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VACUOLAR ATPase OF *NEUROSPORA CRASSA*: ELECTRON MICROSCOPY, GENE CHARACTERIZATION AND GENE INACTIVATION/MUTATION

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Summary

We are using three approaches to investigate the vacuolar ATPase, V-ATPase, from *Neurospora crassa*. (1) Examination in the electron microscope shows the enzyme has a 'ball and stalk' structure like the F-type ATPases. However, the vacuolar ATPase is significantly larger, has a prominent cleft in the head sector, and has extra components associated with the stalk and membrane sectors. (2) Genes encoding three of the major subunits of the vacuolar ATPase and the homologous subunits of the mitochondrial F-ATPase have been isolated. The exon/intron structures of the genes have been analyzed and the chromosomal locations have been determined. Two of the vacuolar ATPase genes map very close to each other, suggesting the possibility of a cluster of ATPase genes. (3) The function of the ATPase is being investigated by isolating strains with altered or inactivated ATPase. We are characterizing strains that are resistant to bafilomycin A_1, a potent and specific inhibitor of the vacuolar ATPase. Initial attempts to inactivate a vacuolar ATPase gene indicate that the enzyme may be essential for growth.

Introduction

The lower eukaryote *Neurospora crassa* is a filamentous fungus characterized by a haploid life cycle, a coenocytic mycelium and a strict dependence on oxygen for growth. The organism contains numerous small (0.2–1.0 μm in diameter when isolated) acidic compartments of about pH 6.1 called vacuoles. These organelles were isolated and extensively studied by R. H. Davis and coworkers during the 1970s and early 1980s, with an emphasis on their role as storage sites for basic amino acids (comprehensively reviewed in Davis, 1986). Vacuoles were found to contain approximately 0.5 mol l^{-1} concentrations of arginine, ornithine and lysine, together with large amounts of the counterion polyphosphate. Apparently bifunctional organelles, the vacuoles also house up to 98% of the cell's degradative enzymes, such as proteases, nucleases and phosphatases, thus functioning like the lysosomes of animal cells.

Critical to both of these functions is the V-ATPase located in the vacuolar membrane (Bowman and Bowman, 1982). By hydrolyzing ATP and pumping protons into the interior of the vacuole, this enzyme generates the acidic interior favorable to degradative activities and provides the gradient required to drive basic amino acid transport across the membrane. Our goal is to determine the structure and function of this enzyme and to

Key words: ATPase, V-ATPase, fungal vacuole, *Neurospora crassa*.

elucidate its role in the cell. Current approaches in our laboratory include ultrastructural analysis of the ATPase, isolation and characterization of the genes encoding the ATPase and alteration of the ATPase by gene inactivation or mutation.

Structure of the vacuolar ATPase

The vacuolar ATPase is a very large enzyme with an unusual shape that allows it to be identified and directly examined in the electron microscope (Bowman *et al.* 1989). Vacuolar membranes from *N. crassa* are thickly studded with ball and stalk structures rather like those of the F-ATPase seen in mitochondria (Fig. 1A). Evidence that the ball and stalks are not due to mitochondrial contamination was obtained by washing both kinds of membranes with 100 mmol l^{-1} KNO_3 plus ATP. Whereas the salt wash had no effect on the mitochondrial ATPase structures, it both released the major subunits of the vacuolar ATPase from the membrane, as analyzed on SDS gels, and stripped the ball and stalk structures off the membranes examined by electron microscopy (Bowman *et al.* 1989). Further analysis has revealed that the mitochondrial and vacuolar ATPases clearly differ in several respects, as described below. The peripheral sector of the ATPase, the 'ball' seen in the electron micrographs, can be recovered from the supernatant after washing vacuolar membranes with nitrate and ATP. In electron micrographs the isolated peripheral sector retains most of the structural features seen in the 'ball' attached to vacuolar membranes. The diameter is about 12 nm (compared to 9 nm seen for the peripheral sector of the F-type ATPase) and a prominent cleft is often observed (Fig. 1B). The stalk that is visible in the membrane-bound enzyme is not apparent in the isolated peripheral portion of the enzyme. When the peripheral sector of the *N. crassa* ATPase is dissociated with SDS and analyzed on polyacrylamide gels, five different polypeptides are observed with approximate relative molecular masses of 69, 59, 48, 30 and

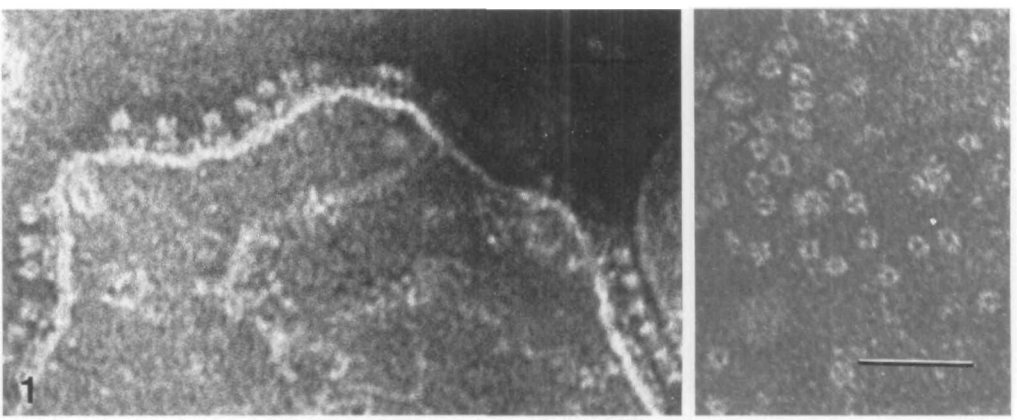

Fig. 1. Electron micrographs of the vacuolar ATPase stained with 1% phosphotungstic acid. (A) Vacuolar membrane vesicles. The head group on the 'ball-and-stalk' structure protruding from the membrane is approximately 12 nm in diameter. (B) The peripheral sector of the vacuolar ATPase has been removed from the membrane by extraction with 100 mmol l^{-1} KNO_3 and 1 mmol l^{-1} ATP as described in Bowman *et al.* (1989). The peripheral sector appears to retain the same size and shape as when it is attached to the membrane. Scale bar, 50 nm.

$17\times10^3 M_r$. A faint band of approximately $51\times10^3 M_r$ is also sometimes seen, but we suspect that this band is a degradation product of the $59\times10^3 M_r$ subunit (Bowman *et al.* 1989). The two largest subunits appear to be at least three times more abundant than the smaller subunits, suggesting that, as in the V-ATPase of the bovine coated vesicle (Arai *et al.* 1988) and in F-ATPases (Walker *et al.* 1990), the subunit stoichiometry may be 3:3:1:1:1. As described below, the genes encoding the two largest subunits have been cloned and sequenced. Amino acid sequences of tryptic peptides have been obtained for the three smaller subunits. The $30\times10^3 M_r$ polypeptide appears to be the homolog of the $31\times10^3 M_r$ subunit indentified in vacuolar ATPases from *S. cerevisiae* (Foury, 1990) and mammalian kidney cells (Hirsch *et al.* 1988). Fragments from the 48 and $17\times10^3 M_r$ polypeptides do not obviously match any protein sequences in the current databases. The membrane sector of the *N. crassa* ATPase cannot be clearly delineated in electron micrographs. Purification of the whole ATPase by detergent solubilization and centrifugation through sucrose density gradients reveals three or four polypeptides in addition to those seen in the peripheral sector. Subunits of approximately 100, 40 and $16\times10^3 M_r$ are easily seen. Analysis on polyacrylamide gels optimized for resolution of peptides of low relative molecular mass indicated that a subunit of approximately 20×10^3 M_r may also be present. The gene encoding the$16\times10^3 M_r$ subunit has been characterized (see below), but no sequence information has been obtained for the others. Fig. 2 shows a model of the *N. crassa* vacuolar ATPase, based on data obtained from electron microscopy and enzyme purification. Although subunits can be assigned to the membrane or peripheral sectors, there are virtually no data to indicate how the subunits are arranged within these sectors. The stalk portion of the ATPase could be composed, at least in part,

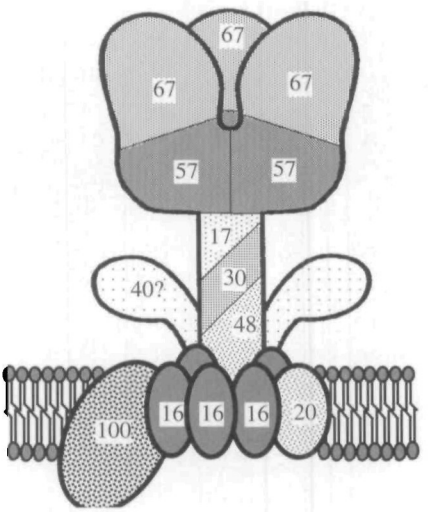

Fig. 2. Model of the vacuolar ATPase from *Neurospora crassa*. The enzyme appears to be composed of at least nine different subunits. Five of them (67, 57, 48, 30, $17\times10^3 M_r$) are in the peripheral sector and four (100, 40, 20, $16\times10^3 M_r$) are associated with the membrane. It should be noted that the subunit composition of the stalk and basal projections of the enzyme seen in the electron micrographs is unknown.

of polypeptides that are anchored in the membrane. It is likely that the V-ATPase will resemble the F-ATPase, but the vacuolar enzyme does have several distinguishing features. It is significantly larger, it appears to have a much more prominent cavity in the center of the peripheral sector, and it has basal appendages unlike anything seen in the F-type enzyme. In recent years, mitochondrial ATPases from both yeast (Norais *et al.* 1991) and bovine cells (Walker *et al.* 1990) have been shown to have at least 12–14 different subunits. It will not be surprising if the larger vacuolar ATPases turn out to have more than the eight or nine subunits currently identified.

Genes encoding ATPase subunits

We have isolated genes encoding three subunits of the vacuolar ATPase: *vma-1*, encoding subunit *A* (E. Bowman *et al.* 1988*b*), *vma-2*, encoding subunit *B* (B. Bowman *et al.* 1988) and *vma-3*, encoding the proteolipid *c* (Sista, 1991). As discussed by Nelson (1992), these three subunits are all highly conserved proteins that are evolutionarily related to subunits of F-ATPases: β, α and the proteolipid *c*, respectively. Genes encoding the homologous mitochondrial subunits have also been isolated in *N. crassa* (*atp-1* and *atp-2* encoding α and β by us, Bowman and Knock, 1992; and *c* by W. Sebald, personal communication), allowing us to compare them directly in this organism.

Because both V-ATPases and F-ATPases are multisubunit enzymes with components present in different stoichiometries, one might expect a mechanism for coordinate regulation of expression of genes encoding various subunits. Northern blot analysis of the V-type genes (for *A*, *B* and *c*) and of the F-type genes (for α and β) shows that all of them are constitutively expressed during mycelial growth but at considerably different levels. Messages for *A* and *B* are about a quarter as abundant as β-tubulin and message for *c* is one-third as abundant (M. Wechser and B. Bowman, unpublished results). By contrast, messages for α and β are about three times more abundant than β-tubulin mRNA (Bowman and Knock, 1992).

Sequence comparisons of the 5′-upstream regions of these genes does reveal some common elements, which are potentially of regulatory significance. Only one element appears to be common to all five genes cloned in our laboratory and this is the GC box sequence identified as an SP-1 binding site in other organisms (Kadonaga *et al.* 1988). The three V-ATPase genes, however, share three other regions (8–17 nucleotides in length) of high sequence similarity positioned at similar distances in each gene from the start of transcription. We are presently trying to determine whether these regions play a role in transcriptional regulation. Although the genes for α and β subunits of the mitochondrial ATPase of *N. crassa* do not appear to share conserved upstream regions, the gene encoding β does have a sequence similar to a putative control motif found in the gene for the human mitochondrial β-subunit gene (Neckelmann *et al.* 1989; Bowman and Knock, 1992).

A prominent feature common to all the ATPase genes in *N. crassa* is the presence of introns. Genes encoding *A*, *B*, α and β have 5–7 introns (Fig. 3), while genes encoding the much smaller V- and F-type *c* subunits have 4 and 2, respectively. For comparison, the homologous genes from *Saccharomyces cerevisiae* have no introns. Genes from

Fig. 3. Structure of the genes encoding major subunits of ATPases in *Neurospora crassa*. *A*, *vma-1*, encoding the $67\times10^3 M_r$ subunit of the vacuolar ATPase; *B*, *vma-2*, encoding the $57\times10^3 M_r$ subunit of the vacuolar ATPase; *α*, *atp-1*, encoding the alpha subunit of the mitochondrial ATPase; and *β*, *atp-2*, encoding the beta subunit of the mitochondrial ATPase. The diagrams include only the protein-coding regions of the genes.

filamentous fungi typically have a few introns, but not usually as many as are found in the genes encoding the ATPase subunits. Two observations suggest that the introns may be important for high expression of the genes. First, in fungi a large number of introns has been found only in highly expressed, constitutive genes (Gurr *et al.* 1987; Bowman and Knock, 1992). Second, the introns tend to be clustered in the 5′ end of the coding region, a position associated with high expression of some plant genes (Callis *et al.* 1987).

In higher eukaryotes, genes are frequently divided into exons with a narrow size distribution peaking at 40–50 codons; the exons often encode folding elements or domains of the protein product (Gilbert, 1985). The ATPase genes in *N. crassa* are quite different. The genes encoding the vacuolar *A* and *B* subunits and mitochondrial *α* and *β* subunits all appear to have evolved from a common ancestor and are likely to encode proteins with similar tertiary structures (Gogarten *et al.* 1989; Iwabe *et al.* 1989). The exons within these genes, however, are quite variable and encode 4–521 amino acids. Furthermore, the position of most of the introns is not conserved. It appears unlikely that the intron/exon structure of the genes will yield much information about domains within the protein. As noted above, the role of the introns in *N. crassa* may be primarily in gene expression rather than in gene evolution.

The chromosomal locations of five of the ATPase genes were determined either by restriction fragment-length polymorphism mapping [genes encoding *A* (E. Bowman *et al.* 1988*b*), *B* (B. Bowman *et al.* 1988), V-type *c* (Sista, 1991) and *β* subunits (Bowman and Knock, 1992)] or by classical genetics (*oli*, encoding F-type *c* subunit; cf. Perkins *et al.* 1982). The genes are scattered on three different chromosomes (in a total of seven chromosomes in *N. crassa*). As we are unable to find a polymorphism for the gene encoding the *α* subunit, we do not yet know its chromosomal location (Bowman and Knock, 1992). Interestingly, *vma-1* and *vma-3*, which encode the V-ATPase *A* and *c* subunits, are close enough (less than two map units or about 40 kb apart) to suggest the

possibility that a cluster of ATPase genes exists. We are eager to learn whether any other ATPase genes map in this region.

Function of the vacuolar ATPase

The activity of the vacuolar ATPase *in vivo* can be illustrated by fluorescence microscopy. Because of their low internal pH, vacuoles and similar organelles accumulate fluorescent weak bases such as acridine orange, quinacrine and chloroquine (Pringle *et al.* 1989). Mycelia of *N. crassa* incubated in the presence of chloroquine are filled with numerous small organelles that glow brightly (Fig. 4). If agents that collapse proton gradients are added, no fluorescence can be seen. To understand the role of the vacuolar ATPase in the cell we are using two genetic approaches. The first is to select for putative ATPase mutations by using the antibiotic bafilomycin A_1, the most potent and specific inhibitor of vacuolar ATPases yet identified (E. Bowman *et al.* 1988*a*). Although bafilomycin A_1 inhibits ATPase activity *in vitro* at a half-maximal concentration of 1–5 nmol l^{-1}, it is a poor inhibitor of growth of *N. crassa* under standard conditions (Fig. 5). However, when the pH of the medium is raised from 5.8 (standard) to 7.5, bafilomycin A_1 becomes an effective inhibitor of growth – with the caveat that relatively high concentrations (approximately $5-10\,\mu$mol l^{-1}) are still needed. These data from

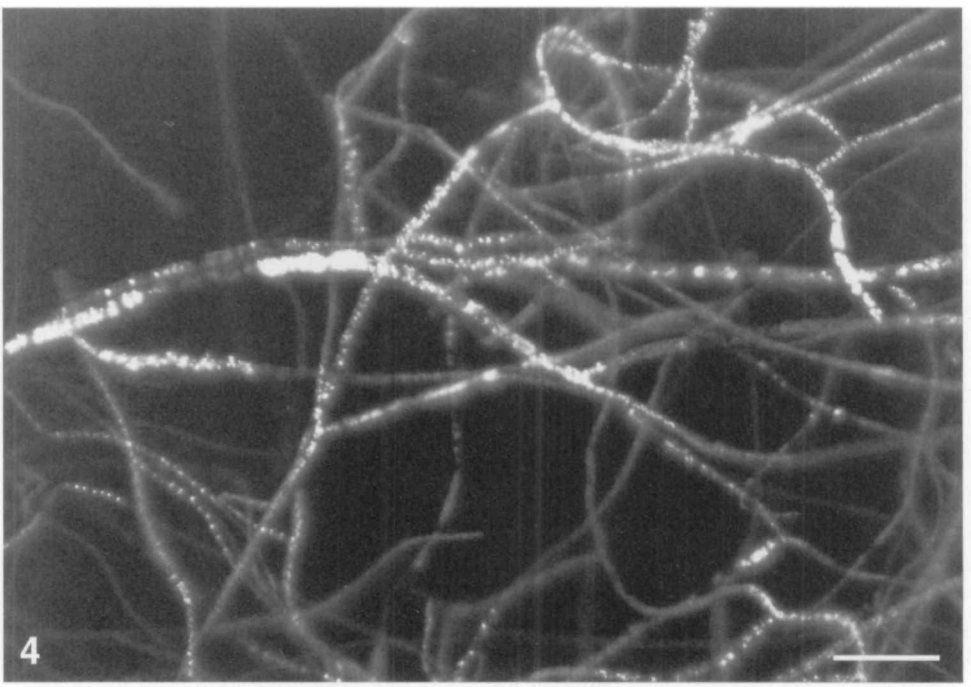

Fig. 4. Fluorescence micrograph of mycelia from *Neurospora crassa*. Mycelia were grown in aerated medium for 15 h and incubated for 10 min in 10 mmol l^{-1} chloroquine. The sample was illuminated with a Leitz Wetzlar type 307 mercury lamp and photographed through a narrow blue-pass filter. Scale bar, 10 μm.

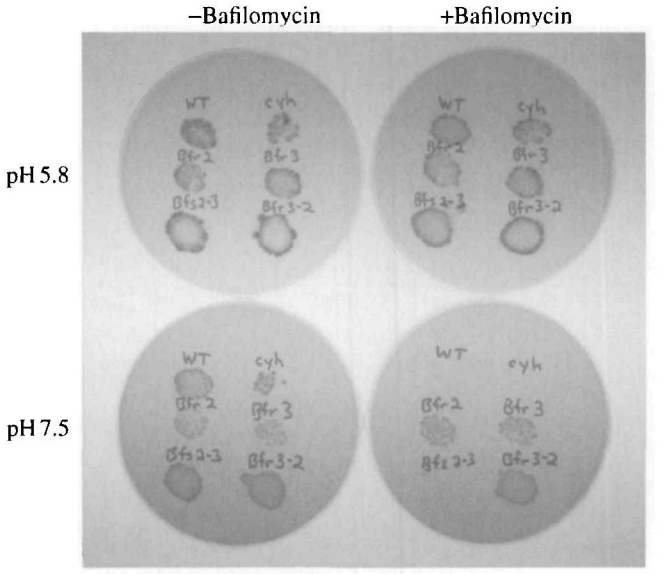

Fig. 5. Effect of bafilomycin A_1 on growth of *Neurospora crassa* at pH 5.8 and pH 7.5. Vegetative spores were spread on plates with minimal medium (unadjusted pH=5.8) containing sorbose to encourage colonial growth. The medium was supplemented with 20 mmol l^{-1} Hepes, pH adjusted to 7.5 with NaOH and 10 μmol l^{-1} bafilomycin A_1 as indicated. Strains tested were: WT, wild-type strain 74A; *cyh*, a cycloheximide-resistant strain used for mapping; *Bfr2* and *Bfr3*, spontaneous mutant strains isolated from 74A; and *Brs2-3* and *Bfr3-2*, progeny from back-crossing the original mutant strains. *Bfr2*, *Bfr3* and *Bfr3-2* are bafilomycin-A_1-resistant strains.

N. crassa are consistent with findings from *S. cerevisiae*, where strains with disrupted vacuolar ATPase genes are characterized by reasonably good growth at pH 5.5 but virtually no growth at pH 7.5 (Nelson and Nelson, 1990).

By selecting for growth on 10 μmol l^{-1} bafilomycin A_1 at pH 7.5, we have isolated several resistant strains of *N. crassa*. In these strains the sensitivity of vacuolar ATPase activity to the inhibitor *in vitro* is unchanged from that of wild-type strains. We do not yet have an explanation for why the cells are resistant. A number of investigators, using yeast and animal cell culture systems, have reported that *in vivo* bafilomycin A_1 can react with specific target membranes and exert its effects on a vacuolar ATPase (Banta *et al.* 1988; Klionsky *et al.* 1992; Yoshimori *et al.* 1991; Umata *et al.* 1990; Sundquist *et al.* 1990). To our knowledge no reports of alternative *in vivo* targets for bafilomycin A_1 have yet appeared. Thus, the bafilomycin-resistant mutants in *N. crassa* could provide an alternative object for investigating both the role of the vacuolar ATPase and the function of the vacuole in the cell.

Our second genetic approach is to inactivate the vacuolar ATPase genes in *N. crassa*. The procedure is not as straightforward as in *S. cerevisiae*, but a novel mechanism discovered by Selker and coworkers has been used to inactivate several genes from *N. crassa*. Selker *et al.* (1987) found that haploid *N. crassa* will not tolerate extra copies of genomic DNA through meiosis. If a second copy of a gene is introduced by

transformation and the strain is put through a sexual cross, the process of RIPing (repeat-induced point mutations) occurs, resulting in inactivation of both the resident gene and the introduced copy. Direct evidence that RIPing has taken place is seen in altered DNA restriction patterns on Southern blots.

Our initial efforts to apply this approach to the vacuolar ATPase genes gave ambiguous results. We were unable to recover strains carrying altered ATPase genes. Thus, either we failed to inactivate the genes or the genes were essential for spore germination. To circumvent this problem, R. Metzenberg and J. Grotelueschen (University of Wisconsin, unpublished results) have attempted to RIP the ATPase gene *vma-1* in a heterokaryon background, where, if essential, the nucleus with the defective ATPase gene could survive. (A useful feature of *N. crassa* is its capacity to grow as a stable heterokaryon, in which two nuclei having different selectable markers co-exist in the cytoplasm and complement each other for their respective deficiencies.) Initial characterization of the heterokaryon obtained from Dr Metzenberg has given intriguing results. To see whether homokaryotic cell lines could be generated, vegetative spores from the heterokaryon were spread on plates. Because each spore typically contains 1–5 nuclei, some of the colonies were expected to be homokaryons, whereas others should be heterokaryons with differing relative amounts of the functional and the inactivated vacuolar ATPase genes. What we have observed is a significant diversity in colony growth and morphology. Some colonies exhibit essentially normal growth characteristics, some are intermediate, and others are very small with growth rates at least 10 times slower than the wild type. We have been able to propagate the slowest-growing strains and analyse the *vma-1* gene by Southern hybridization. These strains appear to contain more of the altered ATPase gene than is seen in the original heterokaryons, but an unaltered copy of the gene is also present.

Our preliminary interpretation of these results is that the vacuolar ATPase is essential for growth of *N. crassa*. Lowering the amount of ATPase in the cell may significantly lower the growth rate and alter the morphology. Inactivation of the vacuolar ATPase gene in *N. crassa* may have more severe effects than have been seen in *S. cerevisiae* because *N. crassa* is an obligate aerobe. *vma* mutants of *S. cerevisiae* have a *pet*$^-$ phenotype (no growth on non-fermentable carbon sources), yet the cells survive (Ohya *et al.* 1991). It may be that, because of secondary effects on mitochondrial function, the vacuolar ATPase is indispensable in most eukaryotic cells.

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