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THE STRUCTURE AND FUNCTION OF THE FUNGAL V-ATPase

BY TOM H. STEVENS

Institute of Molecular Biology, University of Oregon, Eugene, OR 97403, USA

Introduction

The fungal vacuole is a prominent organelle that functions as a storage site for amino acids, Ca^{2+}, storage carbohydrates, inorganic phosphate and numerous hydrolases (Fig. 1) (Klionsky *et al.* 1990). A hallmark of fungal vacuoles is that they are acidic compartments involved in the turnover of cellular macromolecules. In the yeast *Saccharomyces cerevisiae*, the hydrolase activities in the vacuole are required for diploid cells to progress normally through sporulation and meiosis. The goal of this chapter is to present an overview of what is known about the enzyme complex responsible for acidifying the fungal vacuole and to summarize the current view of the function of acidification of the vacuolar network.

The vacuolar H^+-ATPase (V-ATPase) is responsible for acidification of the fungal vacuolar network (Klionsky *et al.* 1990; Raymond *et al.* 1992). This enzyme functions by coupling the hydrolysis of cytoplasmic ATP to the translocation of protons across the vacuolar membrane. The V-ATPase has been isolated from several fungi, and the enzymes isolated from *Neurospora crassa* and the yeast *Saccharomyces cerevisiae* have been extensively characterized. In all cases, the enzyme is a multisubunit complex of at least 500×10^3 relative molecular mass (M_r) (Hirata *et al.* 1989) composed of both membrane-bound polypeptides and peripherally associated subunits. Assigning functions to each of the polypeptides associated with the purified, enzymatically active V-ATPase complex remains a challenge for the future.

The molecular cloning of the genes encoding the V-ATPase subunits has provided an avenue for the detailed characterization of the structure and function of the various polypeptides. Subunit-encoding genes were first isolated and characterized for the *Neurospora crassa* V-ATPase 69 and 59×10^3 M_r polypeptides (Bowman *et al.* 1988*a*,*b*). These investigations were followed by the isolation of the genes for the corresponding yeast subunits (Shih *et al.* 1988; Nelson *et al.* 1989; Hirata *et al.* 1990; Yamashiro *et al.* 1990). The availability of the yeast genes provided an opportunity for the complete genetic dissection of the fungal V-ATPase subunit composition and function through the use of yeast mutants lacking individual subunits as a result of gene disruptions. These genetic analyses have allowed each biochemically defined subunit to be individually scrutinized for its *in vivo* requirement for function and assembly of the V-ATPase complex. These topics are considered in detail in this paper and the other papers of this fungal V-ATPase chapter.

Key words: vacuole, vacuolar ATPase, acidification, fungal vacuole.

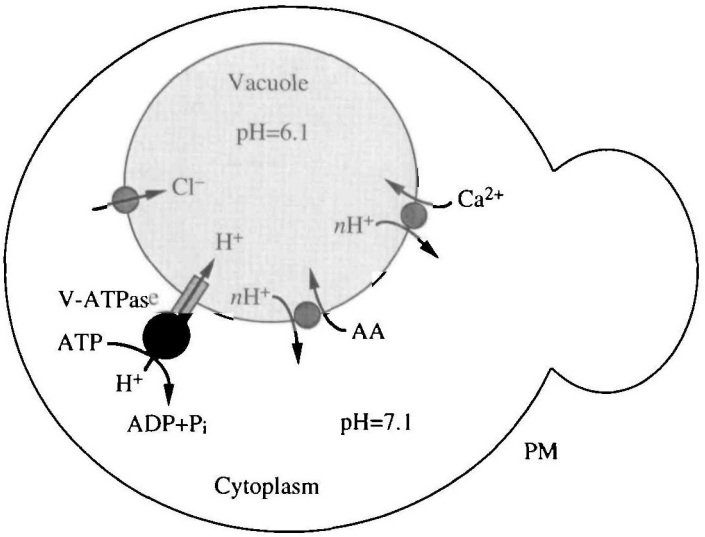

Fig. 1. The vacuole is an acidic storage compartment for amino acids, Ca^{2+} and other molecules. The vacuolar lumenal pH is approximately 6.1 and the cytoplasmic pH is about 7.1. The V-ATPase is represented by a ball and stalk. The chloride ion channel and the Ca^{2+} and amino acid (AA) antiporter proteins are represented by a circle in the vacuolar membrane, and the directions of ion and amino acid movements are indicated by arrows.

Subunit composition of the V-ATPase

The fungal V-ATPase is typically purified beginning with vacuolar membranes that have been separated from cytoplasmic proteins and other organelle membranes, and washed extensively to remove lumenal vacuolar hydrolases (Uchida *et al.* 1985). Following detergent solubilization of vacuolar membranes, most procedures require sedimentation of the membrane proteins through either a sucrose or a glycerol density gradient. These procedures take advantage of the large size of the V-ATPase complex relative to other vacuolar membrane proteins and the fact that this protein is rather abundant in vacuolar membranes. These biochemical separation procedures have been sufficient to define a number of polypeptides that co-purify with V-ATPase activity (Kane *et al.* 1989), and the V-ATPase complexes from *N. crassa* and yeast have been found to contain similar-sized putative subunits (Kane and Stevens, 1992; Bowman *et al.* 1992). The V-ATPase subunits from either fungal source behave as if they are associated with either an integral membrane sector (or V_o) or a peripheral membrane sector (or V_1).

The *N. crassa* V_o membrane sector is composed of 100, 40 and $16\times10^3 M_r$ polypeptides (Bowman *et al.* 1992), whereas the V_o portion of the yeast V-ATPase contains polypeptides of 100, 36, 17 and $16\times10^3 M_r$ (C. M. Bauerle, M. N. Ho, M. A. Lindorfer and T. H. Stevens, in preparation). There are also similarities in the polypeptides that form the V_1 peripheral membrane sector of these two enzymes. When the V_1 sector from *N. crassa* was dissociated from the vacuolar membrane, polypeptides of 69, 59, 48, 30 and $17\times10^3 M_r$ were identified (Bowman *et al.* 1989). By comparison, the peripheral membrane portion of the yeast V-ATPase includes polypeptides of 69, 60,

54, 42, 32 and $27\times10^3 M_r$ (Kane and Stevens, 1992; M. N. Ho, K. Hill, M. A. Lindorfer, and T. H. Stevens, in preparation; M. N. Ho, R. Hirata, N. Umemoto, Y. Ohya, T. H. Stevens and Y. Anraku, in preparation). With the exception of the biochemically well characterized 69 and $60\times10^3 M_r$ peripheral membrane subunits and the $16\times10^3 M_r$ V_o proteolipid, it is has been difficult to assess the requirement and/or function of the remaining polypeptides without the aid of either genetics or biochemical reconstitution. Genetic approaches have been pursued in both yeast and *Neurospora crassa* (see Bowman *et al.* 1992).

Genetic analysis of the V-ATPase has proceeded rapidly in yeast. The first efforts focused on the cloning and disruption of the $60\times10^3 M_r$ subunit (Nelson *et al.* 1989; Nelson and Nelson, 1990; Yamashiro *et al.* 1990). These studies indicated that the $60\times10^3 M_r$ subunit was required for V-ATPase function and that the V-ATPase was required for normal growth rates and for growth over a wide pH range. In particular, yeast cells lacking the $60\times10^3 M_r$ subunit grew about 50% slower than the wild-type cells under optimal conditions, failed to grow at neutral pH, and the vacuoles of the mutants had a neutral pH (Yamashiro *et al.* 1990). Subsequent genetic analysis indicated that V-ATPase mutants failed to grow on non-fermentable carbon sources (Foury, 1990), were sensitive to elevated Ca^{2+} levels in the growth medium (Ohya *et al.* 1991), and contained elevated cytoplasmic Ca^{2+} levels (Ohya *et al.* 1991). Despite all of the physiological effects resulting from loss of the V-ATPase, it has become clear that yeast cells can grow without the V-ATPase and very likely without acidification of the vacuolar network.

Two approaches in yeast have yielded a large number of genes that effect the expression of the V-ATPase. The first was a reverse genetic approach, which involved the biochemical isolation of V-ATPase subunits, determination of peptide sequence by micro-sequencing, and cloning of the gene using degenerate oligonucleotides. This approach has yielded the *VMA2*, *VMA3* and *VMA6* genes, which encode the 60, 16 and $36\times10^3 M_r$ V-ATPase subunits (see Table 1), respectively. The second approach used genetic screens to isolate yeast mutants that exhibited phenotypes predicted for cells lacking a functional V-ATPase, such as a failure to acidify the vacuole (Preston *et al.* 1989; Manolson *et al.* 1992), sensitivity to Ca^{2+} (Ohya *et al.* 1991), failure to grow at neutral pH (M. N. Ho, K. Hill, M. A. Lindorfer and T. H. Stevens, in preparation), as well as sensitivity to the drug trifluoperazine (Shih *et al.* 1988, 1990). These approaches yielded the *TFP1* (also isolated as *VMA1*), *VPH1*, *VMA13*, *VMA5* and *VMA11* (also isolated as *TFP3*) genes, which encode the 69, 100, 54, 42 and $17\times10^3 M_r$ V-ATPase subunits (Table 1), respectively. These approaches also yielded a number of genes that encode polypeptides required for V-ATPase assembly or function; however, these polypeptides may not be actual subunits of the V-ATPase enzyme complex (Ohya *et al.* 1991; R. Hirata, N. Umemoto, M. N. Ho, Y. Ohya. T. H. Stevens and Y. Anraku, in preparation; M. N. Ho, K. Hill, M. A. Lindorfer and T. H. Stevens, in preparation). Table 1 summarizes the genes and polypeptides required for yeast V-ATPase function, which have been identified either genetically or biochemically.

Assembly of the V-ATPase

Evidence that the fungal V-ATPase contains integral membrane (V_o) and peripheral

Table 1. *Summary of genes and polypeptides required for yeast V-ATPase function*

Gene	Molecular mass: Calculated (kDa)[a]	Molecular mass: $10\times^{-3}$ apparent M_r[b]	Component of purified V-ATPase[c]
VPH1[d]	95	95–100	Yes
VMA1[e]/*TFP1*[f]	68	69	Yes
VMA2[g]/*VAT2*[h]	58	60	Yes
VMA3[i]	16	17	Yes
VMA4[j]	27	27	Yes
VMA5[k]	42	42	Yes
VMA6[l]	36	36	Yes
–[m]	–	32	Yes
VMA11[n]/*TFP3*[o]	17	?	?
VMA12[p]	25.3	25	No
VMA13[q]	54	54	Yes
VMA21[r]	8.4	?	?
VMA22,23[r]	?	?	?

[a]Molecular mass was calculated from the deduced amino acid sequence.
[b]Relative molecular mass estimated from SDS–polyacrylamide gels.
[c]Kane *et al.* (1989); M. N. Ho, R. Hirata, Y. Ohya, N. Umemoto, T. H. Stevens and Y. Anraku (in preparation).
[d]Manolson *et al.* (1992).
[e]Hirata *et al.* (1990).
[f]Shih *et al.* (1988); Kane *et al.* (1990).
[g]Anraku *et al.* (1991).
[h]Nelson *et al.* (1989); Yamashiro *et al.* (1990).
[i]Nelson and Nelson (1989); Umemoto *et al.* (1990); Anraku *et al.* (1992).
[j]Foury (1990).
[k]Beltrán *et al.* (1992); M. N. Ho, R. Hirata, Y. Ohya, N. Umemoto, T. H. Stevens and Y. Anraku (in preparation).
[l]C. M. Bauerle, M. N. Ho, M. A. Lindorfer and T. H. Stevens (in preparation).
[m]No gene has been isolated for the 32 kDa V-ATPase subunit.
[n]Umemoto *et al.* (1991).
[o]Shih *et al.* (1990).
[p]R. Hirata, N. Umemoto, M. N. Ho, Y. Ohya, T. H. Stevens and Y. Anraku (in preparation).
[q]M. N. Ho, R. Hirata, Y. Ohya, N. Umemoto, T. H. Stevens and Y. Anraku (in preparation).
[r]K. Hill, and T. H. Stevens (unpublished results).

membrane (V_1) sectors comes from structural, biochemical and genetic investigations. The V_1 polypeptides were shown to be stripped from vacuolar vesicles by procedures involving either washing the membranes with 50–100 mmol l^{-1} KNO_3 in the presence of MgATP (Bowman *et al.* 1989; Kane *et al.* 1989) or cold-inactivation in the presence of MgATP (Beltrán *et al.* 1992). Whether the vacuolar membranes were from *N. crassa* or yeast, a similar set of polypeptides was removed from the membranes by these treatments. Interestingly, the nitrate treatment of *N. crassa* vacuolar membranes not only removed the peripherally associated V-ATPase subunits, but the ball and stalk structures (V_1 complex) that could be visualized by electron microscopy were correspondingly stripped off the membrane by this treatment (Bowman *et al.* 1989, 1992). In an elegant

combination of biochemical and structural approaches, Bowman *et al.* (1992) found that the V_1 complex stripped from vacuolar membranes retained most of the structural features seen when the V_1 sector was attached to the vacuolar membrane. These studies lend solid support to the model that the V_1 and V_o sectors form distinct subcomplexes within the V-ATPase.

Additional support for the two-sector model comes from studies of the state of assembly of the yeast V-ATPase complex in mutants lacking individual subunits of the enzyme. In *vma* mutants lacking an individual subunit of the V_1 sector (69, 60, 42 or $27\times10^3 M_r$), subcellular fractionation and immunolocalization studies indicate that there is a complete failure to assemble any of the V_1 subunits onto the vacuolar membrane (M. N. Ho, K. Hill, M. A. Lindorfer and T. H. Stevens, in preparation; Kane *et al.* 1992; reviewed in Kane and Stevens, 1992). Interestingly, these polypeptides are stable in the cytoplasm of these *vma* mutant cells, but they do not associate with the vacuole. In contrast, *vma* mutants lacking a V_1 subunit localize the 100, 36 and $16\times10^3 M_r$ polypeptides to the vacuolar membrane. These V_o subunits are stable in cells lacking a V_1 subunit, presumably because they are assembled into a V_o complex independently of the V_1 complex.

The state of V-ATPase assembly has also been investigated in mutants lacking components of the V_o sector. Yeast *vma* mutants lacking either the 16 or $36\times10^3 M_r$ subunits (*vma3* or *vma6* mutants, respectively) contained wild-type levels of the V_1 polypeptides, but these V_1 components did not associate with the vacuolar membrane (Noumi *et al.* 1991; Umemoto *et al.* 1991; C. M. Bauerle, M. N. Ho, M. A. Lindorfer and T. H. Stevens, in preparation). These studies also found that the absence of a V_o subunit destabilized the remainder of the V_o polypeptides, suggesting that all of the membrane components are required for the stability of the V_o sector. Interestingly, in the absence of a V_o subunit (*vma3* mutant) the V_1 polypeptides appear to be assembled into a subcomplex of approximately $400\times10^3 M_r$ (Kane, 1992).

An exception to the behavior of cells lacking V_1 sector polypeptides is the yeast mutant lacking the $54\times10^3 M_r$ subunit encoded by the *VMA13* gene (M. N. Ho, R. Hirata, N. Umemoto, Y. Ohya, T. H. Stevens and Y. Anraku, in preparation). *vma13* mutants appear to contain normal levels of all V-ATPase subunits that can be monitored (100, 69, 60, 42, 36, 27 and $16\times10^3 M_r$ subunits) and assemble all of the remaining V_1 polypeptides onto the vacuolar membrane. Whereas the V-ATPase complex appears to assemble in the absence of the $54\times10^3 M_r$ subunit, it is much less stably associated with the vacuolar membrane than wild-type V-ATPase. The role of this VMA13 protein in the function of the yeast V-ATPase has yet to be determined.

Other *VMA* genes have been identified that are required for the function of the yeast V-ATPase (Ohya *et al.* 1991; M. N. Ho, K. Hill, M. A. Lindorfer and T. H. Stevens, in preparation). Whereas little is known about the *VMA22* or *VMA23* genes (Table 1), the *VMA21* gene is predicted to encode a hydrophobic 77 amino acid protein (K. Hill and T. H. Stevens, unpublished data). More is known about the *VMA12* gene and its encoded product (R. Hirata, N. Umemoto, M. N. Ho, Y. Ohya, T. H. Stevens and Y. Anraku, in preparation). The *VMA12* gene encodes a $25\times10^3 M_r$ polypeptide, which is predicted to have two membrane-spanning domains. Yeast cells lacking the VMA12 protein behave

like V_o sector mutants, in that the V_o subunits are destabilized and the V_1 polypeptides fail to associate with the vacuolar membrane. Interestingly, whereas the VMA12 protein is associated with the vacuolar membrane, it does not co-purify with the detergent-solubilized V-ATPase complex, indicating that this polypeptide is not required for ATP hydrolysis and probably is not a subunit of the active ATPase. In summary, these genetic approaches have successfully identified a large number of yeast V-ATPase subunits as well as at least one factor required for V-ATPase function that is not a subunit of the complex.

Function of the V-ATPase

The yeast *S. cerevisiae* is the only organism for which mutants completely lacking the V-ATPase have been reported. Thus, our knowledge of the *in vivo* function of the fungal V-ATPase is limited to yeast, an organism that survives without a V-ATPase. With this limitation in mind, this section will provide a brief summary of the function of the yeast V-ATPase.

Yeast *vma* mutants lacking the V-ATPase have been shown to have no measurable ATPase activity associated with their vacuoles and seem to lack any obvious alternative means to pump protons across the vacuolar membrane. *In vivo* measurements of the vacuolar lumenal pH yield a value of 7.1 (as opposed to a pH of 6.1 for wild-type vacuoles) (Preston *et al.* 1989; Yamashiro *et al.* 1990), indicative of a complete collapse of the pH gradient across the vacuolar membrane. These studies have been interpreted to indicate that the V-ATPase is solely responsible for pumping protons into the vacuole, and that yeast cells survive without an acidified vacuole (Yamashiro *et al.* 1990). An alternative model has been proposed by Nelson and Nelson (1990), in which acidification of the yeast vacuole is required for yeast cell viability, and that *vma* mutant cells acidify their vacuoles by delivering protons to the vacuole by fluid-phase endocytosis. This model was proposed to explain the pH-sensitive growth phenotype of yeast *vma* mutants. Thus far, there has been no experimental test of this alternative model.

Anraku and colleagues (Anraku *et al.* 1992*a*,*b*) have shown that vacuoles isolated from *vma* mutants failed to accumulate Ca^{2+} and basic amino acids in an ATP-dependent manner (Ohya *et al.* 1986, 1991). Because wild-type vacuoles are very efficient at this ATP-dependent uptake (Ohsumi and Anraku, 1981, 1983; Sato *et al.* 1984*a*,*b*; Kitamoto *et al.* 1988), these results have led to the suggestion that *vma* mutant vacuoles are incapable of Ca^{2+} and amino acid storage *in vivo* (Anraku *et al.* 1992*a*). This suggestion has gained experimental support from the observation that cytosolic Ca^{2+} concentration is elevated approximately sixfold in *vma* mutant cells (Ohya *et al.* 1991).

The role of acidification by the V-ATPase in membrane traffic and protein sorting has also been investigated. Studies on the uptake of the yeast peptide pheromone α-factor by receptor-mediated endocytosis indicate that delivery of α-factor to the vacuole, and its proteolytic degradation in this compartment are normal in yeast cells defective for acidification (Singer and Riezman, 1990). These experiments suggest that acidification of the yeast vacuolar network is not required for endocytic traffic from the plasma membrane to the vacuole.

The delivery of newly synthesized proteases to the vacuole is effected in yeast mutants

lacking the V-ATPase. Early studies demonstrated that two soluble vacuolar hydrolases were sorted to the vacuole with 75–80% efficiency (Yamashiro *et al.* 1990), indicating that *vma* cells sort vacuolar proteins much more efficiently than mutants isolated as deficient in vacuolar protein sorting (*vps* mutants; Raymond *et al.* 1992). In addition, the vacuolar membrane protein alkaline phosphatase was found to be present only in the vacuolar membrane in *vma* mutants, when investigated by indirect immunofluorescence (Yamashiro *et al.* 1990). Kinetic and steady-state experiments indicated that the vacuolar forms of the vacuolar proteases were proteolytically activated in *vma* vacuoles (Yamashiro *et al.* 1990).

A more recent investigation of vacuolar protein sorting in *vma* mutants suggested that the defects in vacuolar protein sorting may be more severe than originally reported (Klionsky *et al.* 1992*a*, *b*). These investigators observed a similar degree of missorting of soluble vacuolar hydrolases into the surrounding medium as had been reported previously, but detected a higher level of the intracellular precursor forms of both soluble and integral membrane vacuolar hydrolases. However, the wild-type strain used in the recent investigations exhibited a three- to fourfold slower processing rate for vacuolar hydrolase precursors (Klionsky *et al.* 1992*a*). In addition, subcellular fractionation studies were not conducted to determine whether the intracellular precursors of endogenous vacuolar hydrolases were in fact accumulating in a non-vacuolar organelle in *vma* cells. Therefore, it remains possible that other than secretion of 20–25% of newly synthesized soluble vacuolar hydrolases, *vma* mutant cells exhibit only a kinetic delay in the processing of hydrolase precursors (for further discussion see Klionsky *et al.* 1992).

Conclusions and perspectives

There are a number of important conclusions about the fungal V-ATPase that can be reached with our current level of knowledge. The V-ATPase is structurally very similar to the F_1F_o-ATPase. The V-ATPase is composed of a V_o and a V_1 portion, and each of these sectors appears to be capable of assembling into a sub-complex when the other sector is absent. The yeast V-ATPase is composed of at least ten subunits, and genetic approaches have identified at least four additional factors that constitute either novel subunits or factors required for assembly, targeting and/or regulation of the V-ATPase. Finally, the yeast V-ATPase is absolutely required for acidification of the vacuole, and acidification of the vacuolar network is necessary for efficient vacuolar protein sorting.

Many questions remain to be answered regarding the fungal V-ATPase. The mechanism of assembly and targeting of the V-ATPase to the vacuolar membrane is unknown, as is the subcellular distribution of the V-ATPase through the endomembrane system (endoplasmic reticulum, Golgi complex, endosomes, etc.). Much has yet to be learned about the roles of the various V-ATPase subunits in ATP hydrolysis and proton pumping. There are also a large number of structural issues relating to this multisubunit enzyme that have yet to be addressed. The complex physiological defects associated with loss of the V-ATPase are almost a complete mystery; in particular, why are yeast *vma* mutants incapable of growth on non-fermentable carbon sources? The proteins that function together with the fungal V-ATPase to maintain a vacuolar membrane pH

gradient (such as a Cl^- channel; Wada *et al.* 1992) have yet to be well characterized. A number of the issues raised here are addressed in the five papers that follow in this fungal V-ATPase chapter.

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