Journal of Bioenergetics and Biomembranes, Vol. 24, No. 4, 1992

The Vacuolar ATPase of *Neurospora crassa*

B. J. Bowman,[1] N. Vázquez-Laslop,[1] and E. J. Bowman[1]

Received February 16, 1992; accepted March 16, 1992

The filamentous fungus *Neurospora crassa* has many small vacuoles which, like mammalian lysosomes, contain hydrolytic enzymes. They also store large amounts of phosphate and basic amino acids. To generate an acidic interior and to drive the transport of small molecules, the vacuolar membranes are densely studded with a proton-pumping ATPase. The vacuolar ATPase is a large enzyme, composed of 8–10 subunits. These subunits are arranged into two sectors, a complex of peripheral subunits called V_1 and an integral membrane complex called V_0. Genes encoding three of the subunits have been isolated. *vma-1* and *vma-2* encode polypeptides homologous to the α and β subunits of F-type ATPases. These subunits appear to contain the sites of ATP binding and hydrolysis. *vma-3* encodes a highly hydrophobic polypeptide homologous to the proteolipid subunit of vacuolar ATPases from other organisms. This subunit may form part of the proton-containing pathway through the membrane. We have examined the structures of the genes and attempted to inactivate them.

KEY WORDS: ATPase; vacuole; vacuolar ATPase; fungi.

THE *Neurospora* VACUOLE

As a filamentous fungus, *Neurospora crassa* has unique features which make it a good model system for investigating the vacuolar ATPase. The organism is not "cellular" in the usual sense of the word, but grows as branched filaments, partitioned into multinucleate compartments by incomplete cross-walls. The cells can be grown in logarithmic culture with a doubling time of 2.5 h and harvested in large quantities simply by pouring through cheesecloth. The filaments are easily broken by vigorous mixing with glass beads, a procedure that leaves most of the organelles intact (Cramer *et al.*, 1983; Bowman and Bowman, 1988).

During exponential growth the filaments contain many small vacuoles, about the size of mitochondria. In size, structure, and composition the vacuoles resemble the lysosomes of animal cells. The vacuoles contain acid hydrolases such as proteases, α-mannosidase, and phosphatase (Vaughn and Davis, 1981; Bowman and Bowman, 1982; Klionsky *et al.*, 1990). The internal pH of the vacuole is about 6.0, as estimated by NMR analysis of vacuolar amino acids (Legerton *et al.*, 1983). In two respects, the *Neurospora* vacuoles also resemble chromaffin granules from animal cells. These secretory vesicles serve as storage compartments for phosphate (as ATP) and amines (as catecholamines) (Njus *et al.*, 1981). The vacuoles of *N. crassa* contain high levels of phosphate, in the form of long-chain polyphosphates; and they contain high levels of amines, primarily arginine, ornithine, and lysine (Cramer *et al.*, 1980). Because of their contents the vacuoles are very dense and can easily be separated from mitochondria and other organelles (Cramer *et al.*, 1983).

The vacuolar membranes contain an ATPase that appears to have two major functions. First, the ATPase pumps protons into the organelle, maintaining an acidic internal pH. Second, the ATPase generates an electrochemical gradient that drives the transport of small molecules across the membrane (Zerez *et al.*, 1986). Indeed, the molecular traffic across the mem-

[1]Department of Biology, Sinsheimer Laboratories, University of California, Santa Cruz, California 95064.

0145-479X/92/0800-0361$06.50/0

brane can be quite high. For example, the flux of ornithine across the vacuolar membrane during exponential growth is eightfold higher than the net rate of ornithine accumulation in the vacuole (Bowman and Davis, 1977a, b). The vacuolar ATPase also appears to have an important role in regulating cellular Ca^{+2} levels (Cornelius and Nakashima, 1987; Ohya *et al.*, 1991). These high rates of molecular traffic may explain why the vacuolar ATPase is such a prominent component of the vacuolar membrane.

Several lines of evidence suggest that the vacuolar ATPase is present at high levels in the membranes of *N. crassa*. The specific ATPase activity of isolated vacuolar membranes is approximately 4 μmol/min/mg of protein at 30°C. This is close to the specific ATPase activity of mitochondrial membranes, in which the F-type ATPase is estimated to make up 10–20% of the membrane protein (Sebald and Wild, 1979). If the intrinsic turnover numbers of the vacuolar and mitochondrial ATPases are similar, then the vacuolar enzyme would also comprise 10% or more of the vacuolar membrane protein. In fact, scanning densitometry of membranes separated on SDS polyacrylamide gels has shown that the 67-kDa and 57-kDa polypeptides of the vacuolar ATPase each make up approximately 5% of the stained membrane protein (Bowman *et al.*, 1986; B. Bowman, unpublished data).

Electron micrographs of negatively stained vacuolar membranes provide graphic evidence of the density of vacuolar ATPases (Fig. 1) (Bowman *et al.*, 1989). The membranes are thickly studded with ATPases, which appear as "ball and stalk" structures. The vacuolar enzyme resembles the F_0F_1-ATPase on mitochondrial membranes, but is 25–30% larger in the cross-sectional diameter of the peripheral head. In some regions of the membrane the vacuolar ATPases appear closely packed, but other regions have no visible ATPases. While this mixture of densely packed and no ATPases may represent the distribution of the enzyme *in vivo*, it seems equally likely that the gaps are an artifact of the preparation.

ENZYMATIC PROPERTIES OF THE *N. crassa* VACUOLAR ATPase

To examine the ATPase on vacuolar membranes it is necessary to lyse the vacuoles and separate the membranes from the internal vacuolar enzymes. Whole vacuole preparations are difficult to assay because vacuolar proteases degrade the ATPase, and

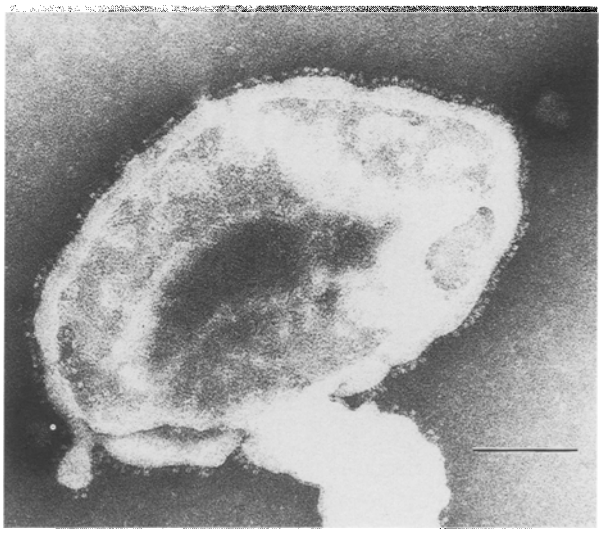

Fig. 1. Electron micrograph of a vacuolar membrane vesicle. Vacuolar membranes were prepared and stained with phosphotungstic acid as described (Bowman *et al.*, 1989). The vacuolar ATPases are visible as projections on the edge of the membrane. The bar in the lower right corner represents a distance of 100 nm.

vacuolar phosphatases hydrolyze ATP at high rates. Vacuolar membranes, which have been washed and resuspended in 1 mM EGTA or EDTA, have high levels of ATPase activity that is stable for at least 12 h at 4°C and for many months at − 70°C (Bowman and Bowman, 1988).

The vacuolar ATPase can be distinguished from other ATPases in the preparation by its insensitivity to the inhibitors azide and vanadate (Bowman and Bowman, 1986) and by its unique inhibition by nanomolar concentrations of the antibiotic bafilomycin (E. Bowman *et al.*, 1988b). A small portion of the ATP hydrolysis catalyzed by vacuolar membranes (0.1–0.3 μmol/min/mg of protein) is inhibited by vanadate. This is apparently due to a vanadate-sensitive phosphatase associated with the membranes rather than to a contaminating P-type ATPase (Bowman and Bowman, 1982).

The *N. crassa* vacuolar ATPase has a K_m for ATP of 0.2 mM. It hydrolyzes GTP and ITP at approximately 30% of the rate obtained with ATP. Mg^{+2} or Mn^{+2} is required at concentrations equimolar to ATP. CaATP is also hydrolyzed, but again at approximately 30% of the rate seen with MgATP. The addition of monovalent salts has little effect on the rate of ATP hydrolysis. KCl or NH_4Cl (10–50 mM) stimulate activity by 20–25% (Bowman and Bowman, 1982; Bowman, 1983). In these characteristics the vacuolar

ATPase resembles the *N. crassa* mitochondrial ATPase (Mainzer and Slayman, 1978) and differs from the plasma membrane ATPase, which has a higher K_m for ATP, is quite specific for MgATP, and shows a somewhat greater stimulation by monovalent salts (Bowman and Slayman, 1977). The reaction mechanism of the vacuolar ATPase is probably very similar to that of F-type ATPases. For the F-type enzyme extensive experimental data support a model in which ATP binding occurs on at least two identical catalytic subunits (Grubmeyer and Penefsky, 1981; Gresser *et al.*, 1982). In one catalytic site ATP undergoes reversible cleavage to bound ADP and P_i until binding at another site causes release of the products. The reversible cleavage at the catalytic site can be demonstrated by measuring the exchange of ^{18}O between P_i and H_2O. The model predicts that as the concentration of ATP is lowered, the time of occupancy in the catalytic site, and thus the number of reversible cleavages, will increase. Experiments by Kasho and Boyer (1989), using vacuolar ATPases from *N. crassa* and *S. cerevisiae*, gave results similar to those obtained with F-type ATPases. Thus, kinetic data, like the structural data described below, suggest that cooperativity between subunits is an integral part of the mechanism of the vacuolar ATPase.

STRUCTURE OF THE VACUOLAR ATPase

An early measurement of the size of the enzyme came from radiation inactivation experiments in which the relative size of several ATPases was assessed (Bowman *et al.*, 1986). The data indicated that the functional unit required for ATPase activity was approximately 15% larger than for the mitochondrial ATPase. Thus, the minimal size of the vacuolar ATPase was predicted to be 500 kDa.

Purification. Purification of the vacuolar ATPase has shown it to be a large complex enzyme. The best preparations of the *N. crassa* enzyme have come from a procedure using two sequential detergent solubilizations (Bowman *et al.*, 1986). Vacuolar membranes are first suspended in 0.4% Triton X-100, pelleted, and then resuspended in 0.4% Zwittergent 3-16 (*N*-hexadecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate). The supernatant from the second detergent treatment is fractionated by centrifugation on a sucrose density gradient. The ATPase activity penetrates deeply into the gradient and copurifies with two abundant polypeptides of approximately 70 and 60 kDa (Fig. 2). These two subunits, named A and B, form the catalytic core of the ATPase, as shown by inhibitor-binding studies and sequence homologies discussed below.

A persistent difficulty with this purification procedure is the lower-than-expected recovery of ATPase activity. On the basis of purification of A and B subunits, the activity of the purified gradient fractions should be 5- to 10-fold higher than the initial membranes. Instead, the purified ATPase typically has approximately the same activity as the initial membranes, 3–5 μmol/min/mg of protein. A likely explanation for this problem is that subunits required for activity are dissociated from the complex during purification.

Vacuolar ATPases from other organisms appear to have 8–10 different subunits (Forgac, 1989). As seen in Fig. 2, purified ATPase from *N. crassa* may also have 8–10 subunits. In addition to the A and B subunits, polypeptides of 100, 51, 48, 40, 30 kDa, and a broad band at 16 kDa copurify with activity. However, the relative amounts of some of these polypeptides varies in different preparations. Whether all of these are *bona fide* subunits of the ATPase is not yet certain.

The V_1 Sector. To further examine the structure of the enzyme, we have separated it into two sectors, a complex of peripheral polypeptides and a complex of integral membrane subunits. By analogy to the terminology used for the F-type ATPase, we refer to these as the V_1 and V_0 sectors. The V_1 sector can be released from the membrane by incubation in 50–100 mM KNO_3 or KSCN together with 5 mM ATP or ADP (Bowman *et al.*, 1989). The A and B subunits and four additional polypeptides (51, 48, 30, and 17 kDa) are solubilized by this treatment. These peripheral subunits stay together as a complex of approximately 450 kDa. In electron micrographs KNO_3-treated vacuolar membranes are devoid of ball and stalk structures. The solubilized V_1 complex appears in the electron microscope as a roughly spherical 12-nm structure, significantly larger than the 9-nm F_1 ATPase from mitochondria (Dschida and B. Bowman, unpublished results).

Interestingly, the release of the V_1 sector appears to require that nucleotide be bound to the catalytic site(s) on the enzyme. For instance, two inhibitors, *N*-ethylmaleimide and 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole, which are believed to bind to the catalytic site, block the release. However, two findings suggest that catalysis *per se* is not required for the release: (1)

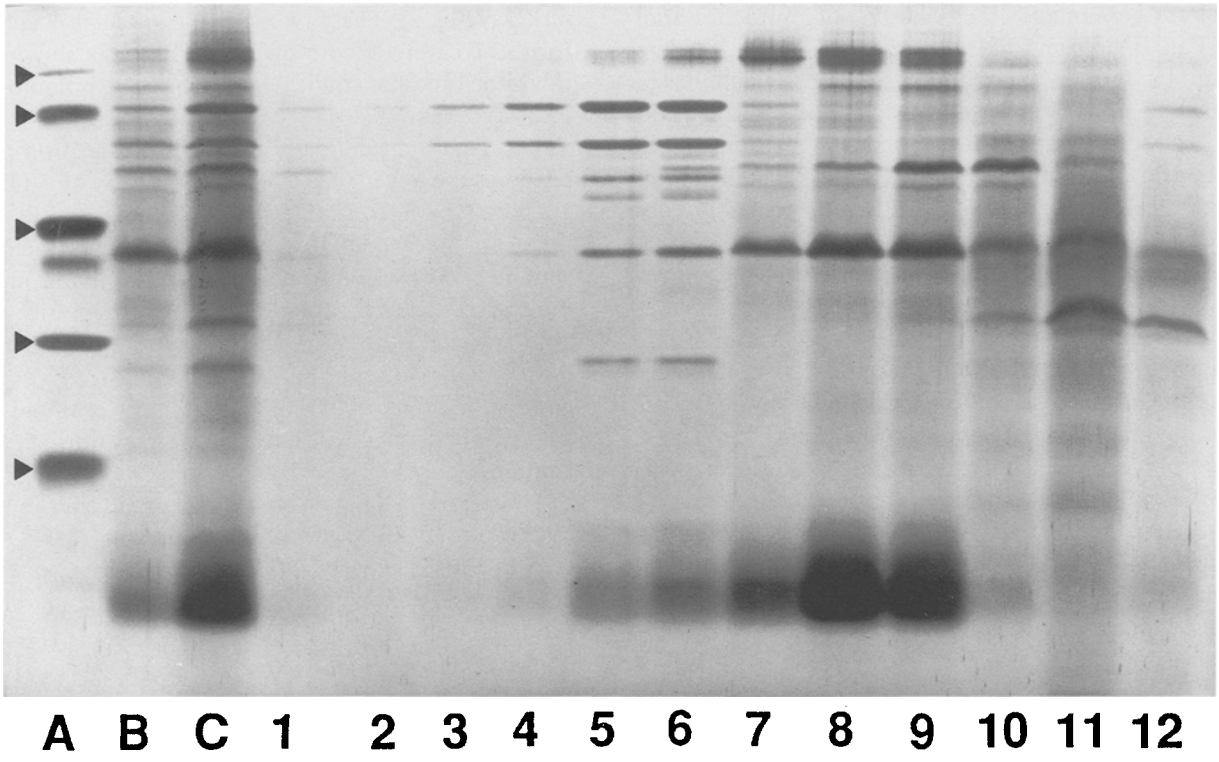

Fig. 2. Purification of the vacuolar ATPase. The vacuolar ATPase was purified by detergent solubilization and sucrose density gradient centrifugation as described (Bowman *et al.*, 1986). Aliquots from the gradient were analyzed by electrophoresis through 12% polyacrylamide gels containing sodium dodecyl sulfate. The gel was stained with silver. Lane A contains molecular weight standards (marked by arrows) of 97, 66, 43, 31, and 22 kDa; lane B, 4 μg of vacuolar membrane protein; lane C, 10 μg of detergent-solubilized vacuolar membrane protein. Lanes 1–12 are fractions from the bottom to the top of the gradient. Note the prominent bands at approximately 70 and 60 kDa in lanes 5 and 6. These two fractions contained 83% of the ATPase activity in the gradient.

inhibition by DCCD, which presumably binds in the membrane sector, does not block release, and (2) ADP is as effective as ATP in promoting release. Thus, NO_3 appears to act on a specific conformation of the ATPase (Bowman *et al.*, 1989). At present there are virtually no data to address the question of whether the V_1 sector can reversibly bind the membrane *in vivo*, but one could speculate that proton pumping across the vacuolar membrane might be regulated in this way.

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                              * *** ******  *** ** * *****
N.c.                          MTAFIKQEAEEK  EIQIKADELFEIEK
S.c.     MSSAITALTPNQVNDELNKMQAFIRKEREEKAKEIQLKAEQEEEIER
bovine       MALSDADVQKQIKHMMAFIEQEANEKAEEIDAKAEEEFNIEK
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Fig. 3. Comparison of peptides from *N. crassa* with the sequence of the 31-kDa subunit of yeast and bovine vacuolar ATPases. The sequences shown are tryptic peptides from the 30-kDa subunit of *N. crassa*, the first 47 residues of the *S. cerevisiae* subunit (Foury, 1990), and the first 42 residues of the bovine subunit (Hirsch *et al.*, 1988). Residues in the *N. crassa* peptides identical to those in the *S. cerevisiae* or bovine subunits are marked with an asterisk.

Of the six polypeptides released with the V_1 sector of *N. crassa*, three can be identified as homologs of vacuolar ATPase subunits in other organisms. A and B subunits are ubiquitous in vacuolar ATPase preparations (reviewed in Forgac, 1990) and show approximately 60–70% amino acid identity in pairwise combinations wherever sequence data are available (e.g., E. Bowman *et al.*, 1988a; Manolson *et al.*, 1988). In addition to the A and B subunits, the 30-kDa polypeptide appears to be the *N. crassa* equivalent of the 31-kDa *Vma-4* gene product of *S. cerevisiae* (Foury, 1990) and of bovine kidney vacuolar ATPase (Hirsch *et al.*, 1988). Although the sequence of this subunit is not highly conserved, two tryptic peptides from the *N. crassa* protein closely match regions of the yeast and bovine proteins (Fig. 3).

Among the other three polypeptides associated with the V_1 sector, analyses of tryptic peptide sequences suggest that two (48 and 17 kDa) may be authentic constituents and one (51 kDa) is likely a proteolytic

fragment (B. Bowman, unpublished data). The 48-kDa polypeptide is always present, although the amount varies. Sequences of tryptic peptides from this protein indicate it is not derived from the A or B subunits, nor do the sequences match any region of the 116-kDa polypeptide from the bovine brain vacuolar ATPase (Perin *et al.*, 1991). Analysis of tryptic peptides from the 17-kDa polypeptide, which is seen easily only when large amounts of V_1 protein are analyzed on acrylamide gels, shows that it is not a fragment of the A or B subunits and that it is not similar to any other vacuolar ATPase subunits for which the sequence has been published. The 51-kDa polypeptide is the most variable and appears to react with antibody raised to the B subunit, suggesting that it is a proteolytic fragment.

The V_0 sector. The membrane sector of the vacuolar ATPase, V_0, appears to have three or four different types of subunits. As seen in Fig. 2, polypeptides of approximately 100 and 40 kDa plus a broad band at 16 kDa are seen in the fractions with highest ATPase activity. These polypeptides remain with the membrane fraction after extraction of the peripheral subunits of the ATPase (Fig. 4, see below). Figure 2 also illustrates another characteristic of the *N. crassa* vacuolar ATPase in these purification experiments—namely, that the V_0 subunits do not exactly copurify with the V_1 components. The 100-, 40-, and 16-kDa bands peak at a lighter density.

The observation that V_1 and V_0 subunits are partially separated on these density gradients could be explained in either of two ways. Vacuolar membranes *in vivo* may have an excess of V_0 components. However, analysis of total vacuolar membrane proteins by SDS-gel electrophoresis (lane 1 in Figs. 2 and 4) does not reveal a great abundance of the V_0 polypeptides. Alternatively, V_0 and V_1 sectors may disassociate during purification, an explanation consistent with the loss of ATPase activity during gradient purification. In fact, the *N. crassa* vacuolar ATPase seems to be particularly easy to disassociate. The concentration of KNO_3, for example, required to release the peripheral subunits (Bowman *et al.*, 1989) is significantly lower than that used for vacuolar ATPases from other organisms (Rea *et al.*, 1987; Arai *et al.*, 1989).

The relative amounts of 100-, 40-, and 16-kDa polypeptides appear to be the same in all fractions obtained from gradient purification, suggesting that these proteins stay together as a complex. Further support for a discrete V_0 complex comes from an experiment using vacuolar membranes stripped of

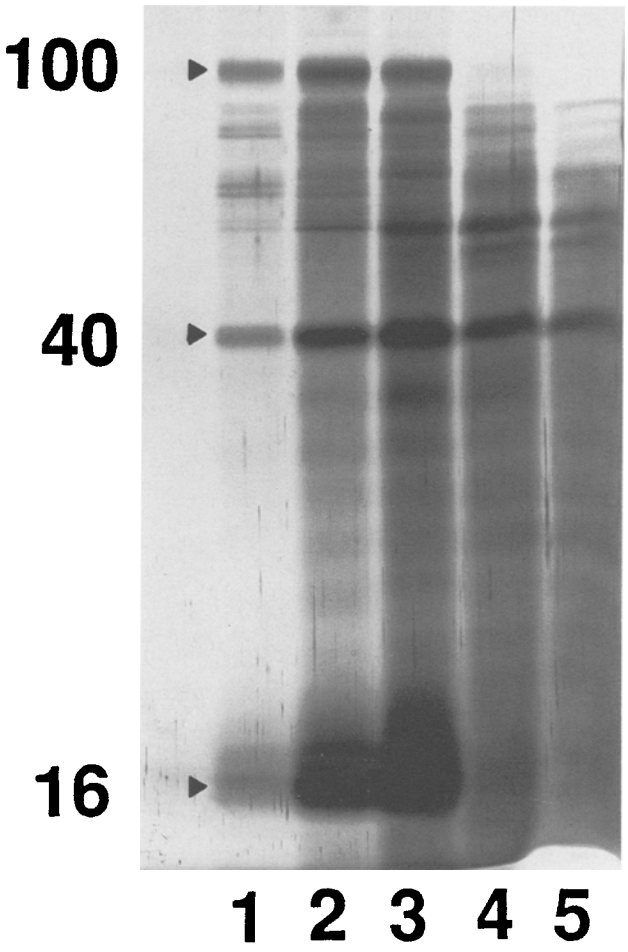

Fig. 4. Purification of the V_0 sector of the vacuolar ATPase. Vacuolar membranes were incubated in KNO_3 and ATP to remove peripheral polypeptides (Bowman *et al.*, 1989). After centrifugation the pellet was solubilized in 14 mM detergent ZW3-14, layered onto a 20–40% glycerol gradient, and recentrifuged for 5.5 h at 230,000 × *g*. Aliquots from the gradient were analyzed by electrophoresis as in Fig. 2. Lanes 1–5 represent the top half of the gradient. No proteins were detected in the bottom fractions which are not shown. Arrows indicate the positions of proteins of 100, 40, and 16 kDa.

peripheral polypeptides (Fig. 4). The V_1 sector was removed with KNO_3 and ATP. The membranes were then solubilized in Zwittergent 3-14 and layered onto a glycerol gradient. After centrifugation, 100-, 40-, and 16-kDa polypeptides were found to have moved well into the gradient. These polypeptides thus behaved not like individual solubilized proteins, but rather like proteins bound to each other in a complex. As judged by staining of gels, the 16-kDa polypeptide is significantly more abundant than the 100- or 40-kDa proteins. This is consistent with data obtained for

the mammalian vacuolar ATPase, which showed that each enzyme contains six 16-kDa subunits for each 100-kDa or 40-kDa subunit (Arai *et al.*, 1988).

Perhaps the strongest evidence that these membrane-associated polypeptides are subunits of the ATPase comes from partial sequencing of the 16-kDa protein. This polypeptide was prepared as shown in Fig. 4, eluted from SDS-polyacrylamide gels, and fragmented by incubation in cyanogen bromide. When a mixture of the fragments was sequenced, five fragments fit exactly to the predicted sequence of a 16-kDa polypeptide, obtained from analysis of a cloned *N. crassa* gene. The gene, described below, encodes a protein highly similar to the 16-kDa subunit of vacuolar ATPases from yeast, plant and mammalian cells (Sista, 1991).

Attempts to sequence the 40-kDa polypeptide have been complicated by the fact that a protein only slightly larger than 40-kDa is one of the most abundant proteins in the membrane. Much of this second protein can be solubilized by extraction with 1 mM EGTA, but a significant amount remains associated with the membrane (see Fig. 2). The sequences of several tryptic peptides from this "contaminant," obtained both from the EGTA extract and from a partially purified V_0 preparation, revealed that the protein is the *N. crassa* homolog of proteinase A (Tenney, Vazquez, and B. Bowman, unpublished data), previously described in vacuoles of *S. cerevisiae* (Ammerer *et al.*, 1986). Not all of the tryptic peptides are obviously derived from the protease, however. We have isolated the gene encoding the *N. crassa* proteinase A and are now sequencing it. This information will allow us to see which of the unidentified peptides from the 40-kDa protein may be from the vacuolar ATPase subunit. Peptide sequence has not yet been obtained for the *N. crassa* 100-kDa protein.

The finding that a protease is one of the major proteins associated with the vacuolar membrane may also explain some of the variability seen in different purified preparations of the *N. crassa* enzyme. For example, the relative amount of 100-kDa polypeptide is sometimes quite low and often does not appear as a single discrete band. Similarly, the B subunit appears to be degraded in some preparations. Other investigators have suggested that some of the subunits, especially the 100-kDa polypeptide, are particularly susceptible to proteolysis (Adachi *et al.*, 1990). We examined this possibility by incubating vacuolar membranes in different concentrations of trypsin. As shown in Fig. 5, most of the membrane proteins, including the 67-kDa A subunit, are not affected by the trypsin. Two polypeptides, however, of 100 and 60 kDa appear to be quickly degraded. It is clear that to prepare vacuolar ATPases from lysosomal compartments like the *N. crassa* vacuole the removal or inactivation of protease activity is essential.

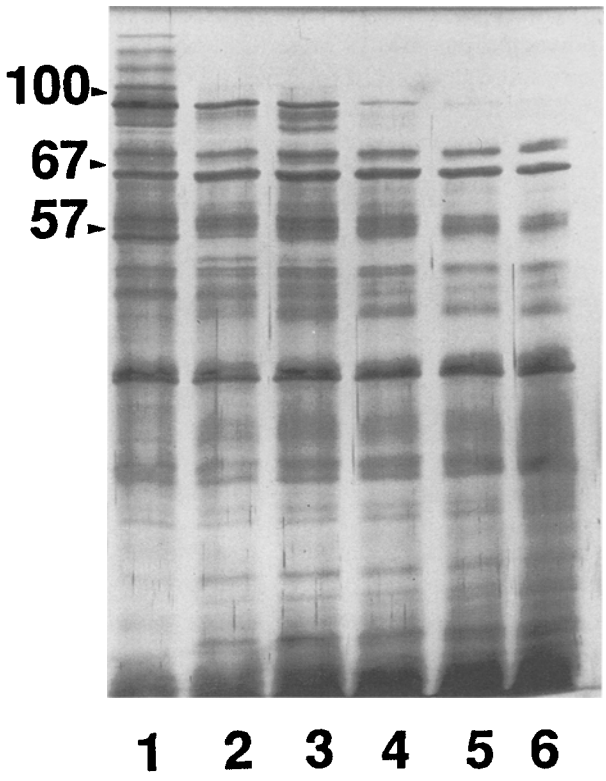

Fig. 5. Trypsin cleavage of proteins in vacuolar membranes. 10 μg of vacuolar membrane protein was suspended in 20 μl of 100 mM Tris-Cl (pH 8.0) and incubated at room temperature with (1) 0.0, (2) 0.2, (3) 0.4, (4) 0.6, (5) 0.8, and (6) 1.0 μg trypsin. The reaction was stopped after 1 min by the addition of 1.0 μg soybean trypsin inhibitor. After centrifugation the supernatants were removed, and the membranes were resuspended and analyzed by electrophoresis as in Fig. 2. Each lane contains 3 μg of protein. Arrows indicate the positions of proteins of 100, 67, and 57 kDa.

ISOLATION OF GENES ENCODING SUBUNITS OF THE VACUOLAR ATPase

In *N. crassa* three genes encoding subunits of the ATPase have been isolated: *vma-1* encoding the A subunit (E. Bowman *et al.*, 1988a), *vma-2* encoding the B subunit (B. Bowman *et al.*, 1988), and *vma-3* encoding the 16-kDa membrane-associated subunit (Sista, 1991). The latter polypeptide has been named subunit

c in several organisms. The *vma-1* and *vma-2* genes were isolated by using antibodies raised against A and B subunits purified on SDS polyacrylamide gels. The identity of the genes was confirmed by amino acid sequencing of peptides derived from the protein. To isolate *vma-3*, primers for the polymerase chain reaction were chosen by selecting regions in which the 16-kDa polypeptide is highly conserved in yeast (Nelson and Nelson, 1989; Umemoto *et al.*, 1990) and mammalian cells (Mandel *et al.*, 1988). A fragment of the gene was amplified using the selected primers, and the complete gene and *c*DNA were isolated by using the amplified fragment. Southern-blot analyses have provided no evidence for other isoforms of any of these three genes in the *N. crassa* genome (Sista, 1991; B. Bowman, E. Bowman, and M. Wechser, unpublished data).

vma-1. The *vma-1* gene encodes a 67,121-Da polypeptide containing 607 amino acids. The amino acid sequence shows a high degree of similarity to A subunits from vacuolar ATPases of plants, mammals, and other fungi (Gogarten *et al.*, 1989; Bowman and Bowman, unpublished observations). In pairwise comparisons approximately 60% of the residues are identical. The A subunit also shares significant sequence similarity (20–25% identity) with the α and β subunits of F-type ATPases (B. Bowman *et al.*, 1988). Several lines of evidence, including sequence similarity, inhibitor labeling, and antibody inhibition, strongly suggest that the A subunit contains the site in which MgATP binding and hydrolysis occurs (E. Bowman *et al.*, 1988a, and references therein). Thus, the A subunit of the vacuolar ATPase and the β subunit of the F-type ATPase appear to be homologous in structure and function.

In general, sequence similarities between the A and β polypeptides are in regions that have been implicated as having important functions. Two regions of the polypeptides contain residues that are universally conserved in all A and β subunits. The first (residues 239–279 in the A subunit of *N. crassa*) contains a motif common to several nucleotide-binding proteins, often called the "Walker A site" (Walker *et al.*, 1982). This area of the $F_1\beta$ subunit has been postulated to bind Mg and the phosphoanhydride portion of nucleotides (reviewed in Senior, 1988). The second highly conserved region (residues 330–335 in the A subunit of *N. crassa*) has the sequence AEYFRD. Relatively little evidence is available which addresses the function of this region, but it has been proposed for $F_1\beta$ that these amino acids also form part of a nucleotide binding site (Vogel and Cross, 1991). The most striking difference between the A and B polypeptides is a region of 90 amino acids in the A subunit (approximately residues 133–222 in *N. crassa*), first noted by Zimniak *et al.* (1988), which has no counterpart in the β subunit. The function of this region is unknown. In comparisons of A subunits from different organisms this region tends to be slightly less conserved than other regions (Gogarten *et al.*, 1989). However, it is present even in the related ATPases from the archaebacteria (Denda *et al.*, 1988; Inatomi *et al.*, 1989). Indeed, the presence of these 90 amino acids appears to be a landmark by which to identify an enzyme belonging to the vacuolar class of ATPases.

vma-2. The *vma-2* gene encodes the B subunit, a 513 amino acid polypeptide with a molecular mass of 56,808 Da (B. Bowman *et al.*, 1988). Comparison with amino acid sequences of B subunits from other organisms shows it to be even more conserved than genes for the A subunit. Pairwise comparisons between plant, fungal, and mammalian B subunits show approximately 70% sequence identity (Gogarten *et al.*, 1989; Bowman and Bowman, unpublished observations). This is surprising since the B subunit does not appear to contain the catalytic site on the enzyme. The observation that relatively few residues in this subunit have changed during the evolution of the ATPase in plants, fungi, and animals suggests that this subunit plays an essential role in the structure and mechanism of the enzyme. In general, the finding that both A and B are highly conserved suggests that all surfaces of these polypeptides are important and that interaction between subunits plays a critical role in function.

Like the A subunit, the B subunit shares sequence similarity to α and β polypeptides of F-type ATPases. It is interesting, however, that unlike the A, α, or β polypeptides, the B subunits do not have an obvious "Walker A consensus sequence." The B subunits do tend to conserve the region that aligns with the AEYFRD site, again pointing to an important function for this part of the polypeptide.

The B subunit of the vacuolar ATPase is likely to have the same function as does the α subunit in the F-type ATPase. The polypeptides are essentially the same size. One specific region of the B subunit is very similar to a region of the α subunit which has been implicated in catalysis by mutational analyses of the α subunit from *E. coli* (Futai *et al.*, 1989). Four different mutations within this region cause the loss of multisite ATPase activity. All four mutations map to amino acid residues that are identical in B and α polypeptides

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                    *                          * **
E.c. α  aa# 348 ITDGQIFLETNLFNAGIRPAVNPGISVSRVG-SA
N.c. B  aa# 347 ITEGQIFVDRGLHNRGIYPPINVLPSLSRLMKSA

N.c. A  aa# 424 IVQVFWGLDKKLAQRKHFPSINTSVSYSKYLTIL
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Fig. 6. Comparison between regions of the α subunit of F_1 ATPase from *E. coli* (E.c.) and the A and B subunits of vacuolar ATPase from *N. crassa* (N.c.). The asterisks are above residues which, when altered by mutation in *E. coli*, cause loss of ATPase activity (Futai *et al.*, 1989).

(Fig. 6). The corresponding region of the A subunit of the vacuolar ATPase is significantly less similar.

vma-3. The *vma-3* gene encodes a 16,465-Da polypeptide with 161 amino acids (Sista, 1991). This subunit, too, is highly conserved. The sequence of the *N. crassa* polypeptide is 79% and 70% identical to the homologous polypeptides from *S. cerevisiae* and bovine kidney cells, respectively. Because this protein is part of the membrane sector of the ATPase, it was expected to be hydrophobic. Analysis of the sequence shows four putative membrane-spanning domains, indicating that half of the polypeptide is likely to be buried within the lipid bilayer. As was pointed out when the first sequences were obtained from bovine and yeast cells (Nelson and Nelson, 1989), the most conserved regions of the 16-kDa polypeptides are the membrane-spanning domains. This curious feature is also true of the *N. crassa* subunit.

Gene Structure. Of the sequences published thus far, those for the vacuolar ATPase genes from *N. crassa* are the only ones to contain introns. (Genomic sequences are not yet available for higher eukaryotes.) Most genes from filamentous fungi, in contrast to *S. cerevisiae*, have a few small introns (Gurr *et al.*, 1987). The size (51–220 bp) and sequence characteristics of the introns in *vma-1*, *vma-2*, and *vma-3* are much like those in other *N. crassa* genes. However, there are more introns in these genes than is typical: four in *vma-3*, five in *vma-2*, and six in *vma-1*. It is interesting that the only other genes with this many introns encode housekeeping enzymes such as β-tubulin (Orbach *et al.*, 1986) or the mitochondrial ATPase (Bowman and Knock, 1992). The genes are constitutively expressed at high levels, and their protein products are abundant. We would like to investigate whether the introns have a role in regulating this high expression of *N. crassa* genes.

PHYSIOLOGICAL ROLE OF THE VACUOLAR ATPase

To further examine the role of the vacuolar ATPase *in vivo*, we are using two approaches. First, we determined the effects of a specific inhibitor of this enzyme, bafilomycin A_1. We identified bafilomycin A_1, a macrolide antibiotic, as a highly potent (half-maximal inhibition at 1–10 nM) and specific inhibitor of vacuolar ATPase activity *in vitro* (E. Bowman *et al.*, 1988b). Experiments with yeast and mammalian cells have shown that bafilomycin acts *in vivo* to prevent the acidification of organelles such as vacuoles (Banta *et al.*, 1988) and lysosomes (Yoshimori *et al.*, 1991). When *N. crassa* is grown on normal medium (minimal supplemented with 2% sucrose, pH 5.8) in the presence of 10 μM bafilomycin A_1, the vacuoles fail to acidify, as indicated by the inability to accumulate fluorescent amines. Control cells grown in the absence of bafilomycin A_1 are filled with fluorescent organelles. These results suggest that the vacuolar ATPase has been inactivated *in vivo* by the bafilomycin A_1.

Somewhat surprisingly, growth of *N. crassa* cells on the normal medium is not significantly affected by bafilomycin A_1. By contrast, when the pH of the medium is raised to 7.5, growth is completely inhibited by 10 μM bafilomycin A_1—if one looks at single colonies growing on agar plates. In liquid medium the density of the conidial inoculum can determine the vigor of the growth seen at pH 7.5. In its sensitivity to high pH, this growth phenotype is similar to the phenotype reported for *S. cerevisiae* strains containing inactivated vacuolar ATPase genes (Nelson and Nelson, 1990). We are intrigued by the effect of bafilomycin on growth but do not yet have an explanation for it. The effect of an inhibitor *in vivo* is difficult to interpret because of the challenge of determining whether the inhibition is complete.

An alternative, second approach to elucidating the *in vivo* role of the vacuolar ATPase is to examine the effects of mutations. With one possible exception (Cornelius and Nakashima, 1987), mutations in vacuolar ATPase genes of *N. crassa* have not been reported. In experiments with *S. cerevisiae* inactivation of genes which encode subunits of the vacuolar ATPase gives rise to a conditional-lethal phenotype. The mutated cells are viable (but not vigorous) if grown in acidic medium, but not viable if grown in alkaline medium (Nelson and Nelson, 1990). Furthermore, the mutants also exhibit a *pet*$^-$ phenotype, i.e., they cannot grow on a nonfermentable carbon source (Ohya *et al.*,

1991). Thus, in some way, inactivation of a vacuolar ATPase gene gives rise to a cell which apparently does not have fully functional mitochondria.

These observations raise an interesting question. Can cells that require oxidative phosphorylation for growth, such as *N. crassa* and higher eukaryotes, be viable under any conditions lacking a functional vacuolar ATPase? We have tried to address this question by inactivating the *vma-1* and *vma-2* genes of *N. crassa*, which is unfortunately not a straightforward procedure in this organism. The method employed was RIPing (an acronym for "repeat-induced point mutations"). RIPing exploits an odd feature of *N. crassa*, discovered by Selker *et al.* (1987). Duplicate DNA sequences, even if unlinked, are somehow detected, and *both* copies are extensively mutated when the organism goes through the sexual cycle. Thus, by transforming a copy of the *vma-1* or *vma-2* gene into a wild-type strain, and then mating the transformant, one expects to recover among the haploid progeny strains lacking a functional copy of the *vma-1* or *-2* gene. We have done this experiment several times and have never recovered a strain lacking the ATPase. A tempting interpretation of these results is that the ATPase genes were RIPed, the enzyme is essential, and the RIPed progeny were all dead. However, because the frequency of RIPing is variable and until the RIPing phenomenon is better understood, we cannot firmly conclude that the ATPase genes were indeed inactivated.

ACKNOWLEDGMENTS

We wish to acknowledge the members of our laboratory whose work has been briefly summarized in this review: William Dschida, Karen Tenney, Hema Sista, and Mark Wechser. Our investigation of the vacuolar ATPase has been supported by National Institutes of Health Grants GM28703 and GM08132.

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