multiple copies. The large soluble domain of Vph1p is thought to be extracytoplasmic, with the transmembrane elements of the protein in contact with the proteolipid (3). Transmembrane helices of both the 16 kDa proteolipid and Vph1p contain charged residues that are essential for proton pumping, and interaction between the two may be a fundamental part of the mechanism of ion translocation.

their C-terminus [16]. In this situation, the affinity of the KDEL receptor is greatly increased by low pH, allowing it to capture endoplasmic-reticulum-resident proteins in the Golgi, in which the V-ATPase is known to be active.

Trafficking within the endosomal/lysosomal compartment is also dependent upon the V-ATPase. Bafilomycin A_1 , a specific inhibitor of the V-ATPase (see section 8.2.), slows down receptor cycling [17], and it has since been found that acidification is essential for the formation of endosome carrier vesicles, which are intermediates between early and late endosomes [18]. Endosome carrier vesicles go on to fuse with late endosomes after first being coated with vesicular coat protein, and the binding of this protein is pH-dependent [19]. The pH-dependence of vesicular coat protein binding is particularly intriguing in that it is to sites on the cytoplasmic face of the endosome carrier vesicles, suggesting the presence of a transmembrane pH sensor.

In plants, the V-ATPase is an active component of the vacuole, which in situations such as citrus fruits can reach pH values as low as 2.2, although other proton pumps may be involved in helping to maintain such a low pH [20]. The plant vacuole is an important component of cellular physiology, acting as a storage compartment and a 'dumping ground' for waste products. In stark contrast with citrus fruits, the V-ATPase is also the electrogenic component used to create the solution of highest pH known in Nature. In the larval midgut of the lepidopteran *Manduca sexta*, the V-ATPase is coupled in some manner to K^+ efflux to create a digestive fluid of pH 12 [21,22]. In this situation, the V-ATPases are located in the goblet-cell membrane, and an increasing number of cells are being found to have V-ATPases at the cell surface. Such cells include the mitochondria-rich cells of transporting epithelia such as the amphibian and reptilian urinary bladder and kidney tubules [23]. In these cells the apically located

V-ATPases perform a vital role in proton secretion and in maintaining the ion balance within the organism. Acid secretion is also required for bone resorption by osteoclasts, and these cells also have high concentrations of cell surface V-ATPases [24].

2. GENERAL STRUCTURE AND COMPOSITION

Electron-microscopic studies have been carried out on vacuoles from *Neurospora crassa* [25,26] and on membrane fragments of mitochondria-rich cells from the amphibian urinary bladder [27], which are highly enriched in V-ATPases to the extent that the ATPases are packed in a honeycomb-like lattice. They both give a similar picture of the V-ATPase, showing it to have a cytoplasmic 'stud' of a stalk and ball complex attached to the membrane by the stalk (Figure 1). By analogy with the F-ATPase, the cytoplasmic stalk and ball complex is termed V₁, while the membrane sector is designated V_o. The V₁ sector, like its F-ATPase counterpart F₁, can be detached from the membrane by treatment of membranes with chaotropic agents [28–33]. Treatment with alkaline carbonate [29,31,34], nitrate and MgATP [31,35] or cold shock in the presence of MgATP [36,37] also results in the release of V₁ subunits. Such dissociation studies have made it possible to assign subunits to either V₁ or V_o.

The V-ATPase can also be solubilized in an active form as a complete holoenzyme by non-ionic detergents. This has allowed measurements of mass by sedimentation, and masses between 560 kDa and 740 kDa have been reported for V-ATPases isolated from fungal, plant and animal sources [38–40]. These values are reasonably close to the predicted size determined from subunit mass and stoichiometry (see below). A similar value of 520 kDa for the yeast enzyme has also been suggested by radiation-inactivation analysis [41].

There was some initial confusion with regard to the number of V-ATPase subunits, with an apparently greater subunit complexity in some mammalian enzymes. For example, the coated-vesicle and kidney microsomal enzymes appeared to contain as many as eight and nine polypeptide species respectively [28,38,42,43], while the chromaffin-granule, plant and yeast enzymes were suggested to contain as few as four [44] or three

[45,46] polypeptides. Subsequent studies, in the light of improved isolation procedures, has led to a narrowing of the differences in composition (see Table 1 and references therein). Enzymes isolated from coated-vesicle membranes [28,32,43,47], chromaffin granules [34,36,48], bovine kidney [38,49], and various plant [29,37] and fungal sources [26,31] all now show a comparable complement of subunits, with ten now appearing to be the consensus number. Confirmation of the subunit composition has come from genetic analysis in a variety of different organisms, as described below (section 3.).

By convention, the subunits in V₁ are designated A–G in decreasing order of mass (see Table 1) and include subunits A (73–67 kDa, depending on species) and B (60–55 kDa), known to be nucleotide-binding polypeptides. Three different subunits comprise the V_o sector, two which are transmembrane (subunit c and a large glycoprotein) and one which is hydrophilic but tightly bound to the integral membrane components (see section 4.).

Stoichiometry measurements of the subunits in the coated-vesicle enzyme by quantitative amino acid analysis indicates a composition of (116 kDa)₁:(73 kDa)₃:(58 kDa)₃:(40 kDa)₁:(38 kDa)₁:(34 kDa)₁:(33 kDa)₁:(19 kDa)₁:(16 kDa)₆ [40], giving a mass of 760 kDa, which is consistent with the mass determined by sedimentation (see above). There is now another subunit to be added to this, subunit G (12 kDa), of which there is thought to be three copies per enzyme [50]. On the basis of staining of polypeptide bands on SDS/PAGE, it seems that the subunit stoichiometry of V-ATPases from other systems will be the same as that of the coated-vesicle membrane V-ATPase. The stoichiometry is similar to that of the F-ATPase, indicating a pseudo-6-fold symmetry of V₁ with subunit A and B possibly forming a dimer. In addition, electron microscopic studies of subunit c isolated in a gap-junction structure (see section 5.1.) show six copies per complex, arranged symmetrically [51]. The stoichiometry of the major subunits is the same as for the F-ATPase [52]. The approximate arrangement of all the known V-ATPase subunits for the yeast enzyme is shown in Figure 1, but, before describing each subunit in turn, we will describe how the genetic analysis of three different organisms has revealed some important information on the composition and importance of the V-ATPase.

Table 1 Composition of V-ATPases from different sources

Source	Subunit ... Yeast gene ...	Molecular mass (kDa)											
		VPH1	A VMA1	B VMA2	VMA13	C VMA5	VMA6	D VMA8	E VMA4	a	c VMA3	F VMA7	G VMA10
Yeast vacuole [31,60]		100	69	60	54 ^a	42	36 ^b	32	27		16	14 ^c	13 ^d
Coated vesicle [28,43,47]		116	73	58	50	40	38	34	33	19 ^e	17		
Red beet tonoplast [35,37]		100	67	55	52	44	42	32	29		16		
Chromaffin granule [34,48,152]		115	72	57		41	39	34	33		16		
Kidney microsomal [38,49]		116	70	56 ^f		45	38	33	31		14	14	12
F-ATPase homologue ^g	a	β				ε		γ	γ		c		b

^a The presence of Vma13p homologues is unconfirmed.

^b The putative Vma6p of yeast, coated-vesicle and plant proteins have similar properties, but identification of a Vma6p homologue is confirmed only in the bovine chromaffin-granule enzyme.

^c The Vma7p homologue in *Drosophila* is encoded by the *vha24* gene.

^d The *Manduca* homologue of Vma10p has been designated subunit G.

^e It is still unclear whether there is an equivalent of subunit a in other V-ATPases.

^f Several species were present of approximate mass 56 kDa.

^g Subunits a, c and b are assigned to F_o.

3. GENETIC ANALYSIS OF V-ATPase SUBUNITS

Quite surprisingly, a eubacterial V-ATPase has been identified in *Enterococcus hirae* [53]; equally surprising is the discovery that the coupling ion is Na^+ and not H^+ . The genes are located on a single operon cluster (*Ntp*) which contains 11 open reading frames, the conceptual sequences of which have similarity in size and sequence to V-ATPase subunits found in eukaryotic organisms. One of the open reading frames is thought to encode a potassium transporter, and so it seems reasonable to suppose that the other 10 subunits form the minimal active core of the V-ATPase.

The yeast *Saccharomyces cerevisiae* has proved particularly useful for all aspects of the analysis of V-ATPase structure and function. The V-ATPase of this organism can be isolated in amounts suitable for biochemical analysis, and gene inactivation can be readily achieved by homologous recombination. Inactivation of any of the genes encoding V-ATPase subunits so far identified in *S. cerevisiae*, with the exception of *Vph1p*, leads to loss of V-ATPase function. The resulting phenotype is not lethal, but there is a failure to grow in medium buffered to pH 7.5, sensitivity to high extracellular calcium concentrations and a dysfunctional vacuole [54–61]. Other genes giving rise to the same phenotype [62] are present that encode polypeptides not yet identified as components of the V-ATPase, but which could possibly be involved in assembly, targeting or regulation of the enzyme. In addition, genes have been found that encode polypeptides with sequence similarity to identified V-ATPase subunits but whose function is not yet clear (see section 5.2.).

Many of the genes encoding V-ATPase subunits have now been identified in *Drosophila melanogaster*. Gene inactivation in *Drosophila* can be achieved by insertional mutagenesis through the transposable P-element, and a library of flies has now been created in which there are P-element insertions on chromosome 2. The library has been selected on the basis that P-element insertion causes lethality in homozygous flies. To date, five such flies have been identified with insertions in loci encoding V-ATPase subunits, including four from V_1 (subunits A, B, E and F) and one from V_0 (subunit c) [63,64]. The lethality occurs in embryonic or larval stages, emphasizing the importance of the V-ATPase in higher eukaryotes.

4. SOLUBLE V_1 SUBUNITS

4.1. Subunit A

The A subunits from a variety of V-ATPase sources have been cloned and sequenced, and range in mass from 73 kDa in the coated-vesicle enzyme [65] to 69 kDa in yeast [66] and 67 kDa in plants [67] and *Neurospora* [68]. Variability in mass arises primarily from differences at the N-terminus, and there is a higher degree of sequence conservation between species than is generally observed for other V_1 subunits (see below); for example the yeast [66] and *Neurospora* [68] proteins show 62% identity with the sequence of the carrot enzyme.

Subunit A is encoded by the *VMA1* gene in yeast [66], a gene also identified as *TFP1*, conferring trifluoperazine resistance [69], and *cls8*, a calcium-sensitivity mutation [62]. The coding sequence of the yeast gene contains a 454-residue intein which appears to be removed only at the post-translational level [66]. The physiological function of this phenomenon remains unclear, but it seems to occur independently of any other cellular machinery, since the mature gene product is generated both *in vitro* and in heterologous expression systems [70].

Both *N*-ethylmaleimide (NEM) and the nucleotide analogue 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole chloride (NBD-Cl) are

specific inhibitors of V-ATPase activity, and both bind to subunit A in a manner that can be blocked by ATP in the presence of bivalent cations [48,71–73] (see section 8.1.). Similarly, azido-ATP is able to bind to subunit A in an ATP-protectable fashion [74], consistent with the conclusion that subunit A contains the site of ATP hydrolysis in the enzyme [75].

The A subunits are characterized by containing a consensus nucleotide-binding 'P-loop' sequence, GXXXXGKT [76]. This sequence also contains a cysteine residue immediately before the GKT motif, which is not an essential component of the active site [77] but which is, however, responsible for conferring sensitivity to NEM [77,78]. Two additional conserved cysteine residues have been shown to be essential for activity [75], and the formation of reversible disulphide cross-links between these residues and the cysteine of the P-loop has been proposed as a possible mechanism of regulation of activity *in vivo* [79,80].

The A subunit shows 25% identity with the β subunit of F-ATPases, concentrated in particular in regions implicated in nucleotide binding, and it seems likely, therefore, that the two subunits may have arisen from an earlier ancestral gene [52,68]. On the basis of this relationship, and bearing in mind the extent to which structure/function relationships in the homologous β subunit have been characterized [6], it seems likely that, in the near future, it will be possible to identify residues of subunit A involved in both ATP binding and hydrolysis.

4.2. Subunit B

Originally identified in various V-ATPase isolates as a polypeptide species of mass ranging from 60 to 55 kDa, subunit B was first cloned from *Neurospora* [81] and *Arabidopsis* [82]. Both sequences, and that of the yeast protein encoded by the *VMA2* gene [83] (also known as *VAT2* [84]), show a high level of identity with each other (> 60%) and contain a consensus sequence for nucleotide binding [35,81,83,85]. Indeed, biochemical studies demonstrated that the B subunit can be photoaffinity labelled by ATP derivatives [74,85,86], identifying it as a nucleotide-binding subunit. Mammalian subunit B is unique among V-ATPase subunits in that it appears to be encoded by a multigene family [87,88]. Differential expression of subunit B subtypes would give rise to tissue-specific isoforms of the enzyme, presumably with differential catalytic or regulatory properties.

The B subunit also shows some 25% sequence identity with both subunit A and the α subunit of the F_1 -ATPase [81–83], further implying evolution from a common ancestral gene. The fundamental similarity in structure and function between F- and V-ATPases makes it reasonable to suppose that subunit B performs the same regulatory function proposed for the α subunit in F-ATPases.

Taking advantage of the published three-dimensional structure of bovine F_1 [6] and the sequence similarity between the α and B subunits, a recent study [89] has identified residues of subunit B likely to be involved in nucleotide binding at both non-catalytic (regulatory) and catalytic sites. On coupling this approach with mutagenesis studies in yeast, two residues were identified that contribute to the V_1 catalytic site and are essential for V-ATPase function. Examination of the P-loop region and residues of the non-catalytic adenine-binding pocket indicated a mutational tolerance which suggests that the precise nature of the side chains in these structures is not critical, but rather that the primary structure is optimized for regulated ATP hydrolysis [89].

Subunit B may have a function outwith the V-ATPase. The *cha55* gene in *Drosophila* encodes subunit B, and P-element inactivation of this gene is embryonic lethal [63]. An unexpected finding was that the gene localizes to a previously identified locus

that suppresses *polycomb*. Flies heterozygous for the P-element insertion in the *vha55* gene exhibit suppression of polycomb, suggesting that subunit B acts as a transcriptional silencer. It is not yet known whether other nearby transcriptional units are affected.

4.3. Subunit C

Subunit C was originally cloned from bovine, and subsequently human, brain libraries [90]. Both cDNAs encode hydrophilic proteins of 44 kDa, differing only at one residue (Tyr-336 → His), which show no identity with any database sequences. The *VMA5* gene, encoding the corresponding 42.3 kDa subunit C from *Saccharomyces*, has 37% identity with the bovine sequence [56,91]. Despite this difference, a chimaeric subunit C containing approx. 60% bovine sequence is able to complement *vma5* disruption in yeast, indicating functional and therefore structural identity between the two species of subunit [91]. Studies on disruption mutants confirmed that the *VMA5* gene product is essential for the assembly and function of the V₁ sector of the ATPase [56,91], but that assembly of the V_o sector remains unaffected [56]. Conversely, the 40 kDa polypeptide in the coated-vesicle enzyme that is likely to be equivalent to Vma5p has been reported to enhance, but not be essential for, activity in a reconstituted system [47], suggesting that this subunit may be somewhat peripheral to V₁.

4.4. Subunit D

This subunit, originally designated as the '32 kDa' subunit, was initially cloned from bovine brain after partial sequencing of peptide fragments [92]. The cDNA encoded a 247-residue hydrophilic protein of deduced mass 28.3 kDa. The gene for the corresponding *Saccharomyces* subunit, designated *VMA8*, was subsequently isolated and sequenced [60,92], and the gene product was shown to be essential for V₁ sector assembly and function.

The yeast gene product is a 256-residue protein of 29.2 kDa showing 51% identity with the bovine protein. A related protein, encoded by the *NtpD* gene, is a subunit of the Na⁺-translocating V-ATPase of *Enterococcus hirae* [53] and retains 27% identity with the yeast protein. Interestingly, subunit D shows regions of similarity with the γ subunit of *Sulpholobus acidocaldarius*, the crucial 'spindle' subunit of F-ATPases. Although the overall extent of conservation appears small (18%), distinct regions of greater conservation are evident towards both the N- and C-termini, leading to the suggestion that *VMA8*/subunit D and γ subunits are structural and functional homologues [92,93]. It is these two regions of the γ subunit of the bovine F-ATPase that form the coiled-coil of two α -helices that contact with the inner surfaces of the α and β subunits.

4.5. Subunit E

Expression cloning of the '31 kDa' subunit E from bovine kidney revealed a 226-amino-acid hydrophilic protein, enriched in charged residues, with a deduced mass of 26.2 kDa and showing no overall similarity with any protein in the sequence database [94]. The *VMA4* gene for the corresponding yeast subunit was found to encode a 26.6 kDa protein, with 34% similarity to the bovine sequence but also containing a characteristically high proportion of charged residues [56,95]. A *vma4* disruption mutant was incompetent for vacuolar acidification [95] and could not assemble V₁, although expression of V₁ subunits and assembly of V_o were unaffected [56]. Cloning of the E subunit of the *Neurospora* [96] and *Drosophila* [97] enzymes revealed a 26 kDa

protein with only a low degree of identity with the bovine protein. However, as in the case of subunit C/Vma5p, lack of similarity at the level of the primary structure does not preclude the possibility that the tertiary structures of the proteins may be very similar.

Subunit E is clearly essential to the assembly and function of the V-ATPase, but its precise function remains uncertain. The protein contains a lysine-rich motif similar to that found in a variety of ion-pumping ATPases which has been postulated to be involved in cytoplasmic cation binding, tentatively suggesting a role in the regulation of cation accessibility to the enzyme. An alternative suggestion, based on structural prediction, is that subunit E (and not subunit D) is a homologue of the γ subunit of the F-ATPase [96]. However, subunit E and the γ subunit do not exhibit even the very limited sequence identity observed between the γ subunit and subunit D. It has been suggested that subunits D and E may functionally associate together to provide a γ subunit homologue in the yeast V-ATPase [93].

4.6. Subunit F

Immunoscreening of a *Manduca sexta* expression library with anti-(V-ATPase holoenzyme) antiserum led to the isolation of a cDNA encoding a 13.8 kDa hydrophilic protein which corresponded to a species present in V-ATPase isolates from goblet-cell apical membranes [98]. cDNAs for related proteins were subsequently cloned from yeast [58,59] and *Drosophila* [99], in which they are encoded by the *VMA7* and *vha14* genes respectively. In contrast with the dissimilarity that characterizes species differences for other single-copy V-ATPase subunits, subunits F from *Manduca* and yeast show as much as 50% identity. Stripping with either chaotropic agents [59,98] or cold shock in the presence of ATP [58] indicates that subunit F is an integral component of the V₁ sector. Disruption of the yeast *VMA7* gene, as for other *vma* mutations, resulted in an inability to assemble the V₁ complex [58,59]. However, unlike other *vma* mutations, *VMA7* disruption has also been reported to disrupt assembly of V_o [59], leading to the suggestion that this subunit may be involved in stabilizing contacts between V₁ and V_o [59]. Intriguingly, pre-incubation of membranes with either monospecific [98] or anti-epitope [58] antibodies resulted in inhibition of ATPase activity and proton pumping. In the case of the *Manduca* enzyme, this phenomenon also appeared to be dependent on the presence of ATP, suggesting a conformational sensitivity. Subunit F has been suggested to have a regulatory role analogous to that of the ϵ subunit of F-ATPases [58].

4.7. Subunit G

Partial protein sequencing of a V-ATPase subunit with an apparent mass of 16 kDa facilitated cloning of the yeast *VMA10* gene [50]. The Vma10p subunit is a 12.7 kDa protein with as many as 40% charged residues and a relatively basic isoelectric point (pH 9) [50]. A related protein, which is a component of the V₁ sector of the *Manduca* and bovine enzymes, has also been cloned [100,101]. This 13.7 kDa subunit also migrates aberrantly on SDS/PAGE with a mass of 16 kDa, probably as a result of its high content of charged residues.

Interruption of the yeast gene gives rise to cells with all the characteristics observed when V-ATPase function is disrupted. Similarly, V₁ subunits cannot be assembled on to the vacuolar membrane. Vma10p does not appear to be stripped from the vacuolar membrane by cold shock, suggesting that it may be associated with the V_o complex [50]. A somewhat contradictory situation was observed in *Manduca*, in which treatment with chaotropes or cold shock resulted in stripping of a V₁ complex

from the membrane including the 'subunit G' [100]. In addition, subunit G is found in V_1 *in vivo* at times when V_1 is dissociated from V_0 (see section 7). Both the yeast and *Manduca* sequences show limited similarity to that of subunit b of the F_0 sector. However, the hydrophobic N-terminal region of F_0 subunit b is absent, and this may account for the stronger association with V_1 than V_0 . In addition, stoichiometric studies of the holoenzyme or of the released V_1 from *Manduca* indicate the presence of three copies instead of the two copies of subunit b found in the F-ATPase.

4.8. *VMA13*

The *VMA13* gene of yeast was isolated (as was the *VMA5* gene for subunit C [56]) by complementation of a pH-sensitive *vma* mutation [57]. The *vma13* mutation, which is the same as the *cls11* calcium-sensitive mutation [62], is characteristic of other *vma* mutations in that mutant cells are defective in vacuolar acidification and are unable to grow at neutral pH [57]. *VMA13* codes for a 54.4 kDa hydrophilic protein that has no significant similarity to any other protein in the sequence database. The gene product was subsequently shown to be essential for activity of the enzyme [57]. However, unlike other *vma* mutations, disruption of the *VMA13* gene does not appear to inhibit assembly of the V-ATPase, but rather destabilizes it, suggesting that this protein may have a somewhat more peripheral position in V_1 [57]. Polypeptides of similar mass are present in both plant and bovine coated-vesicle V-ATPases [37,47], but it remains to be determined whether or not Vma13p is a ubiquitous V-ATPase subunit. An equivalent subunit is not found encoded in the *Ntp* gene cluster of *E. hirae*.

5. MEMBRANE-ASSOCIATED V-ATPase SUBUNITS

5.1. Subunit c

The 16–17 kDa subunit c of the V_0 sector has been widely studied, and the genes from diverse fungal [102,103], plant [104–109], arthropod [7,110–112], mammalian [113–115] and other vertebrate [116] sources have been cloned. The 16 kDa proteolipid is one of the most conserved membrane proteins known, with greater than 65% identity between virtually all species (M. E. Finbow and M. A. Harrison, unpublished work). In addition, eukaryotic subunits c show 25–30% identity with the *NtpK* gene product of *Enterococcus* [53]. Subunit c is encoded by the *VMA3* gene in *S. cerevisiae* and, as might be expected, interruption of the yeast *VMA3* gene encoding the proteolipid results in the loss of V-ATPase activity and the associated dysfunction [55,102,117,118], plus destabilization of V_0 and failure to assemble V_1 [61,119].

Of all the V-ATPase subunits, subunit c is perhaps the most interesting. It forms the principal pathway for proton translocation [120–122] (see section 9.) and, in addition, it has been implicated in cell–cell communication as a component of gap junctions as well as in neurotransmitter release as a component of the mediatothore. It is also a target for a class of unusual viral oncoproteins [123].

The protein is highly hydrophobic, soluble in organic solvents and contains four extended hydrophobic segments that account for two-thirds of the residues [7,102,113,124]. The hydrophobic regions are interspersed by short extramembranous hydrophilic loops, and the loops between helices 1/2 and 3/4 are cytoplasmically exposed in the V-ATPase. Both the N- and C-terminal halves of the 16 kDa proteolipid show significant sequence identity with the 8 kDa proteolipid subunit c of the F_0

sector of F-ATPases [113]. It has been postulated that the two species of proteolipid arose from a common ancestral gene that underwent a duplication event to give rise to the 16 kDa proteolipid [83,113] (Figure 2). Consistent with a gene duplication event is the finding of an intron located halfway through the open reading frame in the *Drosophila* gene (*vha16*) and the human gene [111,114,125]. However, there is no such intron in a putative subunit c gene in *Caenorhabditis elegans*.

NMR studies on the F_0 subunit c from *Escherichia coli* suggest that it forms a hairpin structure of two transmembrane α -helices, with the two helices being separated by a short hydrophilic domain that has contact with F_1 subunits [126,127]. Fourier-transform IR spectroscopic analysis of membrane preparations highly enriched in the V_0 subunit c shows a 60% α -helical content, which is equivalent to four transmembrane α -helices each of 25 or so residues in length [51]. The source of the highly enriched membranes used for the Fourier-transform IR analysis is particularly interesting. These membranes were prepared as gap-junction sheets from the hepatopancreas of the decapod *Nephrops norvegicus*. Gap junctions are sites of cell–cell communication allowing for the movement of low-molecular-mass solutes (< 1000 Da) between cells. The basic channel component of gap junctions is thought by most to be the connexins, but these gap-junction membranes are entirely free of connexins and instead contain the subunit c proteolipid, also known as ductin, as the major protein component. The role of ductin in gap junctions has long been a point of some contention, and is discussed elsewhere at length [123,128,129]. Whatever, the high yield and regular structure of these gap-junctional membranes has proved particularly useful in giving the basic architecture of V_0 . Furthermore, ductin of *Nephrops* gap junctions is able to fully substitute for subunit c in the V-ATPase, showing a degree of functional congruence [130].

Image reconstruction from negatively stained gap junctions has shown the core of the V_0 to be composed of a symmetrical hexameric arrangement of subunit c protomers [51,131]. The length [~ 65 – 70 Å (~ 6.5 – 7.0 nm)], diameter (~ 60 – 65 Å) and shape of the core fit particularly well with each subunit c being organized as a four- α -helical bundle. Of note is the presence of a central pore seen in the images of negatively stained membranes or by atomic force microscopy. This pore is of the order of 15–20 Å in diameter and is far larger than would be expected for proton translocation, but is of a diameter that might be expected for a channel with the permeability properties of a gap-junction channel. It would seem likely that, in the V-ATPase, this pore is occluded, perhaps by the central 'spindle' subunit D.

As mentioned above, *Nephrops* ductin is able to substitute for the *S. cerevisiae* subunit c in the V-ATPase and, because the cysteine-free form is fully active, it has been possible to carry out a detailed cysteine replacement study [132]. This has shown that the central pore is lined by a face of the first transmembrane putative α -helix which is principally composed of neutral or polar amino acids (Figure 3). Cysteine substitutions on this face have no effect on V-ATPase activity, indicating that the pathway for proton translocation is located elsewhere. However, they can be labelled by fluorescein maleimide, suggesting a permeability to larger molecules, as might be expected for a channel with gap-junction-like properties.

By analogy with the F_0 subunit c, it is commonly thought that a highly conserved glutamic acid residue in the fourth helix forms a cation-binding site for translocation of protons [133] (see section 9.1.). This acidic residue is the sole site for reaction with the lipophilic inhibitor *N,N'*-dicyclohexylcarbodi-imide (DCCD) [7,75,104,134], and has been shown both biochemically and mutagenically to be essential for proton translocation [117,135].

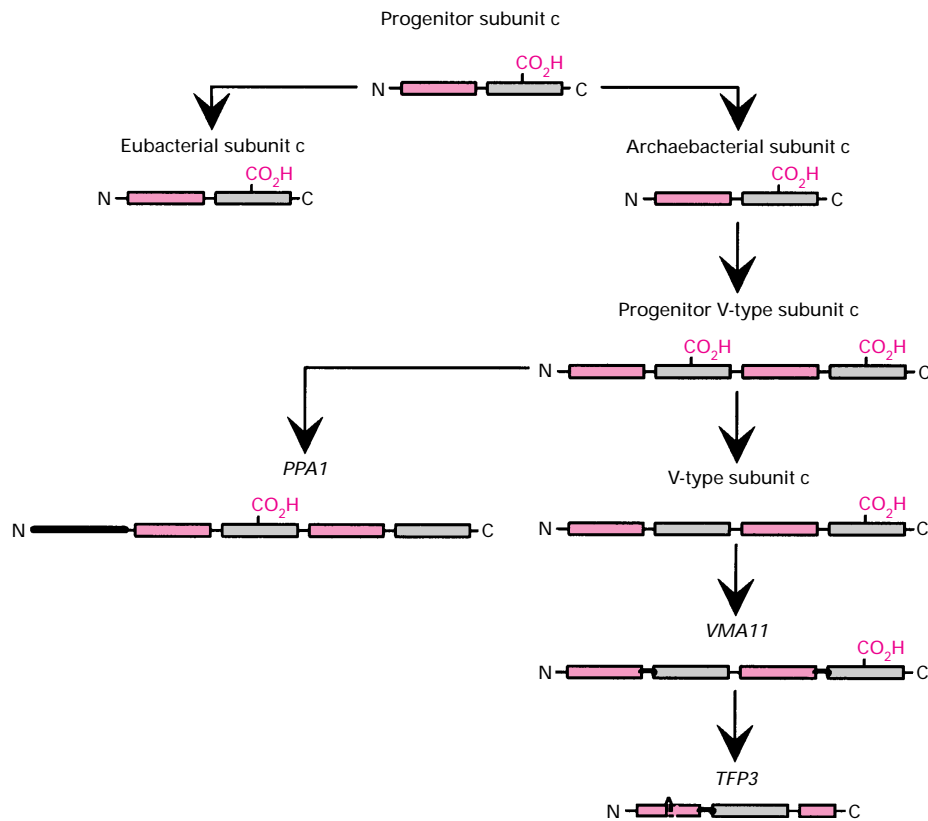


Figure 2 Possible evolutionary pathway of the subunit c family

At the top is shown the progenitor subunit c with two transmembrane domains (pink and grey rectangles), with the second domain containing the critical acidic residue (CO₂H) for cation (H⁺ or Na⁺) translocation. This progenitor gave rise to the eubacterial and archaeobacterial forms of subunit c found in the F-ATPase and A-ATPase respectively. A gene duplication in early eukaryotic cells gave rise to the progenitor V-ATPase subunit c that was a tandem repeat of two archaeobacterial subunit c polypeptides, which consequentially contained two acidic residues in the second and fourth transmembrane domains. One was lost from the second domain, resulting in the modern V-ATPase subunit c present in the V-ATPase (subunit c) and gap junctions (ductin) (see section 5.1.). An earlier duplication event gave rise to the *PPA1* subfamily, in which the critical acidic residue was lost from the fourth domain instead (section 5.2.). The N-terminus of the *PPA1* family is also extended by 30–40 residues that contain a hydrophobic stretch of sufficient length to span the membrane bilayer. The function of the *PPA1* family is unknown, but ongoing sequencing programmes indicate a universal spread throughout eukaryotes. A duplication event gave rise to the *VMA11* gene in *S. cerevisiae*, which encodes a polypeptide that differs primarily at the extramembranous residues thought in subunit c to interact with V₁ subunits. A duplication event of the *VMA11* gene gave rise to the *TFP3* gene, which encodes a truncated Vma11p protein that shows an additional 7-residue deletion in the first domain.

The equivalent acidic residue of the F₀ subunit c is also DCCD-reactive, and this reagent blocks proton translocation irreversibly. The reaction with DCCD suggests that the glutamic acid residue may be partly exposed to a lipid environment, i.e. that it is located on the outer faces of the hexamer complex of subunits c (see section 8.2. and Figure 3).

Because of the seemingly multifunctional nature of subunit c, it might be expected that there are multiple genes encoding different isoforms. However, *Drosophila* contains a single gene locus encoding a single transcript [111] and, although four genes have been identified in humans, three are thought to be pseudogenes [125]. By contrast, higher plants, which do not have gap junctions but have alternative means of cell–cell communication through plasmodesmata, have multiple genes encoding near-identical polypeptides [105,106,108,109]. The significance of this multiplicity in plants is unclear.

As well as binding components of the V-ATPase, subunit c has also been found complexed with the E5 oncoproteins of papillomaviruses (see section 8.2) and with synaptophysin/synaptobrevin [136]. The oncogenic activity of the viral protein is dependent in part upon binding to subunit c, but the significance

and nature of the binding between subunit c and synaptophysin/synaptobrevin complexes is not understood. Subunit c is a component of the mediophore, which has been suggested to be a site of neurotransmitter release [116] (see [123] for references). Indeed, reconstituted subunit c in liposomes is able to form an oligomeric complex that is permeable to acetylcholine and glutamate [116,137]. The question of mediophore and neurotransmitter release has proved as controversial as ductin and gap junctions, although a recent study has shown that quantal release of acetylcholine can be achieved when subunit c is present in the cell membrane [138]. It has been suggested that subunit c could be part of the postulated ‘fusion pore’ complex, which has the potential to provide an efficient pathway for neurotransmitter release from synaptic vesicles [123,136].

Despite its small size and simple construction, subunit c is proving to be a complex and noteworthy protein, and adding to its controversial roles in non-V-ATPase complexes is the finding that it apparently has two orientations in membranes [139,140] (see section 6.). In addition, a number of subunit c-related proteins have now been discovered whose functions are yet to be elucidated.

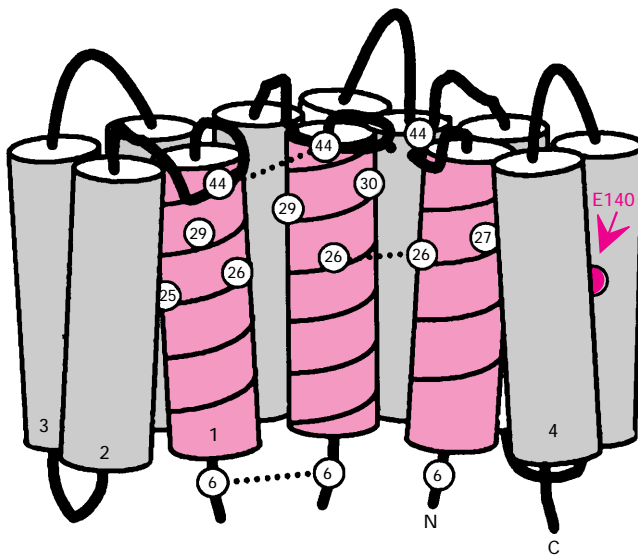


Figure 3 Tentative arrangement of the transmembrane α -helices of subunit c protomers in the V_0 sector

A half-hexamer of subunit c protomers is shown (adapted from [132]). The first putative transmembrane helix is shown in pink and the other three helices in grey. The residues shown in helix 1 and extramembranous regions are ones that have been replaced with cysteine in the subunit c from *Nephrops norvegicus*. Broken lines indicate those residues that are capable of forming inter-subunit c disulphide bonds, hence demonstrating close proximity to one another in the assembled V_0 . The face shown on helix 1 appears to line a central channel which may correspond to a gap-junction-like channel. This channel is presumably occluded in the V-ATPase by the putative spindle subunit, i.e. subunit D (8 in Figure 1). The critical glutamic residue E140 on helix 4 is shown in the crevice formed between helices 3 and 4 and facing the peripheral regions of the hexamer complex possibly exposed to the alkane tails of the lipid.

5.2. Subunit c-related polypeptides

Of all the V-ATPase components, only subunit c has so far been found to have related genes, the products of which are unlikely to be part of the V-ATPase or other subunit c-based complexes. These genes have been found primarily in yeast, but ongoing gene sequencing programmes have shown that one may be universal in eukaryotes [141] (see Figure 2 for evolutionary relationships).

The *VMA11* gene of *S. cerevisiae* [142] encodes a 16 kDa proteolipid with 54% identity with Vma3p (subunit c of *S. cerevisiae*), and although a *VMA11* gene product has not yet been identified, disruption of the gene causes the characteristic phenotype associated with the loss of V-ATPase activity (Figure 2). Despite the high degree of sequence similarity, Vma3p and Vma11p are not able to complement each other, suggesting that they serve different but essential roles in V-ATPase activity [142]. Sequence differences between the two proteolipids are concentrated in the extramembranous loops between the first and second and the third and fourth transmembrane helices, both of which are predicted to interface between V_1 and V_0 . These regions are highly conserved in other species of subunit c and have sequence identity with the equivalent loop region in F_0 subunit c, which is known to be important in contacting F_1 to F_0 [143]. Vma11p is able to complex with subunit c in membranes derived from the endoplasmic reticulum, suggesting it has the potential to form part of V_0 [140]. Genes analogous to *VMA11* have not been found in higher eukaryotes, and the precise function of *VMA11* remains uncertain.

A truncated form of *VMA11* has also been identified in *S.*

cerevisiae. This gene, designated *TFP3*, confers resistance to the drug trifluoperazine, and much of the third and all of the fourth transmembrane region is absent [144], including the acidic residue crucial for proton translocation. In addition, there is a 7-amino-acid deletion (i.e. equivalent to just less than two turns of an α -helix) in the first transmembrane domain. The polypeptide encoded by *TFP3* has not been identified, and little is known about its function.

A third gene, *PPA1*, encodes a proteolipid that is less closely related to the others and which contains a hydrophobic N-terminal extension. It is required for cell viability [141], suggesting an important role; consistent with this, recent genome sequencing programmes have identified related gene sequences in *Arabidopsis*, *Caenorhabditis elegans*, mouse and human. The conceptual translation products of the *C. elegans* and *S. cerevisiae* genes have 50% identity and, interestingly, the critical acidic residue is located in an equivalent position on the second transmembrane domain. It would therefore seem that a second duplication event occurred in early eukaryotes to give rise to two gene families: the *VMA3*-type family and the *PPA1* family (Figure 2). The function of the *PPA1* family remains to be established. However, it is unable to complex with subunit c, suggesting that it is not part of the V-ATPase [140].

5.3. 100–116 kDa subunit

Although originally suggested to be present only in certain V-ATPase types, the 116 kDa protein is now accepted as a ubiquitous subunit and has been cloned from rat brain [145], human [146], yeast [147] and *Dictyostelium* [148]. An equivalent, if somewhat smaller, polypeptide is encoded by the *I*-gene in the *Ntp* gene cluster of *Enterococcus* [53]. A corresponding yeast 96 kDa protein was cloned by complementation of the *vph1* mutation [54] and found to have 42% identity with the rat protein [147], and all 116 kDa-related proteins sequenced to date appear to share a bipartite structure, with extensive N-terminal hydrophilic domain and a C-terminal domain containing up to seven putative membrane-spanning hydrophobic segments. The membrane topology of the protein remains unclear, but its sensitivity to proteolysis has led to the suggestion that the N-terminal domain is cytoplasmically exposed. The coated-vesicle and chromaffin granule proteins are also reported to be glycosylated [32,34], and there is a single N-glycosylation consensus sequence in the loop between putative transmembrane segments 2 and 3 [145]. The presence of such a site would indicate that the N-terminal hydrophilic domain has an intravesicular location [32,40].

Disruption of the *VPH1* gene in *S. cerevisiae* gives partial loss of vacuolar acidification [147]. The incomplete *vma* phenotype is due to the presence of a second gene, *STV1* gene (Similar To *VPH1*), which encodes for a related 102 kDa protein that is 55% identical with *VPH1* [150]. Measurement of mRNA abundance suggests a much lower level of *stv1p* than *vph1p* and inactivation of both genes results in the complete *vma* phenotype. There is also loss of the V_0 complex which is necessary for V_1 attachment to the vacuolar membrane [61,149]. The *stv1p* subunit contains two putative Golgi retention signals suggesting a different cellular localization from *vph1p* [150]. However, overexpression of *STV1* gene results in the polypeptide being found in the vacuole and in the isolated V-ATPase. Overexpression also complements the partial *vma* phenotype of the *vph1* mutant. The existence of 100 kDa subunit isoforms with different cellular localization may suggest the existence of V-ATPase subtypes within the yeast cell, perhaps with distinct roles within different intracellular compartments or in response to stress conditions.

Biochemical studies had suggested that removal of the 116 kDa protein from coated-vesicle enzyme isolates caused abolition of proton translocation [28,32], leading to the suggestion that this subunit may be involved in coupling ATP hydrolysis to proton translocation. Several of the putative transmembrane segments certainly contain conserved charged residues that could be involved in proton translocation, by analogy with the conserved acidic residue of the 16 kDa proteolipid. Recent mutagenesis work in yeast [151] has identified several key charged residues, mutation of which can affect both assembly and proton translocation function. In particular, mutation of conserved glutamates in the first and seventh putative segments, and of conserved histidine and arginine residues in segment 6, have profound effects, indicating a role for the Vph1 protein in the mechanism of proton translocation. The presence of acidic residues in transmembrane helices could account for an early report suggesting modification by DCCD of the 115 kDa subunit of the chromaffin-granule enzyme [44].

5.4. 39 kDa/36 kDa 'accessory' subunit

The 36 kDa polypeptide present in purified V-ATPase fractions from yeast is encoded by the *VMA6* gene [119]. *VMA6* encodes a hydrophilic 345-residue polypeptide of predicted mass 39.8 kDa that has 40% sequence identity with a 39 kDa 'accessory' subunit of the bovine chromaffin-granule enzyme [152]. This latter species was originally predicted to have a mass of 31 kDa, but it is likely that the start codon of the open reading frame was erroneously assigned (see discussion in [119]), and in fact is initiated some 231 bp upstream of the originally designated initiator methionine. The two species share two particularly highly conserved regions of 20–26 residues that also show somewhat greater hydrophobicity, although not sufficiently high to indicate transmembrane structures. Vma6p can also be stripped from the vacuolar membrane by strong chaotropes, confirming that it is unlikely to be an integral membrane protein [119].

Yeast cells in which *VMA6* is interrupted show the usual *vma* phenotype, with failure to assemble V₁ components at the vacuolar membrane [119]. Uniquely, however, disruption of *VMA6* also results in decreases in the levels of both Vph1p and 16 kDa proteolipid in the vacuolar membrane, suggesting that Vma6p is involved in the stabilization of V_o assembly [119]. A tight association between the integral membrane components of V_o and Vma6p was further indicated by assembly of the latter with V_o in a *vma2* mutant in which V₁ assembly was inhibited. Biochemical experiments on a purified V_o complex from the coated-vesicle V-ATPase also clearly show the presence of a 38 kDa polypeptide that is likely to be a homologue of Vma6p [121,122].

5.5. Coated-vesicle V-ATPase 19 kDa subunit

A 19 kDa protein is present in the coated-vesicle enzyme that appears to be tightly bound to V_o [40,121,122]. It has been suggested to be an integral membrane component, even though it is not labelled by lipid-soluble reagents [40]. It is unclear whether or not this polypeptide species is a homologue of Vma7p, which is reported to be tightly associated with V_o [48].

6. ASSEMBLY OF THE V-ATPase

The analysis of *vma* mutants in yeast has greatly increased our knowledge regarding the hierarchy of assembly of the V-ATPase.

It is clear that the V_o domain is assembled independently of the V₁ sector at the endoplasmic reticulum, and requires both the *VMA3* and *VPH1* gene products for stability [61]. Recent studies have shown that subunit c is able to assemble into complexes shortly after insertion into membranes derived from endoplasmic reticulum [140]. As might be expected from the hydrophobic character, synthesis of subunit c is dependent upon the presence of the signal-sequence recognition particle, but, quite unexpectedly, the polypeptide appears to be inserted in both orientations. This unusual feature has been suggested to segregate those subunit c polypeptides destined for the V-ATPase from those to be sequestered into connexon channels for gap junctions [139,140].

Although the V-ATPase is assembled at the endoplasmic reticulum [49], it is the case that both cytosolic, fully assembled, V₁ [49] and free V_o [121] are present within cells, and dissociation of the two may be an important regulatory mechanism (see section 7.).

While interruption of genes for the V₁ subunits does not in general disrupt the correct assembly and targeting of the V_o sector to the vacuolar membrane (see section 2.), a stable V_o is required for membrane assembly of the V₁ sector [61,118,153]. Assembly of V_o (and therefore V₁) does, however, require the presence of two additional integral membrane proteins, identified by complementation of *vma* or *cls* mutations, neither of which is a structural component of the V-ATPase. The product of the *VMA21* gene is an 8.5 kDa integral membrane with a C-terminal di-lysine motif that is required for retention in the endoplasmic reticulum [154], and disruption of the gene causes failure to assemble a stable V_o, rapid turnover of Vph1p and consequent loss of V-ATPase function. Disruption of the *VMA12* gene also results in failure to assemble V_o [155], although this 25 kDa membrane protein is reported to be present in the vacuolar membrane and may, therefore, be involved in targeting of the assembled enzyme [155]. A variety of other genetic loci giving rise to the *vma* phenotypes have been identified using the aforementioned genetic screens, but their precise function awaits evaluation.

Studies on cytoplasmic V₁ sub-complexes in yeast mutants in which single *VMA* genes have been knocked out have also provided information regarding hierarchical interactions between individual V₁ subunits. Both immunoprecipitation [156] and non-denaturing PAGE [93] studies indicate that Vma1p, Vma2p and Vma4p are able to associate together in a stable cytoplasmic complex. This complex can also associate with Vma8p, suggesting that these four proteins, in a stoichiometry (Vma1p)₃:(Vma2p)₃:(Vma4p)₁:(Vma8p)₁, represent a V₁ 'core' complex, which may also include as many as three copies of Vma7p [93]. Vma5p, although required for association of V₁ with V_o [56,91], was found not to be essential for the assembly of this complex, suggesting that it may be somewhat peripheral to the V₁ sector and involved specifically in coupling of the soluble and membrane domains.

Removal of subunits A–E from the coated-vesicle V-ATPase with chaotropic agents and subsequent reconstitution provides for an almost complete recovery of initial ATP-dependent H⁺-pumping activity [33]. This activity can be at least partially recovered even in the absence of the 40 kDa subunit C (homologous to Vma5p) [47]. As in the yeast system, the 40 kDa protein appears to be required only for stability and optimal activity, again implying that the function of this protein is not at the core of V₁. Some limited information regarding subunit contacts is also available. Interaction between the 33 kDa subunit E (Vma4p equivalent) and the 40 kDa subunit C has been demonstrated by immunoprecipitation [47], and cross-linking

studies indicate that the three polypeptides C, D and E (homologous to Vma5p, Vma8p and Vma4p respectively) are all in contact with the 16 kDa proteolipid of the V_o sector [32].

7. REGULATION

Because of the importance of the V-ATPase in vacuolar and cellular physiology, it might be expected that its activity would be tightly regulated at different levels. One level of regulation is the assembly/disassembly of the V-ATPase. Unlike the situation with F_o , which is freely permeable to protons [157], V_o free of V_1 is incompetent for proton translocation [121,122,158]. Similarly, cytoplasmic pools of V_1 appear to be unable to hydrolyse ATP. Uncoupling of the two domains is, therefore, an efficient method of controlling activity without futile ATP hydrolysis or collapse of membrane potential. Although it is often difficult to make a distinction between 'unassembled' and 'disassembled' domains with systems studied *in vitro*, studies *in vivo* have been much less ambiguous. One such situation is the larval midgut of the lepidopteran *Manduca sexta*. Here the loss of transepithelial voltage across the midgut of *Manduca*, which drives K^+ secretion, occurs as a consequence of disassembly of V_1 from V_o , and this disassembly is tightly controlled during stages of the larval development cycle [159]. At such times when V-ATPase is disassembled, the V_1 sector can account for as much as 2% of the total cytosolic protein [160].

Disassembly is not limited to specialized cell types. Shifting the carbon source from glucose to less favoured sources, such as raffinose or galactose, in *S. cerevisiae* results in a rapid dissociation of V_1 from V_o , a phenomenon that is fully reversible when the glucose supply is restored and which does not require *de novo* synthesis of proteins [161]. The intracellular signals that lead to disassembly and reassembly in *Manduca* and yeast remain to be determined, but they are clearly central to the integration of V-ATPase function into the physiological requirements of the organism. As a further complication, the intracellular signal appears to be suppressed under stress conditions, as indicated by the maintenance of V-ATPase function when *S. cerevisiae* cells are grown at non-optimal pH with galactose as the sole carbon source [130].

Regulation might also be achieved by redistribution of the V-ATPase. In a variety of secretory epithelia of vertebrates, V-ATPases are located in the apical membranes of MR (mitochondrially rich) cells [23]. Electron microscopic studies on such cells have suggested that V-ATPases might be shuttled between intracellular membranes and the apical surfaces in order to modulate proton secretion from the cells. The identity of the transport vesicles involved in this process remains unclear.

Salt stress also gives rise to an increase in the abundance of mRNAs encoding V-ATPase subunits in higher plants [109,162]. The subunit c transcript appears to be most increased in response to elevated salt. Increased V-ATPase activity also occurs after exposure of neutrophils to phorbol esters, indicating the involvement of protein kinase C [10]. In neutrophils it appears that the V-ATPase is important in regulating the cytosolic pH in response to activation by the 'switching on' of host defence systems upon microbial infection. Regulation can also be achieved directly by allosteric inhibition through binding of nucleoside diphosphates (see below). This might be important for turning off ATPase activity at times of energy shortage when ADP concentrations would be high. Finally, a protein activator is present in the kidney which appears specifically to increase V-ATPase activity at the brush border, but not in lysosomal membranes [121].

8. MOLECULAR BASIS OF INHIBITOR ACTION

A variety of molecules have been found to interact with the V-ATPase and cause inhibition of both ATP hydrolysis and proton translocation activities. Some of these substances are specific for the V-ATPases, and in many cases have provided information regarding not only the catalytic mechanism of the enzyme but also its molecular architecture and the disposition of individual subunits.

8.1. Soluble-domain inhibitors

The inhibitory effect of NBD-Cl is based on its structural analogy to ATP. This compound covalently modifies subunit A of V_1 , resulting in decreased ATP-hydrolysing activity [45,48,72], with an IC_{50} of approx. $1 \mu M$ [72]. Labelling of subunit A with a ^{14}C -labelled derivative of NBD-Cl originally identified this subunit as the site of ATP binding and therefore as the primary component of the active site.

The phosphate analogue vanadate is extremely effective at inhibiting P-type ATPases, with IC_{50} values in the sub-micromolar range. Although this property has been used to discriminate between the ATP-hydrolysing activities of V- and F-ATPases and that of the P-type enzyme, it is now clear that V-ATPase is also sensitive to vanadate [88,130,158], albeit at much higher concentrations (IC_{50} of 0.5–0.6 mM; 90% inhibition at 5 mM [130,158]). ADP and exogenous phosphate can also act as a competitive and non-competitive inhibitors respectively of ATP hydrolysis [73,163], indicating that the ordered release of the products of ATP hydrolysis is an important component of the catalytic cycle of the enzyme.

Inhibition of V-ATPase activity by nitrate has been widely documented in a variety of systems [25,29,31,36,46,164–167], and appears to occur as the sum effect of two distinct phenomena [165,166]. At high concentrations, nitrate may act as a chaotropic agent, with inhibition resulting from the dissociation of peripheral V_1 subunits from the membrane [25,31,36,165]. However, half-maximal inhibition by nitrate can occur with the anion at concentrations in the region of 40 mM [31], a concentration which should be too low to exert a strongly chaotropic effect. Furthermore, not all V-ATPases dissociate in the presence of nitrate, even though activity is inhibited [168,169].

A more subtle effect is therefore suggested, and it appears that this effect results from the oxidizing effect of nitrate. Protection from the effects of nitrate can be conferred by sulphite or other reducing agents [170], but inhibition is not reversed by the same agents. It has been proposed that the inhibitory effect of nitrate (or other oxidizing agents) results from promotion of the formation of disulphide cross-links between cysteine residues in V_1 , which subsequently becomes inactivated and released from the membrane [170]. In a similar vein, there is considerable evidence that disulphide-bond formation may contribute to a mechanism by which V-ATPase activity can be regulated *in vivo* in response to the redox state of the cytoplasm [79].

The presence of cysteine residues close to the active site of V_1 is also the crucial factor in the sensitivity of the V-ATPases to NEM. Uniquely amongst the three classes of ATP-driven proton pumps, V-ATPases are acutely sensitive to NEM, with inhibition of ATP hydrolysis occurring at low micromolar concentrations [28,44,48,71,171,172]. Inhibition arises through modification of a cysteine residue in the conserved P-loop sequence of subunit A [77,78]. Since this sequence contributes to the structure of the nucleotide-binding site (see section 4.1.), the inactivation of ATP hydrolysis by the incorporation of the NEM adduct is likely to be largely a steric effect. Consistent with this assumption is the observation that nucleotides are able to efficiently block in-

activation by NEM [73,174]. Introduction of a cysteine residue by mutagenesis into the equivalent position in the β subunit sequence transforms the F-ATPase into an enzyme that is sensitive to NEM [175].

Kinetic analysis of the reconstituted chromaffin granule enzyme has suggested the occurrence of allosteric inhibition by nucleoside diphosphates [174]. Fitting of kinetic models to the initial rate of proton pumping suggests two conformational states, R and T, of which the R state is catalytically active. Whereas nucleoside diphosphates bind to both catalytic and regulatory sites in both states, binding to the regulatory site is much tighter in the inactive T state. The advantage of such allosteric regulation is that activity would be down-regulated in the presence of high ADP concentrations, thus conserving energy.

The V-ATPases show no sensitivity to the F-ATPase inhibitors azide and oligomycin, a property that is useful for discriminating between F- and V-ATPase activities.

8.2. Inhibitors acting at membrane sites

The sensitivity of the V-ATPases to DCCD has been widely documented [45,46,120,176,177], and the effect of this inhibitor has been shown to be exerted through covalent modification of the 16 kDa proteolipid [75,104,134]. Micromolar concentrations of DCCD are sufficient to give complete and irreversible inhibition of proton pumping, although only 60–80% of ATP-hydrolysing activity may be lost. DCCD reacts with the side chain of the conserved acidic residue present in transmembrane segments of both the 16 kDa proteolipid [7] and the related 8 kDa proteolipid of F_0 . A reaction between carboxy groups and DCCD yields a stable dicyclohexyl-*O*-acylisourea [178,179]. It is not clear whether inhibition results from loss of the carboxy side chain, steric hindrance brought about by the introduction of the bulky adduct, or a combination of the two. It is known, however, that modification of only a single site per enzyme is sufficient to abolish proton translocation entirely [75], indicating some form of allostery or co-operativity in the enzyme.

The chemistry of DCCD provides some indirect clues about the structures and mechanisms involved in proton translocation. Firstly, DCCD is highly lipophilic and reacts only with protonated carboxy side chains [178,179], a state that is implicit in the transmembrane location of the acidic residues. Secondly, DCCD will only form a stable end-product in environments from which water is precluded. Taken together, these observations point to a structural model in which the essential glutamate residue of the 16 kDa proteolipid is lipid-exposed and protonated under normal conditions (Figure 3). Furthermore, they imply that the pathway of proton translocation may be at a protein–lipid or protein–protein interface on the external lipid-exposed surface of V_0 [135].

Both F- and V-ATPases show sensitivity to alkyl tin compounds [135,180–184]. These lipophilic compounds appear to interact with V_0 at or close to the site of DCCD action [135], and presumably therefore inhibit proton translocation specifically, further indicating the proximity of the proton-translocating pathway to the lipid phase.

The bafilomycins, and the related compound concanamycin, are macrolide antibiotics consisting of a 16- or 18-membered lactone ring which are highly specific inhibitors of the V-ATPases [185,186]. Reversible inhibition of proton translocation occurs at sub-micromolar concentrations [31,185,187,188], and involves interaction with at least one protein component of the V_0 sector [122,188,189]. This has been proposed to be the 116 kDa/Vph1p protein [122] or the 16 kDa proteolipid [189], but the interaction could equally well involve a contribution from both proteins.

Because of the highly specific nature of their inhibitory effect, bafilomycins have proven to be invaluable in a wide variety of studies seeking to dissect out cellular processes that involve V-ATPase activity [10,139,190–195]. The cyclic peptide mycotoxin destruxin B has also been demonstrated to be a highly specific and efficacious inhibitor of V-ATPases [196], but its site of action remains uncertain.

The synthetic oestrogen diethylstilboestrol is a lipophilic compound that exerts an inhibitory effect on a number of ATPases [197]. In the case of V-ATPases, 100 μ M diethylstilboestrol completely abolishes proton translocation and inhibits 80% of ATP-hydrolysing activity [198]. This inhibition is reversible and, given the lipid solubility of diethylstilboestrol, is likely to occur through non-covalent interactions with V_0 . It has been proposed that diethylstilboestrol may cause perturbation of the local lipid environment of the enzyme, implying that specific interactions between lipid and protein may be essential for the proton translocation function. Studies on *Nephrops* gap junctions containing subunit c (ductin) show a tight annulus of lipid with a preference for negatively charged phospholipid [131].

A specific interaction between transmembrane elements of subunit c and the E5 oncoprotein of papillomaviruses has been demonstrated in a number of studies [199–203]. In cultured cells this interaction can give rise to a partial inability to acidify the endosomes, suggestive of impairment of V-ATPase function [204]. This specific interaction appears to be essential for the transforming activity of the oncoprotein, and can apparently be stabilized by the charge interaction between the conserved glutamate of the proteolipid and the introduction of a transmembrane arginine residue in E5 [202]. In addition, two mutant forms of subunit c, one which is truncated at the end of the third transmembrane domain and another where the crucial glutamic acid is replaced by arginine, have transforming activity. Expression of E5 also results in almost complete loss of cell–cell communications via gap junctions [205,206], leading to the possibility of a ‘dual hit’ on cellular transformation by these viral oncoproteins [200]: (1) up-regulation of growth-factor stimulation due to loss of V-ATPase function in the early endosome, and (2) loss of cell–cell interactions due to loss of gap-junction-based communication.

9. MECHANISM OF ENZYME ACTION

There is little direct information on the mechanisms of ATP hydrolysis and proton pumping in the V-ATPase. However, much is now known about the F-ATPase and, because of the similar composition and identity between the subunits, it is likely that the mode of action of the V-ATPase is similar.

9.1. Specificity of the translocated cation

Although F_0 shows proton conductance in the absence of F_1 , there remains some doubt as to whether V_0 can passively translocate protons in the absence of V_1 . An early reconstitution study of organic-solvent-extracted subunit c in liposomes by Stone and co-workers did show the DCCD-sensitive translocation of protons [120]. However, a number of subsequent studies using either vacuolar membranes stripped of V_1 [121,130,158] or reconstituted V_0 [121], or reconstituted ductin from gap junctions [130], have failed to show passive translocation of protons. This apparent discrepancy might be explained by studies showing that passive proton translocation can be achieved with intact V_0 if the complex is first treated with low-pH buffers [188], or if the complex is dissociated into its

constituent subunits and subsequently reconstituted [122]. In light of the available information, it seems likely that subunit c itself does contain all the structural elements necessary for proton translocation, but that (as might be expected) V_o is normally impermeable when dissociated from V_1 . In addition, reconstituted subunit c has unexpected transport properties (e.g. acetylcholine), perhaps through the presumptive central channel described in section 5.2.

Cations other than protons have been shown or suggested to be translocated by forms of V-ATPase. The *E. hirae* V-ATPase translocates Na^+ in preference to H^+ , a situation analogous to that with the F-ATPase of *Propionigenium modestum*, which likewise couples ATP synthesis to Na^+ translocation. Using hybrid enzymes comprising F_1 and F_o from H^+ - and Na^+ -translocating F-ATPases respectively, it has been possible to show that cation discrimination is solely the property of F_o [207]. Mutational analysis has furthermore shown that subunit c contributes to cation discrimination [208], as might be expected from its pivotal role in ion translocation. Similar reconstitution and mutation studies have yet to be carried out on H^+ - and Na^+ -pumping V-ATPases. Unfortunately, sequence comparisons of subunits c from H^+ and Na^+ V- and F-ATPases provide no clues regarding the identity of residues that might be involved in cation discrimination. In addition, cations are for the large part translocated in opposite directions by the F- and V-ATPases, and it might therefore be expected that sites of cation discrimination in the two classes of pump would be located on opposite sides of the membrane.

As well as Na^+ , it has been suggested that iron might also be translocated by one form of V-ATPase [209]. Reconstituted preparations of reticulocyte V-ATPase have demonstrated the translocation of iron, with a preference for Fe(II) over Fe(III), that was partially inhibited by DCCD. However, it was not established unambiguously whether or not the movement was a *bona fide* activity of the V-ATPase, and the possibility remains that it is a contaminating activity that may use proton gradients generated by the V-ATPase.

9.2. H^+ /ATP stoichiometry

For the F-ATPase, it is commonly thought that on average three to four protons are translocated for the synthesis of each ATP molecule [133]. Such a stoichiometry is consistent with the three copies of the α/β dimer in F_1 and 10 ± 2 copies of subunit c in F_o (providing 12 potential proton-binding sites) [210]. For the V-ATPase, the stoichiometry is thought to be lower, i.e. two protons translocated for the hydrolysis of an ATP molecule [133]. Again, this is consistent with three copies of an A/B dimer in V_1 and six copies of subunit c in V_o providing six potential proton-binding sites. Such a stoichiometry suggests that the active unit of V_o is a dimer of subunit c and, in fact, subunit c does form stable dimers in SDS [211]. A recent analysis of two-dimensional arrays of subunit c in detergent also suggested that the unit cell is most likely a dimer of subunit c [212].

However, a study on red beet vacuoles showed that the coupling ratio of the enzyme is more variable than expected [213]. The authors measured proton flux by $\Delta\Psi$ in the absence and presence of bafilomycin. They found that the variability is a function of both cytosolic and luminal pH. In the presence of moderate differences between cytosolic and luminal pH (e.g. 2.2 units), a ratio as high as 3.2 H^+ /ATP hydrolysed was measured, but with greater pH differences (e.g. 4.7 units) the ratio was lowered to 1.75. This implies that there may be co-operativity between the subunits c. Such variability might be an advantage in creating the low pH found in, for example, the vacuoles of

citrus fruits. The non-integer ratios also indicate that ATP hydrolysis and proton translocation are not tightly coupled events. In extending the study, the authors found evidence for three H^+ -binding sites per ATP hydrolysis event, two of which are negatively charged. The identity of these two sites is not known, but it is interesting to speculate that they may be an active dimer of subunits c which would correspond to the A/B dimer.

9.3. Coupling of ATP hydrolysis to proton pumping

In the case of the F-ATPase, it is now generally thought that the translocation of protons through F_o transmits long-range conformational changes to the F_1 -ATPase, effecting changes in nucleotide binding. The crucial linking subunit is the γ polypeptide, which forms a spindle of extended α -helices at the centre of the pseudo-hexameric arrangement of alternating α and β subunits in the F_1 sector [6]. There is now experimental evidence to show that, during the catalytic cycle, the γ subunit rotates about its long axis with respect to each α/β dimer in the F_1 sector, in a so-called 'entropic motor' [173,214–216]. The γ subunit also interfaces with the ϵ subunit [217], and both of these subunits in turn contact both the β subunit in F_1 [218] and subunit c in the F_o sector [143,219,220]. Both subunits certainly undergo conformational changes during the catalytic cycle [210,221,222].

The mechanism by which proton movement is coupled to changes in nucleotide binding in the F_1 ATPase remains less certain. What is clear is that subunit c forms much of the pathway of proton translocation in F_o , and it is also thought that subunit a may contribute [223,224]. This has led to the proposal of a mechanism in which side-chain protonation/deprotonation events at the interface between subunits a and c cause changes in their mutual interaction, driving rotation of the subunit c oligomer relative to subunit a [223]. In the proposed model, subunit a is fixed relative to the α_3/β_3 complex of F_1 by a 'stator' comprising two copies of subunit b. A structure consisting of the subunit c oligomer and the γ and ϵ subunits would be able to rotate relative to this fixed unit, inducing conformational changes in F_1 and effecting the changes in nucleotide binding.

Several observations make it attractive to suppose that the V-ATPases operate by a similar rotational mechanism. Firstly, it seems clear that the A and B V_1 subunits equate functionally to the β and α F_1 subunits respectively (see sections 4.1. and 4.2.). Secondly, on the basis of limited sequence identity and structural prediction, we can postulate that subunit D/Vma8p similarly equates to the γ subunit of F_1 . It is possible, therefore, that subunit D/Vma8p (perhaps in conjunction with subunit E/Vma4p) likewise forms a spindle of coiled-coil α -helices and rotates relative to V_1 during the catalytic cycle of the V-ATPase (see Figure 4). The central pore in V_o created by the symmetrical arrangement of six subunit c protomers, which may be as much as 2 nm in diameter, could provide an attachment for a centrally disposed γ -like subunit, although such a contact has yet to be proven experimentally. Alternatively, the conserved loop between helices 3 and 4 of the V-ATPase subunit c could form the point of contact between a γ subunit analogue and V_o .

The Vph1p subunit of V_o is reported to contain charged residues that are essential for proton translocation [151], leading to the supposition that Vph1p plays a role similar to that of subunit a in F_o . Residues from both the 16 kDa proteolipid subunit c and Vph1p would contribute to a pathway of proton translocation. By analogy with the mechanism proposed for the F-ATPase, Vph1p would be fixed relative to V_1 by a 'stator' (Figure 4), a function that could be performed by the Vma10p polypeptide, which has limited sequence similarity to subunit b

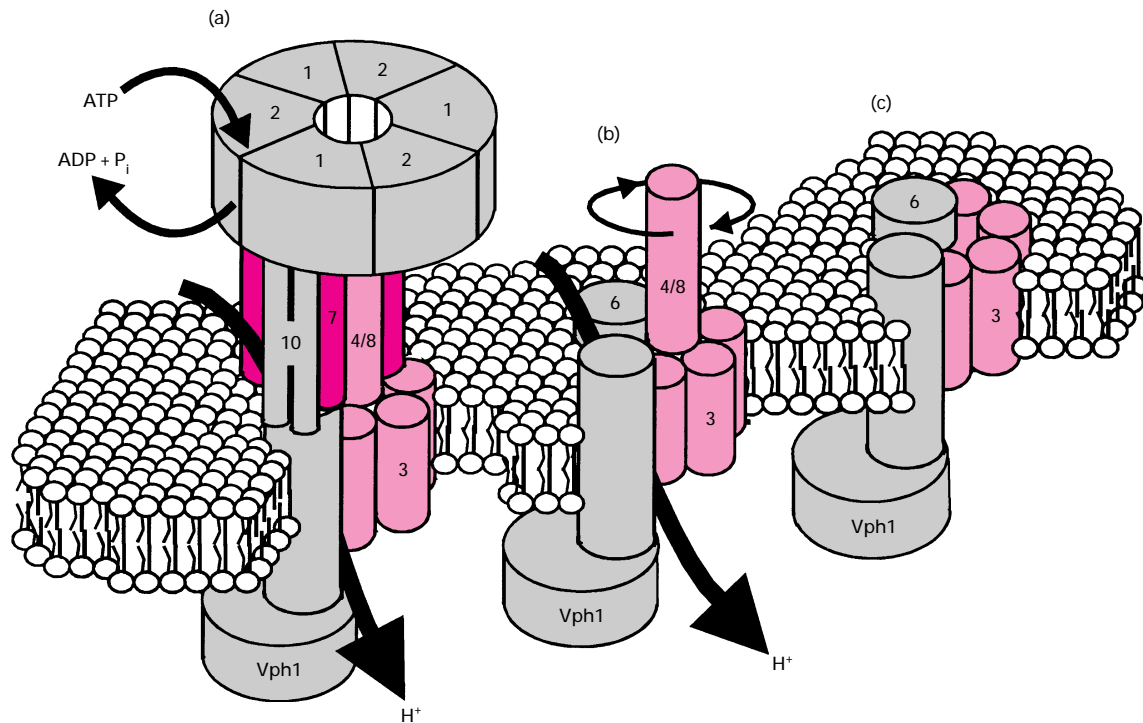


Figure 4 Functional organization of the yeast V-ATPase

A speculative model for the mechanism of action of the V-ATPase is presented, based on analogy with that proposed for the F-ATPase (see section 9.3.). Individual subunits are numbered according to their gene-product designations in *Saccharomyces* (see legend to Figure 1). Changes in nucleotide binding in V_1 (a) induce conformational changes that translate into rotation of the subunit γ homologue Vma8p/Vma4p relative to the (Vma1p)₃/(Vma2p)₃ structure. If Vma8p/Vma4p is fixed relative to the 16 kDa proteolipid (Vma3p) hexamer in V_0 , then ATP hydrolysis will drive rotation of this hexameric assembly relative to Vph1p (b; subunits removed for clarity). Vph1p and the (Vma1p)₃/(Vma2p)₃ structure constitute a static element, fixed relative to each other by their respective interactions with the subunit b homologue Vma10p (a). A mechanism for the transfer of protons can be described in simplistic terms: interaction between Vph1p and the Vma3p proteolipid facilitates deprotonation of side chains of residues at the protein-protein interface, with concomitant release of the proton into the vacuolar lumen. The conserved glutamate residue of the Vma3p proteolipid (see section 5.1.) must re-acquire a proton from the cytoplasmic phase (either directly or indirectly) before the next step in rotation exposes it to the lipid phase, thus effecting sequential transfer of protons from cytoplasm to vacuole. An alternative mechanism is that the whole of V_0 remains static with respect to the rotation of Vma8p/Vma4p, which ratchets around the central channel of the core hexamer of Vma3p subunits rather like a camshaft. This could induce conformational change in Vma3p, so leading to cycles of protonation and deprotonation. In this instance, the transmembrane pathway for proton translocation might not require Vph1p. Under normal circumstances, removal of the V_1 subunits from the enzyme yields a V_0 consisting of (Vma3p)₆/(Vma6p)₁/(Vph1p)₁, which is incompetent for proton translocation (c). The remaining structure can be presumed to be locked in an inactive state in the absence of the structural elements that drive rotation. The involvement of the additional subunits (a; shown in dark pink) in the proposed mechanism is undefined, but Vma5p and Vma7p are likely to be in contact with the Vma3p hexamer.

of F_0 . Conformational changes occurring as a consequence of ATP hydrolysis would drive rotation of Vma8p and the subunit c hexamer relative to the V_1 /Vph1p structure. The resultant transient interactions between Vph1p and subunit c would allow deprotonation of the glutamate residue of subunit c and release of the proton into the lumen of the vacuole.

There is, at the present time, no direct evidence for rotational proton pumping in either V_0 or F_0 . However, two recent studies have shown that single copies of defective mutant proteolipids incorporated into either reconstituted F_0 [225] or V_0 expressed *in vivo* [135] are able to block virtually all proton translocation. Such results would be entirely consistent with the notion of rotational proton pumping.

Rotational pumping might also be achieved if the spindle subunit acted as a rotating cam in the central channel of the subunit c hexamer, which remains stationary relative to V_1 . A rotating cam could transmit conformational changes by 'deforming' each subunit c in turn. Such a mechanism would imply that Vph1p does not necessarily form part of the proton translocation pathway unless it too rotates. On this point, it is interesting to note that, if the F_0 subunit c dodecamer is organized

in the same manner as the V_0 subunit c hexamer, then six of the potential proton-translocating sites in F_0 would be internal and not involve subunit b.

While it is interesting to draw analogies on the basis of similarities between subunits of the F- and V-ATPases, one should add the caveat that mechanistic models such as the ones described above must remain highly speculative. There is an obvious requirement for firm structural data relating to subunit interactions in the V-ATPase.

Note added in proof (received 30 April 1997)

The two related vma3p related proteolipids, vma11p and ppa1p (now designated vma16p), have been shown to be present in the V-ATPase and inactivation of the genes encoding either results in the vma phenotype in *S. cerevisiae* [226]. In addition, a recent study has directly visualized the rotation of the γ subunit (V-ATPase homologue, subunit D) with respect to the trimeric complex formed from α/β dimer (V-ATPase homologues, subunits B and A) of F_1 during ATP hydrolysis [227].

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