J. exp. Biol. **172**, 93-103 (1992)
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BIOGENESIS OF THE YEAST VACUOLAR H^+-ATPase

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Summary

Achieving an understanding of the biosynthesis, assembly and intracellular targeting of the vacuolar H^+-ATPase is critical for understanding the distribution of acidic compartments and the regulation of organelle acidification. The assembly of the yeast vacuolar H^+-ATPase requires the attachment of several cytoplasmically oriented, peripheral subunits (the V_1 sector) to a complex of integral membrane subunits (the V_o sector) and thus is not easily described by the established mechanisms for transport of soluble or vacuolar membrane proteins to the vacuole. In order to examine the assembly of the enzyme complex, yeast mutants lacking one of the subunit genes have been constructed and the synthesis and assembly of the other subunits have been examined. In mutants lacking one subunit, the remaining ATPase subunits seem to be synthesized, but in many cases are either not assembled or not targeted to the vacuole. Immunofluorescence and subcellular fractionation experiments have revealed that deletion of one peripheral subunit prevents the other peripheral subunits, but not the integral membrane subunits, from reaching the vacuole. In contrast, the absence of one of the integral membrane subunits appears to prevent both the peripheral subunits and another integral subunit from reaching the vacuole and also results in reduced cellular levels of the other integral membrane subunit. These data suggest that transport of integral and peripheral membrane subunits to the vacuole may employ somewhat independent mechanisms and that some assembly of the V_1 and V_o sectors may occur before the two sectors are joined. Current models for the assembly process and the implications for organelle acidification are discussed.

Structure of the yeast vacuolar H^+-ATPase

The yeast vacuolar H^+-ATPase closely resembles the V-ATPases from other fungi, plants and animals, both in its overall structure and in the sequences of the subunit genes that have been cloned (reviewed in Kane and Stevens, 1992). The yeast enzyme has been partially purified by density gradient centrifugation of solubilized yeast vacuoles and the fraction exhibiting ATPase activity contains eight polypeptides of relative molecular masses (M_r) 100, 69, 60, 42, 36, 32, 27 and 17×10^3 (Uchida *et al.* 1985; Kane *et al.* 1989*b*). The same collection of polypeptides was immunoprecipitated by a monoclonal antibody recognizing the 69×10^3 M_r subunit, suggesting that all of these polypeptides are part of the active complex (Kane *et al.* 1989*b*). Further support for this subunit composition has been provided by genetic studies, which indicate that mutagenesis of any one of the genes for the 100, 69, 60, 42, 27 and 17×10^3 M_r subunits can totally abolish

Key words: acidification, ATPase, yeast vacuole, assembly.

ATPase activity in isolated vacuolar vesicles and elicit a set of phenotypes which has been associated with loss of vacuolar H^+-ATPase activity (Manolson *et al.* 1992; Kane *et al.* 1990; Hirata *et al.* 1990; Yamashiro *et al.* 1990; Nelson and Nelson, 1990; Beltran *et al.* 1992; Foury, 1990). Cloning of the genes for the 36 and $32\times10^3 M_r$ polypeptides present in the glycerol gradient has not yet been reported.

Biochemical studies have revealed that the yeast vacuolar H^+-ATPase is composed of both peripheral and integral membrane subunits. The 69, 60 and $42\times10^3 M_r$ subunits can be stripped from the vacuolar membrane by potassium nitrate, indicating that they are peripheral membrane proteins (Kane *et al.* 1989*b*). The $27\times10^3 M_r$ subunit also appears to be a peripheral membrane protein based on its predicted amino acid sequence (Foury, 1990). The 100 and $17\times10^3 M_r$ subunits are integral membrane proteins that remain in the membrane through either potassium nitrate or alkaline sodium carbonate treatment (Kane *et al.* 1992). Both the nitrate-stripping data and analysis of mutants lacking one peripheral subunit (described below) suggest a structural association between the 69, 60 and 42×10^3 M_r subunits (Kane *et al.* 1989*b*, 1992; Noumi *et al.* 1991; Beltran *et al.* 1992). These three subunits along with other peripheral subunits appear to form a peripheral complex, called the V_1 sector of the vacuolar H^+-ATPase by analogy with the F_1 sector of the F_1F_o-ATPases (Bowman *et al.* 1989; Puopolo and Forgac, 1990). The V_1 sector of the vacuolar H^+-ATPase contains the catalytic sites for ATP hydrolysis (Uchida *et al.* 1988) but, unlike the F_1-ATPase, the V_1 sector has no activity when dissociated from the membrane subunits in yeast or in any other system that has been studied (Kane *et al.* 1989*b*; Bowman *et al.* 1989; Moriyama and Nelson, 1989; Adachi *et al.* 1990). The 100 and $17\times10^3 M_r$ subunits have been proposed to form the V_o membrane sector of the enzyme that is thought to contain the proton pore, based on labeling of the $17\times10^3 M_r$ subunit with dicyclohexyl carbodiimide (Uchida *et al.* 1985), indirect evidence suggesting a structural association between the 100 and $17\times10^3 M_r$ subunits (Kane *et al.* 1992) and by analogy with the F_1F_o-ATPases. The current working model for the structure of the yeast vacuolar H^+-ATPase is shown in Fig. 1.

Analysis of mutant yeast cells lacking one vacuolar ATPase subunit

A variety of methods have been developed that exploit homologous recombination in yeast cells in order to replace the chromosomal copy of a wild-type gene with a copy of the cloned gene that has been mutagenized *in vitro* (reviewed in Rothstein, 1991). These techniques have been used to construct a variety of mutant yeast strains that fail to produce one subunit of the vacuolar H^+-ATPase. Studies of mutant cells lacking one of the subunits of the vacuolar H^+-ATPase have not only provided support for the biochemically determined subunit composition, but have also generated a number of insights about the biosynthesis and assembly of the enzyme complex. The genes for the 100, 69, 60, 42, 27 and $17\times10^3 M_r$ subunits (called *VPH1*, *TFP1/VMA1*, *VAT2/VMA2*, *VATC*, *VMA4* and *VMA3*, respectively) have been identified and cloned (Manolson *et al.* 1992; Shih *et al.* 1988; Nelson *et al.* 1989; Beltran *et al.* 1992; Foury, 1990; Nelson and Nelson, 1989) and the corresponding mutant strains have been constructed. Deletions in any of these subunit genes yield a well-defined set of phenotypes, which includes a

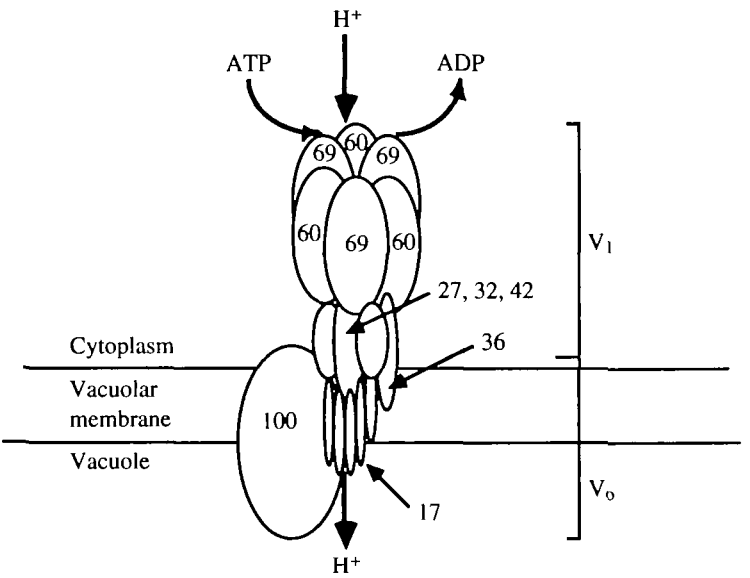

Fig. 1. Structural model for the yeast vacuolar H^+-ATPase. The 69, 60, 42 and $27\times10^3 M_r$ subunits are shown as part of the V_1 sector and the 100 and $17\times10^3 M_r$ subunits are shown as part of the V_o sector, based on their behavior as peripheral and integral membrane subunits, respectively. The 32 and $36\times10^3 M_r$ subunits are shown as part of the V_1 sector, based on evidence that polypeptides of approximately this size can be removed from the membrane during cold-inactivation (Noumi *et al.* 1991). The overall model is revised from Kane *et al.* (1989*a*).

complete loss of vacuolar acidification, absence of all ATPase activity in isolated vacuoles and failure to grow in media buffered to neutral pH (Nelson and Nelson, 1990; Yamashiro *et al.* 1990; Ohya *et al.* 1991). Lack of one subunit does not necessarily affect the synthesis or stability of the other subunits (see below).

Deletion of any of several of the peripheral subunits, including the 69, 60, 42 and $27\times10^3 M_r$ subunits, has a very similar effect on the other subunits of the vacuolar ATPase. In general, the other peripheral and integral membrane subunits for which a means of detection is available (including the 100, 69, 60, 42 and $27\times10^3 M_r$ subunits) are present in whole-cell lysates of the mutant cells at nearly the same levels as in wild-type cells (Kane *et al.* 1992; Beltran *et al.* 1992; M. N. Ho and T. H. Stevens, personal communication). Isolated vacuolar membranes from the mutant cells contain none of the peripheral subunits when one of the peripheral subunits is missing, suggesting that, although these subunits are synthesized in the mutant cells, they are not properly assembled and/or targeted to the vacuolar membrane (Kane *et al.* 1992; Beltran *et al.* 1992; M. N. Ho and T. H. Stevens, personal communication). In contrast, both the 17 and $100\times10^3 M_r$ subunits are present in the vacuolar membranes of these mutant cells, indicating that they are not dependent on the peripheral subunits for targeting to the vacuole (Kane *et al.* 1992; Noumi *et al.* 1991; Umemoto *et al.* 1990). These results have been confirmed by immunofluorescence microscopy using antibodies recognizing the 60

and $100\times10^3 M_r$ subunits. In wild-type cells, the $60\times10^3 M_r$ subunit appears as a ring around the vacuolar membrane. However, in cells lacking the $69\times10^3 M_r$ subunit, the $60\times10^3 M_r$ subunit appears to be diffusely distributed in the cytoplasm, while the antibody recognizing the $100\times10^3 M_r$ subunit stains the vacuolar membrane (Kane *et al.* 1992).

Deletion of the $17\times10^3 M_r$ subunit gene (*VMA3*), which encodes an integral membrane subunit of the ATPase, has somewhat different effects on assembly and targeting of the other subunits. The peripheral subunits, including the 69, 60 and $42\times10^3 M_r$ subunits, are present in the cells at near-normal levels, but are not present in isolated vacuolar vesicles (Umemoto *et al.* 1990; Noumi *et al.* 1991; Kane *et al.* 1992). Antibodies against the $69\times10^3 M_r$ subunit (Umemoto *et al.* 1990) or the $60\times10^3 M_r$ subunit (Kane *et al.* 1992) appear to stain the cytoplasm when viewed by immunofluorescence microscopy. The $100\times10^3 M_r$ subunit, another integral membrane subunit, is present at reduced levels (about 10% of wild-type) in whole-cell lysates from *vma3Δ* mutants, but it has not yet been established whether changes in the rate of synthesis or the stability of this subunit cause the reduced cellular levels. Isolated vacuolar vesicles from *vma3Δ* cells contain no $100\times10^3 M_r$ subunit, suggesting that even the small amount of the $100\times10^3 M_r$ subunit that is synthesized in the mutants is not transported to the vacuole (Kane *et al.* 1992). Therefore, the $17\times10^3 M_r$ subunit appears to be necessary for the vacuolar targeting of both the $100\times10^3 M_r$ integral membrane subunit and the peripheral subunits of the ATPase.

The yeast mutants lacking one ATPase subunit have been analyzed in more detail to determine whether the remaining subunits that do not reach the vacuolar membrane are still partially assembled. This type of analysis has previously yielded information about the steps involved in assembly of other multisubunit proteins because assembly can often proceed in the mutant cells up to the point where the missing subunit is required (Crivellone *et al.* 1988; Manolios *et al.* 1991). Wild-type and mutant cells lacking one subunit were subjected to high-speed centrifugation in order to separate the ATPase subunits into an insoluble fraction (containing membrane-bound proteins and large protein aggregates) and a soluble fraction. Even in wild-type cells, the 69, 60, 42 and $27\times10^3 M_r$ subunits were found partially in the soluble fraction, while the $100\times10^3 M_r$ subunit of the ATPase and another vacuolar membrane protein, dipeptidyl aminopeptidase B, were found entirely in the insoluble fraction. The distribution of the subunits between the soluble and insoluble fractions varied in the different mutant cells, with *tfp1Δ* and *vat2Δ* mutant cells exhibiting the smallest proportion of the peripheral subunits in the soluble fraction and the *vma3Δ* cells exhibiting a large proportion in the soluble fraction (R. D. Doherty and P. M. Kane, unpublished data). Further fractionation of the soluble fractions by glycerol density gradient centrifugation showed that all of the strains contained the peripheral subunits at least partially in a low-density fraction, corresponding to a relative molecular mass of less than $100\times10^3 M_r$ when compared to molecular mass standards run in parallel. The subunits in this fraction are probably unassembled or associated to only a very limited degree. In the *vma3Δ* and wild-type cells, the 69, 60, 42 and $27\times10^3 M_r$ subunits are also found in a second region of the gradient of much higher density, corresponding to a relative molecular mass of roughly

400×10^3 compared with the molecular mass standards. The subunits fractionating in this region of the gradient may be associated into partially assembled sub-complexes and the predicted size of these complexes is large enough to constitute most or all of the V_1 sector (R. D. Doherty and P. M. Kane, unpublished data). The absence of the high-density peak in the *tfp1Δ* and *vma4Δ* mutants suggests that partial assembly into this complex cannot occur in the absence of the 69 or the $27\times10^3 M_r$ subunits. The high proportion of the peripheral subunits in the insoluble fraction from *tfp1Δ* and *vat2Δ* cells may be attributable to insoluble protein aggregates resulting from a failure to assemble, since the initial analysis of these mutants (described above) provided little evidence for association with the integral membrane subunits. A model for assembly of the peripheral subunits into the V_1 sector, which includes the results of the experiments with mutant cell lines, is presented in the cartoon in Fig. 2.

Involvement of other proteins in assembly of the vacuolar H^+-ATPase

The experiments described above focus on the subunit composition of the final, active form of the vacuolar H^+-ATPase and the role played by those subunits in assembly of the complex. However, it has become increasingly clear that even complexes that can self-assemble *in vitro* often utilize other cellular proteins to assist their assembly *in vivo* (reviewed in Ellis and van der Vies, 1991; Gething and Sambrook, 1992). The general class of 'assembly factors' covers a range of functions, encompassing both chaperone proteins, which assist a wide variety of proteins in attaining tertiary or quaternary structure, and very specific assembly factors, which appear to act on a single protein

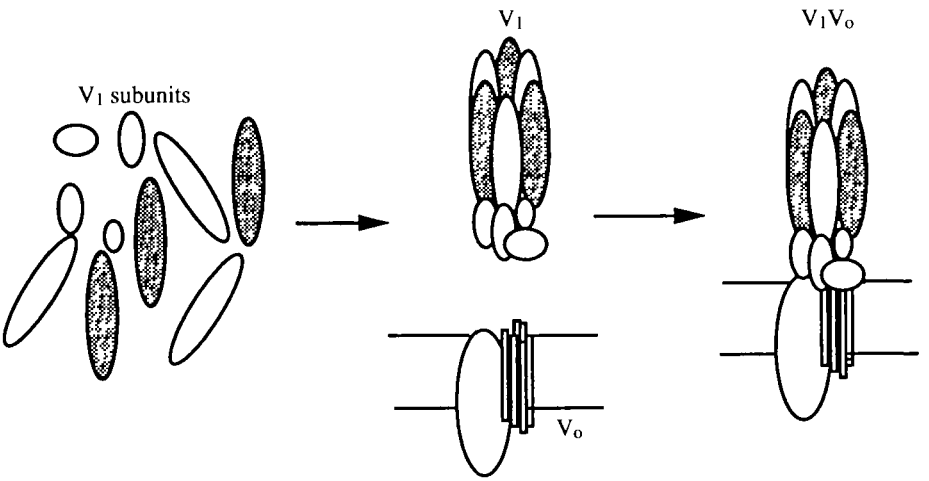

Fig. 2. Assembly of peripheral subunits of the vacuolar H^+-ATPase. The V_1 sector is shown as being assembled from the individual peripheral subunits prior to attachment to the integral membrane V_o sector. In the mutant cells examined, the *tfp1Δ* and *vma4Δ* cells appear to be arrested at the first step, because the peripheral subunits do not become attached to the vacuolar membrane and no complexes large enough to correspond to the V_1 sector appear to be formed. The *vma3Δ* cells may proceed to the second step and form a V_1 sector, or a similar larger complex, but the complex does not attach to the vacuolar membrane.

complex. Candidates for both types of assembly factor have been identified for the yeast vacuolar H^+-ATPase.

The *VPS3* and *VPS6* gene products appear to influence the assembly of the yeast vacuolar H^+-ATPase, but have pleiotropic effects on the cell, suggesting that they may act on a number of proteins unrelated to the ATPase or acidification. A number of *vps3* and *vps6* mutants were initially identified as part of a large set of mutants defective in sorting of soluble vacuolar proteases to the vacuole (*vps*=*v*acuolar *p*rotein *s*orting; Rothman and Stevens, 1986; Rothman *et al.* 1989*a*). The *vps3* and *vps6* mutants were subsequently found to be distinct from the rest of the collection in that they failed to accumulate the lysosomotropic amine quinacrine, indicating that they had an acidification defect as well as the protein-sorting defect (Rothman *et al.* 1989*b*). Further analysis of these mutants revealed that the specific activity of the vacuolar ATPase in isolated vacuoles was greatly decreased (6–15% of wild-type activity) and the levels of the 69 and $60\times10^3 M_r$ subunits in vacuolar membranes were comparably diminished, even though these subunits were present at near-normal levels in whole-cell lysates from the mutant cells (Rothman *et al.* 1989*b*). However, comparison of the phenotypes of a *vps3Δ* mutant and a *vat2Δ* mutant revealed significant differences, suggesting that the *VPS3* gene product plays a number of cellular roles in addition to its potential role in ATPase assembly (Raymond *et al.* 1990). For example, the dramatic defect in protein sorting seen in the *vps3* mutants appeared to be distinct from the defect in acidification (Raymond *et al.* 1990). Similarly, the vacuolar pH of a *pep12Δ* mutant (*PEP12* is the same gene as *VPS6;* Rothman *et al.* 1989*a*) appeared to be lower than that of mutants lacking an ATPase subunit, indicating a less severe acidification defect (Preston *et al.* 1989), but the effects of the mutation on other cellular functions, including vacuolar protein sorting and zymogen activation, were more severe than those seen in the vacuolar ATPase mutants (Yamashiro *et al.* 1990). Thus, both the *VPS3* and *VPS6* gene products appear to affect a number of cellular functions, only one of which is assembly of the vacuolar H^+-ATPase. It is also entirely possible that some of the even more general yeast chaperone proteins (Gething and Sambrook, 1992) are involved in vacuolar H^+-ATPase assembly, but there is no direct evidence of this at present.

In contrast, a number of other gene products have now been identified that appear to be specifically necessary for ATPase activity even though they are not part of the final ATPase complex and these proteins are good candidates for the highly specific assembly factors described above. Mutations in the *VMA12* and *VMA13* genes yield a set of phenotypes identical to that of the *tfp1Δ*, *vat2Δ* and *vma3Δ* mutants (Ohya *et al.* 1991). However, the predicted sizes and sequences of the *VMA12* and *VMA13* gene products do not correspond to those of any of the previously identified polypeptides in the glycerol-gradient-purified vacuolar H^+-ATPase, indicating that even though these proteins are indispensable for vacuolar ATPase activity they may not be part of the active complex (Anraku *et al.* 1992; Kane and Stevens, 1992). Another gene, *VMA11*, has been identified from a mutant strain exhibiting the characteristic set of phenotypes for vacuolar H^+-ATPase mutants (Ohya *et al.* 1991). The *VMA11* gene is highly homologous with *VMA3* and may encode a second proteolipid ($17\times10^3 M_r$) subunit of the ATPase (Umemoto *et al.* 1991). However, the protein sequence obtained for the chloroform:methanol-extracted

proteolipid from vacuolar membranes corresponded to the predicted sequence of the *VMA3* gene (Anraku *et al.* 1991), so it is not yet clear whether the *VMA11* gene product is present in vacuolar membranes or the active vacuolar H^+-ATPase complex. The exact functions of the *VMA11–13* gene products are still under investigation.

Models for assembly

No multisubunit protein of the yeast vacuolar membrane has been extensively studied previously, so the requirements for assembly and vacuolar targeting of the yeast vacuolar H^+-ATPase are largely unknown. However, the proposed structure for the yeast vacuolar H^+-ATPase shown in Fig. 1 has immediate implications for its biogenesis and the experiments with mutants described above provide further clues about these processes.

The biogenesis of the peripheral subunits, which are on the cytoplasmic face of the vacuolar membrane, may bear more resemblance to the synthesis and transport of cytoplasmic proteins than to those of vacuolar proteins. Two types of experimental evidence indicate that the peripheral subunits never enter the secretory pathway. First, the sequences of cloned genes of the 69, 60, 42 and $27\times10^3 M_r$ subunits contain no evidence of signal sequences or potential transmembrane domains. Second, there are several potential sites for N-linked glycosylation in the predicted amino acid sequence of the 60 and $69\times10^3 M_r$ subunits, but neither subunit receives N-linked glycosylation (P. M. Kane and T. H. Stevens, unpublished data). These results suggest that the peripheral subunits that make up the V_1 sector remain in the cytosol prior to attachment to the integral membrane subunits and leave open the possibility that they may partially or completely assemble into the V_1 sector before they become attached to the V_o sector. The presence of high-molecular-mass complexes in the supernatants from *vma3Δ* and wild-type cells provides experimental support for this possibility.

The structure shown in Fig. 1 suggests that the integral membrane subunits may behave like other integral membrane proteins of the yeast vacuole. The fact that the integral membrane subunits can reach the vacuole independently of assembly with the peripheral subunits also suggests that they are targeted as vacuolar membrane proteins. The transport of several integral membrane proteins, including dipeptidylaminopeptidase B (Roberts *et al.* 1989), alkaline phosphatase (Klionsky and Emr, 1989) and the vacuolar glycoprotein vgp72 (Nishikawa *et al.* 1990), to the vacuole has been well defined. All of these proteins follow the initial stages of the secretory pathway in their transit to the vacuole. They enter the endoplasmic reticulum and travel to the Golgi apparatus along with proteins destined for secretion and are separated from these proteins by some type of sorting step in the Golgi apparatus. If the integral membrane proteins of the vacuolar H^+-ATPase also follow this pathway, then the failure of the $100\times10^3 M_r$ subunit to reach the vacuole in the absence of the $17\times10^3 M_r$ subunit could be caused by a requirement for the V_o sector to assemble in the endoplasmic reticulum. A large number of membrane protein complexes that travel through the secretory pathway appear to assemble in the endoplasmic reticulum (Hurtley and Helenius, 1989). It is still very unclear at what point the peripheral subunits become associated with the integral membrane subunits, but the point of association between the two sectors of the ATPase could be very important in determining which organelles are acidified (see below).

The overall structural resemblance of the vacuolar H^+-ATPases to the F_1F_o-ATPases has frequently been noted and suggests that there might also be similarities in the assembly of the two types of complexes (Nelson and Taiz, 1989). There is evidence that the F_1 portion of both the *E. coli* and the mitochondrial F_1F_o-ATPase can assemble in the absence of the membrane sector (Klionsky and Simoni, 1985; Schatz, 1968). In *E. coli*, the F_o subunits also appear to be able to assemble in the absence of the F_1 subunits (Aris *et al.* 1985). There is evidence that the F_o and F_1 portions of the yeast enzyme each have associated assembly factors (Ackerman and Tzagoloff, 1990*a*,*b*). However, the membrane topology and organellar location of the V-type and F-type ATPases indicate that other aspects of their biogenesis may be quite different (Fig. 3). Subunits of the chloroplast or mitochondrial enzymes that are encoded by nuclear genes are synthesized in the cytoplasm with an appropriate organelle targeting signal and individually imported into the organelle, where they are assembled into the enzyme complex. In some cases, including the yeast mitochondrial F_1F_o-ATPase, assembly of the full complex also requires association of subunits encoded by the organellar genome with nuclearly encoded and imported subunits. Although assembly of the F_1F_o-ATPase is still under study, it is clear that subunit import may be an important regulatory step in the assembly pathway (Burns and Lewin, 1986). In this respect, the vacuolar H^+-ATPases of eukaryotic cells resemble the prokaryotic F_1F_o-ATPase more closely than they do the

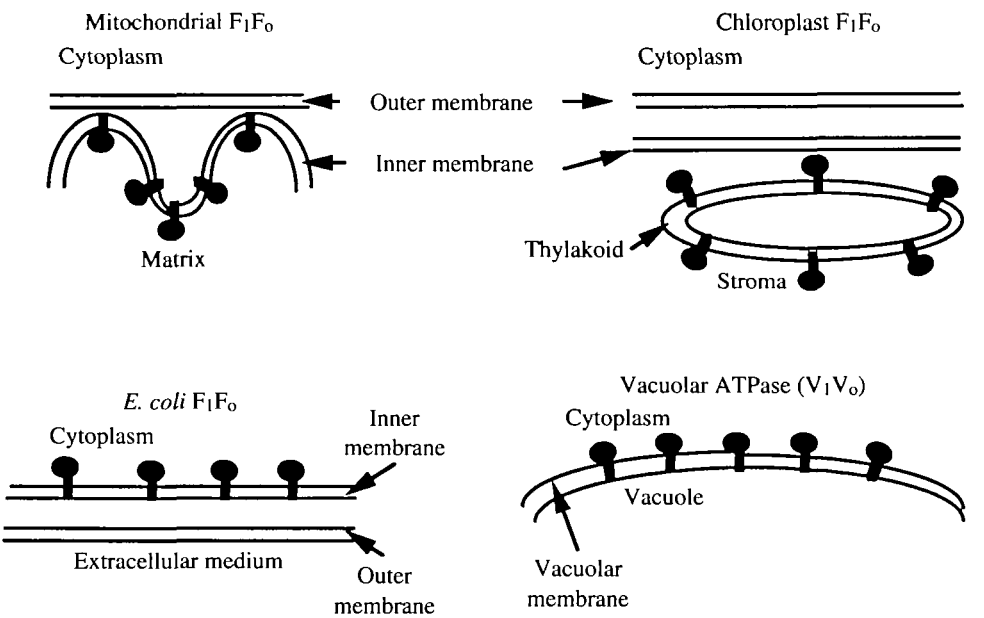

Fig. 3. Membrane topology and organelle targeting of the F-type and V-type ATPases. The topology of the ATPases in mitochondria, chloroplasts, bacteria and the yeast vacuole are compared. The F_1 subunits of the mitochondrial and chloroplast enzymes that are encoded by nuclear genes must be imported through at least two membranes following their synthesis on cytoplasmic ribosomes. In both the *E. coli* and the yeast vacuolar ATPases, the peripheral subunits end up facing the compartment where they are synthesized.

mitochondrial or chloroplast enzymes (see Fig. 3), because the subunits of the V_1 sector may be immediately available for assembly after synthesis.

Assembly of V-type ATPases and regulation of acidification

One of the central questions surrounding the acidification of intracellular compartments is the issue of how different organelles of the biosynthetic and endocytic pathways, including the lysosome (or vacuole), endosomes, the Golgi apparatus, clathrin-coated vesicles and secretory vesicles, can be acidified to different extents (Mellman *et al.* 1986). One means of regulating organelle pH is the regulation of other ion permeabilities of the organelle membrane (Fuchs *et al.* 1989). However, it is impossible to dissociate the question of how the V-ATPases are assembled in eukaryotic cells from questions about the distribution of acidic compartments and the regulation of acidification. Evidence that the V_1 and V_0 sectors of the yeast vacuolar H^+-ATPase may be assembled and targeted to the vacuole independently suggests that the stage at which the V_1 sector becomes attached to the V_0 sector could be an important factor in determining what intracellular compartments are acidified. For example, if V_1 attached to V_0 in the Golgi apparatus, then the active complex could travel from the Golgi apparatus to the vacuole and both compartments might be acidified to the extent of their occupancy by the vacuolar ATPase. Alternatively, identical V_1 sectors, assembled in the cytoplasm, could attach to multiple organelle-specific V_0 sectors, which regulate acidification of the compartments where they reside. The existence of two potential proteolipid genes (*VMA3* and *VMA11*; Umemoto *et al.* 1991) and two homologous genes that could encode $100\times10^3 M_r$ subunits (*VPH1* and *STV1*; Manolson *et al.* 1992) fits well into this model. (To date, only one gene for each of the peripheral subunits has been discovered in yeast.) It is clear that experiments aimed at understanding the assembly of vacuolar H^+-ATPase complexes in yeast and other eukaryotes may not only yield insights into the assembly of multisubunit complexes, but also have much broader implications for cell physiology.

This work was supported by a grant from the Howard Hughes Medical Institute to the College of William and Mary and a National Science Foundation Presidential Young Investigator Award to P.M.K.

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