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*Topical Review*

# $H^+$-ATPases from Mitochondria, Plasma Membranes, and Vacuoles of Fungal Cells

Barry J. Bowman and Emma Jean Bowman
Department of Biology, University of California, Santa Cruz, California 95064

## Introduction

A remarkable number of cellular activities are directly dependent on ion-pumping ATPases. In addition to the fundamental processes of active transport and ATP synthesis, recent evidence has shown an essential role for ion-pumping ATPases in cell motility, development, ligand-receptor uncoupling, and protein sorting [71, 111, 147]. Despite the multiplicity of functions for ATPases, the basic design of these enzymes has been highly conserved. In both eucaryotic and procaryotic organisms, most ion-pumping ATPases that have been characterized fit into one of three structural types. In this review we wish to compare these three types of ATPases, drawing upon recent work with fungal cells.

## Why Fungi?

The fungi are clearly worthy of study as a diverse and ecologically important class of organisms. But in addition the fungi are particularly good organisms for the study of ion-pumping ATPases. First, there are obvious advantages of working with microorganisms: the capability to control the growth of the cells and to manipulate the extracellular environment. Second, a wealth of genetic information is available and molecular techniques can be readily applied. Third, the large cell size and rigid wall of some fungi permit the insertion of microelectrodes, which can be used to directly measure the electrical and chemical gradients generated by the ATPases in vivo. Futhermore, the three types of ATPases referred to above are all well represented in the fungal cell. As will be documented below, membrane fractions from fungal cells are relatively rich in ATPase activity compared to membranes from other sources.

Figure 1 shows the sub-cellular location of three ATPases described in fungal cells. The inner mitochondrial membrane contains an $F_0F_1$ ATPase, typical of the type found in other mitochondria, thylakoid membranes of chloroplasts, and bacterial membranes [10, 59, 72, 131, 158]. The ATPase in fungal plasma membranes closely resembles the ATPases of plant cell plasma membranes, animal cell plasma membranes ($Na^+$,$K^+$-ATPase, $H^+$,$K^+$-ATPase), and sarcoplasmic reticulum of muscle cells ($Ca^{2+}$-ATPase) [20, 39, 64, 79, 135, 136, 151]. Because they cycle through two conformational states, this class of enzymes is often referred to as $E_1E_2$ ATPases [9]. The vacuolar membrane ATPase represents a third class. Primarily on the basis of sensitivity to inhibitors, this ATPase resembles the ATPases of plant vacuolar membranes [31], lysosomal membranes [128], secretory granule membranes [98, 112], and endosomal membranes [60]. All three ATPases appear to be proton pumps in fungi. Using primarily *Neurospora crassa*, *Schizosaccharomyces pombe*, and *Saccharomyces cerevisiae*, recent work has given us a fairly complete picture of the properties of each of these $H^+$-ATPases.

## The Mitochondrial ATPase

Actively growing fungal cells are a rich source of mitochondrial ATPase, because mitochondria are by far the most abundant of the organelles. In our fractionation procedure for *N. crassa*, we obtain for each mg of vacuolar membrane protein, 15 mg of plasma membrane protein and about 200 mg of mitochondrial protein [29]. Consequently, it is essential to be able to distinguish the mitochondrial ATPase; if even 1% of the mitochondrial mem-

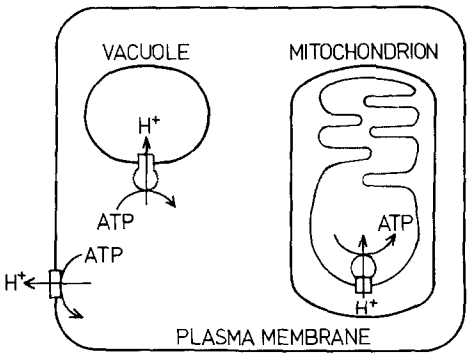


**Fig. 1.** Intracellular location of H+-ATPases

branes copurify with other membranes, they can contribute significant ATPase activity.

The function of the mitochondrial ATPase is to synthesize ATP. In fungi, there is no evidence that the reverse reaction, ATP-dependent proton pumping, occurs in vivo. The mitochondrial ATPase is the best characterized of the three types of ATPases, and its structure and mechanism have been reviewed extensively [10, 59, 72, 75, 131, 132, 158]. It belongs to the family of $F_0F_1$-ATPases, so named because they can be dissociated into two parts: a water-soluble component, $F_1$, which contains the ATP-binding sites and retains the capacity to hydrolyze ATP at high rates, and an integral membrane component, $F_0$, which contains the proton-conducting channel.

The structure of the $F_1$ sector appears to be essentially the same in all organisms, consisting of five different types of polypeptides ($alpha_3$, $beta_3$, $gamma_1$, $delta_1$, and $epsilon_1$) of mol wt 59,000–8,000 [86, 131]. The $F_0$ sector is more variable. In procaryotes it is composed of three types of polypeptides, of mol wt 5,400–24,000 [59, 75], while eucaryotes contain additional small subunits. The $F_0$ sector of the *N. crassa* ATPase has at least four types of polypeptides [129], and in *S. cerevisiae* there may be six different types of subunits [113]. Like other eucaryotes, the fungal mitochondrial ATPases have a polypeptide named OSCP (mol wt 22,000), which is involved in conferring sensitivity to the inhibitor oligomycin, and a small protein (mol wt 7,000), which may function as an in vivo inhibitor [103]. Because some of the polypeptides exist in multiple copies, the whole ATPase contains at least 20 subunits with an aggregate mol wt of approximately 500,000.

The gene for the beta subunit, which contains the catalytic site, has been cloned and partially sequenced for both *S. cerevisiae* and *S. pombe* [18, 114]. The amino acid sequence of the beta subunit is highly conserved, and, in fact, a plasmid carrying the beta subunit gene from *S. cerevisiae* was shown to complement a mutant of *S. pombe* that lacked its own beta subunit. The resulting strain produced hybrid mitochondrial ATPase with approximately 20% of the activity of the normal enzyme [17].

In fungi, genes coding for the ATPase polypeptides appear to be scattered throughout the nuclear and mitochondrial genomes. All the genes for the $F_1$ sector and at least two of the genes for the $F_0$ sector are in the nucleus. Mapping of the genes for the alpha and beta subunits of *S. pombe* has shown that, although both are on the same chromosome, they are not genetically linked [155]. The mitochondrial genome codes for three subunits of the mitochondrial ATPase, presumably all components of the $F_0$ sector [33, 157]. Most intriguing is the location of the gene for the $M_r$ = 8,000 proteolipid subunit of the $F_0$ sector. In mammalian cells this gene is in the nucleus, while in *S. cerevisiae* it is in the mitochondrion. *N. crassa*, however, has two slightly different copies of this gene, one in the nucleus and one in the mitochondrion. The nuclear gene appears to be the one expressed, because its DNA sequence matches the amino acid sequence of the purified proteolipid. Furthermore, mutations in the nuclear gene alter the proteolipid of the mitochondrial ATPase [75]. The mitochondrial gene is transcribed; whether it is translated into a functional protein is not yet known [15*a*].

Because it is so well characterized, the mitochondrial ATPase is an important model for understanding the biochemical mechanism of proton pumps. This ATPase can pump protons in vitro, and the homologous enzyme in procaryotic cells can function as a proton pump in vivo [59, 132]. In comparing the mitochondrial ATPase to plasma membrane and vacuolar membrane ATPases, we want to focus on two questions. First, what is the nature of the proton channel? Second, what is the mechanism of ATP hydrolysis at the active site?

The proton channel of the mitochondrial ATPase is in the $F_0$ sector. The small hydrophobic polypeptide of $M_r$ 8,000, sometimes referred to as the proteolipid, appears to be the key component. Proton movement through the enzyme can be blocked by the antibiotic oligomycin or by dicyclohexylcarbodiimide (DCCD) [75, 130]. In *N. crassa* and *S. cerevisiae* DCCD binds a specific glutamyl residue located in the middle of a sequence of about 25 hydrophobic amino acids. Because mutations affecting DCCD binding also affect oligomycin binding, it has been proposed that oligomycin binds at the same site [75]. The binding of one DCCD molecule per six proteolipid polypeptides completely inhibits the ATPase, suggesting a hexamer of proteolipids forms an essential part of the proton

channel [129, 130]. By contrast, more recent experiments with the *E. coli* ATPase indicate 9 or 10 proteolipid subunits per enzyme [61].

In mammalian mitochondria, and presumably fungal mitochondria as well, ATP synthesis and hydrolysis occur on the $F_1$ sector, specifically at a site on each of the three beta subunits [32]. Interestingly, the three alpha subunits also bind nucleotides, but the function of these subunits is not understood. It has been shown in experiments with mammalian mitochondria that the beta subunits interact with each other. The rate of hydrolysis at a single site is increased 20-fold by the binding of ATP to a second site [68]. Rates of ATP hydrolysis and formation are approximately equal on the surface of the enzyme. Apparently it is the binding and release of substrates and products that is coupled to proton translocation [32, 104, 105]. Unlike the plasma membrane ATPase described later, the mitochondrial ATPase does not form a covalent phosphoenzyme intermediate as part of its reaction cycle.

**Table.** Comparison of inhibitor sensitivities of three H+-translocating ATPases from *N. crassa*[a]

| Inhibitory | Concentration giving 50% inhibition | | |
|---|---|---|---|
| | *VM* ATPase | Mito ATPase | *PM* ATPase |
| | | (μM) | |
| Vanadate | NI[b] | NI | 1.0 |
| Azide | NI | 30 | NI |
| DCCD | 0.7 | 0.3 | 8.0 |
| Tributyltin chloride | 0.06 | 0.01 | 0.6 |
| NBD-Cl | 1.4 | 3.0 | 14 |
| TNP-ATP | 10 | 6.0 | 100 |
| AMP-PNP | 2.0 | 2.0 | 100 |
| Quercetin | 22 | 170 | 24 |
| N-ethylmaleimide | 50 | NI | 50 |
| | | (mM) | |
| $KNO_3$ | 50 | NI | NI |

[a] Data from refs. 26 and 28.
[b] NI, not inhibited.

## Plasma Membrane ATPase

### Identifying Characteristics

A distinctive type of ATPase has now been identified on the plasma membrane in several types of fungi: *N. crassa* [24, 124], *S. pombe* [48], *S. cerevisiae* [5, 108, 133, 166], *Candida tropicalis* [13], *Candida albicans* [77], and *Dictyostelium discoideum* [87, 109, 137]. Although the plasma membrane in these organisms has many functions and contains may different proteins, it is rich in ATPase activity (1–8 μmol/min/mg protein), having levels almost as high as that seen in specialized plasma membranes from brain or kidney cells. While explained in part by a higher intrinsic turnover number for the fungal ATPases, the high activity also reflects the fact that, even in these very generalized cells, the ATPase is a major component of the plasma membrane. An excellent review of the characteristics of the fungal plasma membrane ATPases appeared in 1981 [64]. In the present review we will mainly focus on the work from the last five years. Also of interest are two recently published reviews comparing fungal and plant plasma membrane ATPases [135, 136].

The simplest method for distinguishing the ATPase on the plasma membrane from the other ATPases is by assaying the effect of inhibitors (Table). The plasma membrane ATPase can be completely blocked by vanadate but is unaffected by azide and oligomycin, diagnostic inhibitors of the mitochondrial ATPase, or by nitrate, an inhibitor of the vacuolar ATPase [13, 23, 26, 48]. Vanadate inhibition is not an unambiguous indication of ATPase activity, however, because fungal cells also contain vanadate-sensitive phosphatases able to hydrolyze ATP [27]. Another characteristic, which distinguishes the plasma membrane ATPase both from mitochondrial and vacuolar ATPases and also from phosphatases, is its specificity for MgATP as substrate. Other trinucleotides, including GTP and ITP, are hydrolyzed at less than 10% of the rate measured with ATP [13, 30, 53, 108].

Important characteristics of the plasma membrane ATPase are its high $K_m$ for MgATP, its acidic pH optimum, and its relative insensitivity to monovalent salts. With two exceptions [5, 133] the $K_m$ values for the fungal plasma membrane ATPase have fallen in the range of 0.8–4.8 mM MgATP [13, 16, 19, 53, 76, 109]. This is quite high compared to $K_m$ values for the ATPases on other fungal membranes, as well as to values for plasma membrane ATPases of animal cells [39]. It is not unreasonably high, however, given the observation that the cytosolic ATP concentration in fungal cells is about 3 mM [140]. Conceivably, the high $K_m$ may allow the plasma membrane ATPase to be regulated by cellular ATP concentrations. The fungal plasma membrane ATPase also differs from other ATPases in its relatively low pH optimum for ATP hydrolysis, ranging from 6.7 for *N. crassa* [25] to 5.0 for *S. cerevisiae* [108]. For *N. crassa* this value is close to the measured cytosolic pH of 7.1 [117]. Because fungal cells usually secrete acid and grow very well in media of pH 4–5, the external face of the plasma

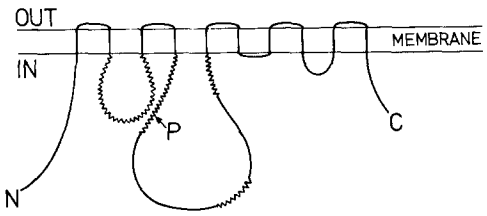


**Fig. 2.** Model for the structure of the polypeptide chain of the plasma membrane $H^+$-ATPase. The jagged lines represent areas of highly conserved sequenced. The position marked with *P* is the site of phosphorylation

membrane is generally exposed to a lower pH in vivo. Another important distinction between the ATPases on fungal plasma membranes versus animal cell plasma membranes is that the fungal enzyme appears to require no specific ions (other than $Mg^{2+}$) for activity. The inclusion of $K^+$ or $NH_4^+$ in the assay medium increases activity generally less than twofold [30, 48]. This small stimulation is presumably a reflection of the fact that the cytosol, in which part of the ATPase normally resides, contains about 200 mM $K^+$ [145].

## Primary Structure

The plasma membrane ATPase has been purified from *S. pombe*, *N. crassa*, and *S. cerevisiae* [1, 22, 52, 83, 90]. In all cases the enzyme has been solubilized by detergent, followed by fractionation *via* density gradient centrifugation. The most active preparations have specific activities of 25–95 μmol/min/mg protein [22, 52, 146]. No major improvements in this purification procedure have appeared since the first report for *S. pombe* [52]. Unfortunately, methods that produced highly purified ATPase from *S. pombe* proved less effective when applied to *S. cerevisiae*, an organism highly amenable to the manipulations of molecular genetics.

The purified plasma membrane ATPase is composed of a single type of polypeptide of mol wt approximately 100,000 [1, 22, 52, 90]. Lipids are required for enzymatic activity. There is no evidence for carbohydrate on the enzyme or for associated low mol wt polypeptides as reported for the $Na^+$,$K^+$-ATPase and $Ca^{2+}$-ATPase in animal cells [39, 127]. Considerable homology exists between the 100,000 mol wt polypeptides from different fungal sources, and antibodies to the plasma membrane ATPase react between species. For example, antibodies to the *S. pombe* enzyme inhibit the *C. tropicalis* ATPase [12]. Antibodies to the *N. crassa* enzyme bind to the 100,000 mol wt polypeptide of *S. pombe*, *S. cerevisiae*, and *Endothia parasitica*. Crossreactivity with nonfungal ATPases is less striking. Antibody to the *N. crassa* enzyme failed to bind to $Na^+$,$K^+$-ATPase or $Ca^{2+}$-ATPase of animal cells but appeared to crossreact weakly with the $H^+$,$K^+$-ATPase of gastric mucosa (K. Hager and C.W. Slayman, *personal communication*). In an analysis of plant plasma membranes the antibody raised to the *N. crassa* enzyme did not bind oat or potato membranes, but appeared to bind weakly to the plasma membrane ATPase in tomato [148, 150], and antibody to the *S. pombe* enzyme also appeared to bind to plant membranes [42].

The primary structure of the plasma membrane ATPase is now known for *S. cerevisiae* [138] and *N. crassa* [69]. In the last year, the genes coding for these enzymes have been cloned and sequenced. For both organisms cloning was achieved by using antibody to screen DNA inserted into the expression vector λgt11. The *N. crassa* gene contains four introns, while the *S. cerevisiae* gene has none. The two genes are highly homologous (74%) and code for proteins of 918 amino acids (*S. cerevisiae*) and 920 amino acids (*N. crassa*). For *N. crassa* this predicts a polypeptide of 99,886 mol wt, in good agreement with the earlier estimates derived from mobility on polyacrylamide gels.

The cloned DNA has been used to map the chromosomal location of the ATPase genes. In *N. crassa* the gene lies about two map units to the right of the mating-type locus and does not obviously correspond to any locus previously defined by mutation. In *S. cerevisiae*, however, the ATPase maps to the *pma* locus, which had earlier been suggested as the structural gene for the ATPase [63*a*, 154]. The *pma* locus had been identified by mutations that altered the plasma membrane ATPase, yielding enzyme with an elevated $K_m$ for MgATP and increased resistance to the inhibitor vanadate. Similar mutations have also been characterized in *S. pombe* [153].

By examining the hydrophobicity profile of the ATPase, a hypothesis for higher order structure has been proposed [69, 138], shown in Fig. 2. The polypeptide appears to have 8–10 membrane-spanning segments with very few residues exposed on the external surface. Three major portions of the polypeptide protrude into the cytosol: an N-terminal tail of about 115 amino acids and two large loops of about 130 and 300 amino acids. The larger loop contains the site which is phosphorylated during the reaction cycle and is therefore thought to be involved in ATP binding and hydrolysis. The two cytosolic loops are the most highly conserved parts of the protein. They contain the regions most homologous to the $Na^+$,$K^+$-ATPase and $Ca^{2+}$-ATPase of animal cells and to the $K^+$-ATPase of *E. coli* [88, 73*b*, 139]. Sequence data from all of the $E_1E_2$-type enzymes can be fit to essentially the same structural

model, suggesting the enzymes share a common mechanism of transport even though they pump different ions.

## Oligomeric Structure

Evidence for oligomeric structure has come primarily from data obtained with the *N. crassa* ATPase. Several different types of experiments indicate the *N. crassa* enzyme functions *in situ* as a dimer of two identical $M_r$ = 100,000 polypeptides [20]. Oligomeric structure is strongly suggested by the kinetic behavior of the ATPase, discussed below in the section on mechanism. The kinetic data have been interpreted as showing positive cooperativity between at least two substrate binding sites [19, 25]. Experiments in which the putative substrate binding site was labeled with *N*-ethylmaleimide indicated only one substrate binding site per $M_r$ = 100,000 polypeptide [35]. Therefore, we have hypothesized that the *N. crassa* enzyme is a dimer.

Two other types of data support this hypothesis. Freeze-fracture electron microscopy of vesicles reconstituted with ATPase showed particles with a diameter of 116 Å [106]. A sphere of this size would be more than large enough to account for two $M_r$ = 100,000 polypeptides. The electron micrographs should be interpreted with caution, however, because a substantial part of the particles observed can come from the metal used for shadowing. A direct measurement of the size of the ATPase was done by radiation inactivation [21, Fig. 3]. By irradiating plasma membranes with high energy electrons or gamma rays and using target theory, the size of the ATPase was determined *in situ*. The results showed a mol wt for the functional unit of approximately 220,000.

For the yeast plasma membrane ATPase there is less evidence that addresses oligomeric structure. Most reports show the yeast ATPase to exhibit simple Michaelis-Menten kinetic behavior [13, 16, 51, 53, 135] (but *see* discussion of ref. 83 below). During purification the *S. pombe* ATPase behaves as an octomer or decamer, but this is presumably because of detergent-induced aggregation [53]. After reconstitution of the *S. pombe* enzyme in lipid vesicles, maximal ATPase activity was observed at a calculated ratio of one vesicle per 100,000 mol wt polypeptide [56]. A recent study from G. Scarborough's laboratory also concluded that the reconstituted *N. crassa* ATPase could function as a monomer [64*a*].

## Function, in Vivo

The primary function of the fungal plasma membrane ATPase is to generate an electrochemical proton gradient across the membrane. The electrical component of the gradient can be very large in fungi; in fact, the first good evidence for the existence of the plasma membrane $H^+$-ATPase came from electrophysiological analysis. Using *N. crassa*, C.L. Slayman and collaborators have shown that fungal cells typically maintain an electrical potential of about 200 mV (interior negative) and a pH gradient of about 1.5 units (outside acidic) [65, 142]. By experimentally controlling the pH of the medium, they showed the electrical component and pH component of the gradient to be interconvertible (e.g., as the pH gradient gets smaller, the electrical potential gets larger); consequently, the total gradient is maintained at about 300 mV [118]. An electrochemical potential of this magnitude permits cells to transport nutrients against a concentration gradient of 100,000 : 1. Indeed, the major physiological role of the plasma membrane $H^+$-ATPase is probably to provide energy for nutrient-proton cotransport systems [58, 116, 119].

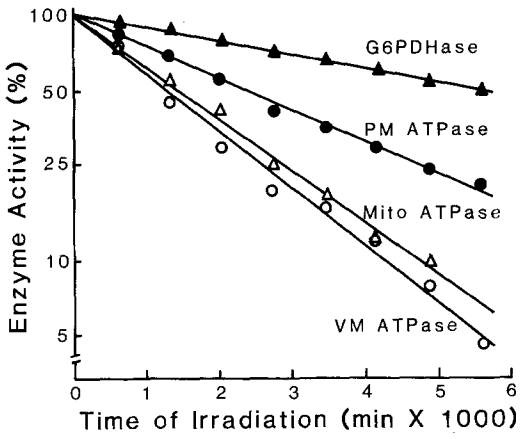


**Fig. 3.** Radiation inactivation of *N. crassa* $H^+$-ATPases. Glucose-6-phosphate dehydrogenase (G6PDHase), mol wt = 103,700, was used as a standard. For details *see* refs. 21 and 31

By inserting microelectrons into *N. crassa*, the rate of proton pumping has been measured in vivo. The ATPase generates a current of about 20 $\mu A/cm^2$, corresponding to 200 pmol of protons per $cm^2$ [65]. The density of pumps in the membrane can be estimated by using the turnover rate of the purified enzyme. The best preparations of $H^+$-ATPase have a specific activity of ~100 $\mu$mol/min/mg of protein or 330 protons per sec per enzyme (assuming a dimeric enzyme and a stoichiometry of one proton per ATP hydrolyzed) [22]. If we picture the membrane as a grid of 100 Å squares and assume the ATPase is about 100 Å in diameter, one-third of the squares would be occupied by ATPases to account for the measured current. Given that a number of simplifying assumptions are made in this calcula-

tion, this is a reasonable conclusion and suggests that the purified ATPase is working at rates comparable to those measured *in situ*. It is also consistent with the observation that the ATPase is one of the most abundant proteins in isolated plasma membranes [22, 30].

Another plausible function of the plasma membrane $H^+$-ATPase is to control intracellular pH. Using microelectrons to monitor both the pH gradient and the electrical potential, Sanders and Slayman [117, 118] showed that, while proton flux through the $H^+$-ATPase is an important component of the acid-base balance in the cell, regulation of pH *per se* does not depend on the $H^+$-ATPase. For example, inhibiting the $H^+$-ATPase with vanadate caused a rapid depolarization of the membrane potential but had little effect on intracellular pH. These investigators concluded that oxidative metabolism, and not the direct transport of protons, exerts the major control over cellular pH. Because the $H^+$-ATPase may consume 38–52% of the ATP produced by the cell [65], this is not surprising. There may simply be insufficient ATP for the cell to get rid of excess acidity by increasing the activity of the $H^+$-ATPase.

The function of the yeast plasma membrane $H^+$-ATPase has been more difficult to analyze in vivo, because the small size of the cells generally precludes the use of microelectrodes. Other types of experiments, however, which used NMR or measured the distribution of permeant ions, indicate that the $H^+$-ATPase generates an electrochemical gradient much like that observed in *N. crassa* [73*a*, 74, 97]. Furthermore, as in *N. crassa* the $H^+$-ATPase is a major component of the yeast plasma membrane [64].

## Function, in Vitro

Analysis of transport in vitro, first performed with purified plasma membranes, demonstrated the dependence of proton pumping on ATP, with characteristics matching ATP hydrolytic activity of membranes [4, 14, 38, 123]. The conclusion that the ATPase is a proton pump has been reinforced by recent experiments with purified yeast ATPase reconstituted into liposomes. An examination of the specificity of the lipid requirement for maximal ATP hydrolytic activity revealed that most phospholipids work well and mixtures such as asolectin yield nearly maximal rates of activity [54–56]. Using this information, the *S. pombe* plasma membrane ATPase was reconstituted into liposomes by both freeze thaw and cholate dialysis procedures [55, 57, 160]. Measured by either pH electrodes or quenching of the fluorescent dye ACMA, the reconstituted ATPase behaved like an electrogenic proton pump. In the presence of permeant anions, which prevented the build-up of an electrical potential, the ATPase was observed to acidify the interior of vesicles by as much as 3.6 pH units [55]. Electrogenic proton pumping was also observed with reconstituted ATPase from *S. cerevisiae*, as assayed by $^{32}$P-ATP exchange [91].

Initially, the $^{32}$P-ATP exchange experiments were also interpreted as providing evidence for an electroneutral $K^+$-$H^+$ exchange reaction because of the inability of CCCP to inhibit completely [91]. Subsequent experiments with different uncouplers showed complete inhibition of $^{32}$P-ATP exchange, however, and are thus consistent with the ATPases transporting only protons [135]. There has also been one report of ATP-driven $K^+$ efflux from liposomes reconstituted with purified ATPase from *S. pombe* [159]. These data were interpreted as evidence for direct involvement of the ATPase in $K^+$ transport, but it is difficult to rule out passive $K^+$ movements in response to membrane potential.

The plasma membrane ATPase of *N. crassa* has been reconstituted into asolectin liposomes, and the orientation of the enzyme evaluated by electron microscopy [106] or tryptic digestion [127]. Most of the reconstituted vesicles appear to be everted, i.e., with the catalytic moiety of the ATPase on the exterior. By quantitatively assessing the amount of active enzyme and the amount and size of radioactive polypeptide incorporated, it was concluded that only 100,000 mol wt polypeptide is required to reconstitute the proton pump [127].

As with the yeast ATPases, the *N. crassa* enzyme appears to be an electrogenic proton pump. The fluorescent dyes ACMA and acridine orange were used to monitor acidification of the interior of vesicles; a pH gradient of about 2.5 units was observed [106, 127]. The fluorescence of oxonol V was used to demonstrate the generation of an electrical membrane potential [106]. Fluorescence changes were also exploited to determine the stoichiometry of the pump in vitro [107]. After establishing a steady state in which the rate of proton pumping was balanced by the rate of proton leakage, EDTA was added to chelate Mg, thus depleting the assay of MgATP. The pump stopped immediately, and the rate of proton leakage was determined by measuring the change in fluorescence. The data showed one proton pumped for each ATP hydrolyzed, in agreement with the estimate made by electrophysiological measurements on living cells [65, 162].

## Mechanism

Much of the current work with the plasma membrane ATPase is directed toward a biochemical de-

scription of the coupling of ATP hydrolysis to ion translocation [125, 126]. Understanding the fungal enzyme may provide information broadly applicable to many eucaryotic plasma membrane ATPases, because these enzymes are so similar in structure and mechanism. All the $E_1E_2$ ATPases go through a reaction cycle during which the phosphate from ATP is covalently bound to an aspartyl residue at the active site [6, 7, 46, 62, 90]. The working hypothesis is that phosphorylation at the active site causes the enzyme to become energetically charged, and that a subsequent conformational change in the protein allows the actual movement of the ion.

When compared to phosphorylation of other ATPases, the phosphorylation of fungal plasma membrane ATPases differs in some details. Relatively high concentrations of $^{32}$P-ATP (above 1 mM) are required to phosphorylate the enzymes in vitro, a reflection of the relatively high $K_m$ of the fungal plasma membrane ATPases for MgATP [7, 46]. Another important difference is that, unlike the $Na^+$,$K^+$-ATPase or $Ca^{2+}$-ATPase, the reaction cycle cannot be stopped after the phosphorylation step, because there is no effective way of removing the transported ion, the proton, from the reaction mixture. Thus, in a population of ATPases, only a small percent can be trapped in the phosphorylated state. The fact that after phosphorylation the enzyme immediately moves through subsequent steps in the reaction cycle may also explain why it has been difficult to phosphorylate fungal ATPases with $^{32}$P-phosphate (as compared to $^{32}$P-ATP) [7].

By measuring the exchange of $^{18}$O between water and phosphate catalyzed by the plasma membrane ATPase from *S. pombe*, it has been possible to study the binding of phosphate to the enzyme and to describe several additional steps in the reaction cycle [8, 9]. Phosphate labeled with $^{18}$O was shown to bind to the ATPase, but with a low affinity ($K_m$ = 117 mM). In the active site the phosphate can tumble and repeatedly form and break covalent bonds (presumably with the carboxyl of the aspartyl residue) as evidenced by the loss of $^{18}$O from the phosphate molecule. The rate of $^{18}$O exchange was stimulated sevenfold when assayed during ATP hydrolysis. These data were interpreted as supportive of the reaction cycle shown in Fig. 4. The ATPase behaves as though it cycles through two isomeric forms, one that binds ATP and the other that binds phosphate. Rate constants were derived for several steps in the pathway [8].

The rate equation for the above reaction cycle yields a hyperbolic curve when reaction velocity is plotted against substrate concentration; indeed, empirical measurements of $v$ versus [S] have generally fit hyperbolic curves for yeast plasma membrane

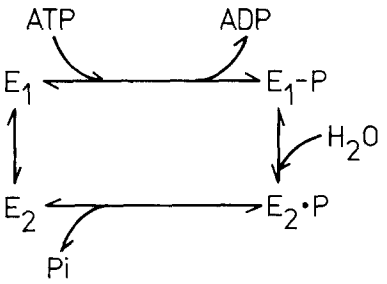


**Fig. 4.** Reaction cycle of the plasma membrane ATPase

ATPases [64]. Furthermore, varying ATP concentrations during measurements of $^{18}$O exchange gave no indication of cooperativity [8, 9].

A recent reexamination of the kinetic behavior of the *S. cerevisiae* ATPase, using purified enzyme reconstituted with phosphatidylserine, showed the enzyme to exhibit sigmoid $v$ versus [S] curves [83]. The data were fit well by a model with cooperativity between active sites, or alternatively, by a model assuming different conformational states of the enzyme, which could hydrolyze ATP by parallel pathways. These results are very similar to data obtained with the *N. crassa* plasma membrane ATPase. The $v$ versus [S] curve for the *N. crassa* enzyme is sigmoid, and the Hill coefficient of the data is 2, indicative of an enzyme with two or more interacting active sites [19]. ATP not complexed with $Mg^{2+}$ is not hydrolyzed but appears to activate the *N. crassa* ATPase. (The true substrate for the ATPases is MgATP [37, 83].) The effects of vanadate on the *N. crassa* enzyme also suggest cooperative behavior [19, 25]. Vanadate is a potent inhibitor [$K_i$ = 1 μM] of ATPase activity when assayed at saturating MgATP concentrations [23, 25]. However, when assayed at MgATP concentrations below the $K_{0.5}$, vanadate has a surprising biphasic effect, stimulating activity at low vanadate concentrations (1 μM) and inhibiting at higher concentrations. If vanadate binds to the active site of the ATPase, its stimulatory effect is readily explained if the ATPase has two active sites. At low concentrations of MgATP, vanadate presumably binds one active site and promotes the binding and hydrolysis of MgATP at a second active site [19].

Further evidence of subunit interaction comes from analysis of DCCD binding. Complete inactivation of ATPase activity occurred when only 0.4 molecules of $^{14}$C-DCCD were bound for each 100,000 mol wt polypeptide [149]. Conceivably, a rearrangement reaction could have displaced the radioactive label from the enzyme, but the most straightforward interpretation is that the *N. crassa* ATPase is a dimer and interaction between polypeptides is obligatory for catalysis. This hypothesis

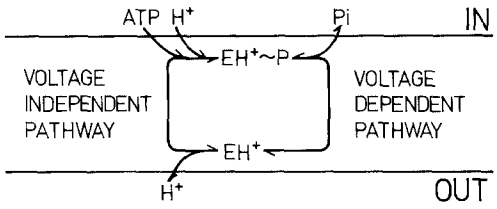


**Fig. 5.** Two-state reaction-kinetic model for the plasma membrane ATPase

would also explain the need for relatively high concentrations of $^{32}$P-ATP in phosphorylation experiments. The question of whether the ATPases have one or more active sites is being hotly debated among workers studying the $Na^+$,$K^+$-ATPase and $Ca^{2+}$-ATPase of animal cells (*see* references in 19, 20), and this controversy is obviously mirrored in the literature on the fungal plasma membrane ATPases.

A major complication in the kinetic analysis of all ATPases is that $Mg^{2+}$ is required as substrate (MgATP) but may also affect activity by binding to other sites on the enzyme. In fact, the sigmoid behavior of the *N. crassa* ATPase can be fit fairly well to a model with one MgATP binding site and two $Mg^{2+}$ binding sites [37]. A good illustration that $Mg^{2+}$ *per se* can significantly affect the activity was the observation that $^{18}$O exchange between water and phosphate, described above, did not require ATP but did require $Mg^{2+}$ [8, 9]. Although the exact nature of the $Mg^{2+}$ bindings sites on the ATPase is not known, a consensus has developed as to their kinetic descriptions. First, both yeast and *N. crassa* ATPases have a high affinity site ($K_m$ = 5–20 $\mu$M), which activates ATPase activity and protects against inhibition by N-ethylmaleimide. Second, the ATPases have a low affinity site ($K_m$ 0.4–3.5 mM), which lowers ATPase activity and promotes inhibition by N-ethylmaleimide [3, 5, 36, 37, 76, 83]. $Mg^{2+}$ binding also appears to facilitate the binding of vanadate [16].

The best evidence that binding of $Mg^{2+}$ and substrate actually cause a conformational change in the ATPase comes from studies of trypsin fragmentation of the enzyme. For example, the addition of 10 mM $Mg^{2+}$ significantly protects the *N. crassa* ATPase from inactivation by trypsin and causes the appearance of a unique sized fragment of about 91,000 mol wt [36]. In another study using ATPase from the *slime* mutant of *N. crassa*, clearly different fragmentation patterns were seen when the enzyme was digested in the presence of $Mg^{2+}$ plus vanadate or a nonhydrolyzable analog of ATP, as opposed to digestion in the presence of MgADP [2]. These experiments strongly suggest that ATPase can exist in several discrete conformational states, dependent on the type of ligands bound.

By using ligands that bind covalently and inhibit the ATPase, the structure of the active site has been investigated. The data thus far point to the involvement of three amino acid residues. First, the aspartyl residue phosphorylated by ATP is almost certainly involved. Second, analysis of the inhibition caused by N-ethylmaleimide indicates that a cysteine residue may be at the active site [34–36]. The binding of $^{14}$C-N-ethylmaleimide to a single specific tryptic fragment of the *N. crassa* ATPase occurs with psuedo-first order kinetics. A quantitative assessment of the concentrations of MgATP and MgADP needed to prevent NEM binding yielded dissociation constants essentially equal to the $K_m$ for MgATP as substrate or the $K_i$ for MgADP as competitive inhibitor. A third candidate is an arginine residue. Phenylglyoxal and 2,3-butanedione, arginine-specific reagents, inactivate the ATPase with kinetics that suggest one molecule bound per active site. The binding can be prevented by the presence of MgATP [49, 82].

For the *N. crassa* plasma membrane ATPase the biochemical analysis of mechanism has been complemented by electrophysiological analysis. To understand the ATPase it is essential to take into account the fact that the transport reaction is electrogenic and takes place in vivo by moving a proton against an opposing electrical field. By inserting microelectrodes into living *N. crassa* cells, C.L. Slayman and colleagues [15, 66, 70, 162] have simultaneously measured the electrical potential across the plasma membrane and the pH on both sides. Furthermore, they have used the electrodes to pass current or inject acid, thus permitting a precise and fast variation in the components of the electrochemical potential [117, 118]. The rate at which the ATPase pumps protons under varying conditions can be derived from measurements of the current across the plasma membrane.

To determine how the electrical potential affects the ATPase, the current was measured over a range of membrane potentials [66, 70, 115, 144]. The data, expressed as a current-voltage curve, could be described by a simple reaction kinetics model shown in Fig. 5; the enzyme can exist in two states and the transition between states occurs via voltage-sensitive and voltage-insensitive pathways. Several important predictions have come from this model. First, the energy-requiring step in the reaction cycle is the transport of charge. Second, the fast step is the release of the proton on the external surface. Third, the rate-controlling step is recycling the enzyme, i.e., ATP or proton binding.

Slayman et al. have extended the analysis by changing additional parameters, perhaps most importantly the internal and external pH [115, 143, 144]. Current-voltage curves from these experiments were described by models with additional states for the enzyme in the reaction cycle. From the models, tentative values have been calculated for the equilibrium dissociation constants of the proton from the ATPase. At the membrane inner surface the $pK_i$ = 5.4. (Cytosolic pH is typically 7.2.) At the outer surface the $pK_o$ = 2.9. (Medium in which *N. crassa* grows is typically pH 5.) Thus the enzyme exists predominantly in the undissociated form at both surfaces of the membrane.

Biophysical experiments in vivo have yielded information that would be very difficult to obtain with the isolated ATPase. Because what we have summarized here does not do justice to the analysis presented, two very readable reviews, refs. 115 and 144, are recommended.

Overall, the view of the enzyme most consistent with both biochemical and biophysical analysis is the following. Phosphorylation via MgATP yields a high-energy conformation of the enzyme. A proton binds on the cytosolic side, and a conformational change carries the charge through the membrane (or moves the membrane field around the charge [144]). The proton is immediately released at the outer surface. Through slower processes the enzyme is dephosphorylated and relaxes back to the conformation that binds ATP.

### Regulation

The electrophysiological data contain the clearest indication that the rate of proton pumping can be quickly regulated. For example, exposure of *N. crassa* to osmotic shock causes a rapid decline in the electrical current across the membrane, apparently because the $H^+$-ATPase slows down. Within 30–40 sec the rate of $H^+$ leakage across the membrane also declines, thus restoring the membrane potential to its previous level [141]. Earlier data, which showed the membrane potential could be made to oscillate, strongly suggest the pump is subject to some kind of feedback regulation [67]. The biochemical mechanism of regulation is not understood. There are several indications of phosphorylation of $E_1E_2$-type ATPases by kinases [96, 122, 168], and the activity of the ATPase appears to change with different growth conditions [134]. The significance of the phosphorylation and the mode of regulation are major unanswered questions concerning the plasma membrane $H^+$-ATPase.

## The Vacuolar Membrane ATPase

During the last five years good methods have been developed for the isolation of vacuoles from *S. cerevisiae*, *S. carlsbergensis*, and *N. crassa* [27, 29, 44, 80, 101*a*]. Two functions are known for the vacuoles. First, they store high concentrations of polyphosphate, arginine, ornithine, and lysine, and have an important role in the regulation of amino acid metabolism [43, 45, 47, 78, 156, 163–165, 169]. Second, the vacuoles presumably function as lysosomes, because they contain most of the proteases and other hydrolytic enzymes found in the cell [95, 156]. By lysing vacuoles and washing with chelators such as EDTA or EGTA, the membranes can be cleanly separated from the internal contents, and membrane proteins can be analyzed without significant proteolysis [27, 29, 152]. The yield of membranes is relatively low. In *N. crassa* 1 mg of vacuolar membrane protein is obtained for each 5 g (dry wt) of cells; however, vacuolar membranes have high levels of ATPase activity, 3–5 μmol/min/mg, indicating the ATPase is a major component [29].

### Identifying Characteristics

Careful examination of pH optima, substrate specificity, and inhibitor sensitivities has shown that the vacuolar ATPase is distinctly different from plasma membrane and mitochondrial ATPases. The vacuolar enzyme is most active at neutral pH (7.0 to 7.5 [27, 80]) in contrast to the plasma membrane ATPase, which has an acidic pH optimum (5.0 to 6.7, ref. 64) and the mitochondrial ATPase which has an alkaline pH optimum (8 to 9 [48, 89]).

In its ability to hydrolyze different nucleotides the vacuolar ATPase is fairly specific for ATP but, like the mitochondrial ATPase, also appears to hydrolyze GTP and ITP and, to a lesser extent, UTP and CTP. The membrane-bound form, for example, of the vacuolar ATPase from *S. cerevisiae* has essentially equal activities with GTP and ATP [80]. With *N. crassa* vacuolar membranes GTP and ITP are hydrolyzed at 30% the rate for ATP (C. Franklin and E.J. Bowman, *unpublished*). In partially purified vacuolar ATPase the preference for ATP is more pronounced. Data obtained with *S. carlsbergensis* vacuolar membranes argue that the ATPase and GTPase activities can be partially separated, suggesting they are due to separate enzymes [84, 85, 101*a*]. However, the most highly purified preparations of vacuolar ATPase from *S. cerevisiae* [152] and *N. crassa* (C. Franklin and E.J. Bowman, *unpublished*) still retain significant levels of GTPase activity, 25–50% of that seen with ATP. Conceiv-

ably, in the intact membranes part of the GTPase activity is due to phosphatases shown to be present in vacuoles [27].

As with other ATPases the true substate of the enzyme is probably ATP complexed to $Mg^{2+}$. In vitro either $Mg^{2+}$ or $Mn^{2+}$ is required for activity [27, 80]. Activity is about 50% less with $Co^{2+}$ and about 70% less with $Ca^{2+}$ or $Zn^{2+}$. This order of preference for divalent cations is similar to that seen for the mitochondrial ATPase [89]. By contrast, the plasma membrane ATPase is more specific in both nucleotide and metal requirements [64].

The sensitivity of the vacuolar ATPase to inhibitors clearly defines it as a unique type (Table and refs. 85, 152). Oligomycin and azide, inhibitors of the mitochondrial ATPase, and vanadate and miconazole, inhibitors of the plasma membrane ATPase, have no effect on the vacuolar ATPase. Like the other proton-pumping ATPases, the vacuolar enzyme is inhibited by dicyclohexylcarbodiimide (DCCD) and tributyltin chloride, agents reported to bind to the putative proton channel in other ATPases. Several other molecules inhibit the vacuolar ATPase presumably because they resemble ATP. These include NBD-Cl, an adenine analog; TNP-ATP, a fluorescent derivative of ATP; and AMP-PNP, an ATP analog. Although the mode of action of quercetin is not understood, conceivably it inhibits because its flavanoid ring mimics part of the structure of ATP. Diethylstilbestrol inhibits the vacuolar ATPase; again, its mode of action is not understood.

For the compounds described above, inhibition of the vacuolar ATPase is quantitatively different from inhibition of plasma membrane or mitochondrial ATPases, but none of the inhibitors is specific for the vacuolar enzyme. The molecules closest to being specific inhibitors are $KNO_3$ and KSCN [26]. In *N. crassa*, for example, 50 mM $KNO_3$ inhibits the vacuolar ATPase by 50% but has no effect on plasma membrane or mitochondrial ATPases. Unfortunately, high levels (above 100 mM) of $KNO_3$ are required to give complete inhibition, thus limiting the usefulness of this compound. Nitrate would seem to be an unlikely effector of the ATPase in vivo, because fungi are routinely grown in media containing 25 mM nitrate [161].

## Structure

The vacuolar ATPase appears to be firmly embedded in the membrane, and ATPase activity can be solubilized only with the use of detergents [31, 85, 152]. Purification of the enzyme is difficult, apparently because delipidation by detergents causes a loss of activity that cannot, as yet, always be restored by adding back lipids [31]. Uchida et al. [152] have achieved the best results. After solubilizing the vacuolar ATPase from *S. cerevisiae* and purifying it on a glycerol gradient, they were able to reactivate the enzyme by adding asolectin. The peak gradient fraction in their procedure had a fivefold higher specific activity, compared to the initial washed membranes (18 μmol/min/mg versus 3.3 μmol/min/mg).

Three reports of purification of the vacuolar ATPase have appeared, all with similar protocols. The membranes are washed in EDTA or EGTA, solubilized in detergent (zwitterionic detergents seems to be most useful) in the presence of glycerol, and fractionated by centrifugation on glycerol or sucrose gradients [31, 85, 152]. The *S. carlsbergensis* enzyme was purified further by nondenaturing gel electrophoresis [85]. In all three preparations the major components of the vacuolar ATPase are two large polypeptides (89 and 64 kD in *S. cerevisiae*, 70 and 62 kD in *N. crassa*, and 75 and 64 kD in *S. carlsbergensis*) and a small DCCD-binding polypeptide (*see below*). The extent to which these major polypeptides really differ in size cannot be determined until they are directly compared in the same gel system.

The vacuolar ATPase may contain additional polypeptides. It is noteworthy that in purified preparations of mitochondrial ATPase, which contain at least 10 subunits, some of the polypeptides produce very faint bands on polyacrylamide gels [102]. All the vacuolar ATPase preparations contain minor components, as assayed by staining of polyacrylamide gels. In our preparations from *N. crassa*, for example, bands at 52, 29, 27, and 16 kD consistently copurify with activity [31]. The data for *S. carlsbergensis* show copurification of several small polypeptides [85], and the most highly purified plant vacuolar ATPases also contain minor components [92–94, 110]. By contrast, Uchida et al. [152] reported that in *S. cerevisiae* the minor components are found in different stoichiometries in different preparations and can be eliminated by chromatography with Blue Sepharose CL-6B.

In addition to its being the most prominent subunit, several other lines of evidence argue in favor of the largest polypeptide as a component of the vacuolar ATPase. In experiments with the *N. crassa* enzyme $^{14}C$-labeled N-ethylmaleimide and NBD-Cl, inhibitors of vacuolar ATPase activity, were found to bind covalently to the 70 kD polypeptide [31]. The amount of inhibitor bound was reduced if the incubation mixture contained MgATP or MgADP. Thiocyanate also prevented binding of NBD-Cl.

Comparison of the purified fungal vacuolar ATPases with ATPases purified from plant vacuoles is consistent with polypeptides of approximately 70 and 60 kD as major subunits of the enzyme. Four different laboratories have reported purification of the plant vacuolar ATPase, and all report subunits of approximately 70 and 60 kD as the major components [92–94, 110]. A side-by-side comparison on an SDS polyacrylamide gel of the vacuolar ATPases from *N. crassa* and *Zea mays* verified that the two large subunits are very similar in size [31].

Perhaps the strongest evidence that the largest polypeptide is part of the ATPase comes from immunological studies. A polyclonal antibody, raised against the 72 kD polypeptide from *Z. mays*, has been shown to inhibit *Z. mays* vacuolar ATPase activity [92*a*]. By immunoblot analysis, this antibody reacts with the *Z. mays* 72 kD polypeptide and, most strikingly, reacts specifically with the 70 kD polypeptide of the *N. crassa* vacuolar ATPase [31]. (The antibody does not inhibit the *N. crassa* enzyme.) Antibody raised to the 70 kD polypeptide from *N. crassa* also crossreacts with the 72 kD polypeptide from *Z. mays* on immunoblots (E.J. Bowman, *unpublished results*). Antibodies to the 62 kD polypeptides have been prepared for both organisms; these antibodies show no crossreactivity between species. As part of the immunological studies, antibodies to the plasma membrane ATPase and mitochondrial ATPase from *N. crassa*, and the $F_0F_1$ ATPase from *Escherichia coli*, were tested for crossreactivity with the *N. crassa* vacuolar ATPase. Immunoblot analysis revealed no binding of these antibodies to any component of the vacuolar enzyme [31]. The activity of the *S. cerevisiae* vacuolar ATPase was also found to be unaffected by antibody raised against and inhibitory to *S. cerevisiae* mitochondrial ATPase [152].

As mentioned earlier the vacuolar ATPase is inhibited by DCCD (26, 27, 80, 85]. Experiments with mutants of *N. crassa* indicated the DCCD binding component of the vacuolar ATPase must be different from that of the mitochondrial ATPase [26]. The *C93* mutation rendered the mitochondrial ATPase essentially resistant to inhibition by DCCD. A second mutation, *oli 16-16*, located in the structural gene for the DCCD-binding polypeptide of the mitochondrial ATPase, caused an eightfold decrease in the amount of DCCD required to inhibit the mitochondrial ATPase. Neither mutation had any effect on the sensitivity of the vacuolar ATPase to DCCD. The DCCD-binding polypeptide in the vacuolar ATPase has been identified by incubating membranes or purified enzyme with radioactive DCCD and subsequently separating the polypeptides on polyacrylamide gels [26, 31, 85, 152]. The DCCD binds covalently to a small polypeptide that is difficult to see in Coomassie-stained gels. In *N. crassa* the polypeptide has an apparent size of ~15 kD, and in *S. cerevisiae*, of ~19 kD. A smaller polypeptide of ~9 kD was reported to be the most prominently labeled band in *S. carlsbergensis*, although in some preparations from this yeast a 16-kD band was also strongly labeled [85]. Whether multiple copies of these small subunits are present, as occurs in the mitochondrial ATPase, is not known.

Overall, the emerging picture for the structure of the vacuolar ATPase is that of a large, multisubunit complex. One of the few ways to measure the size of the complex *in situ* is by radiation inactivation. This technique has been applied to both the membrane-bound form and the purified preparation of the *N. crassa* vacuolar ATPase [31]. As shown in Fig. 3, the vacuolar ATPase appears to be the largest of the three $H^+$-ATPases. Using the average value from ten experiments, we found it to be about 13% larger than the mitochondrial ATPase, with an approximate mol wt of 520,000. Similarly, the vacuolar ATPase from plants appears to be a large enzyme ($M_r$ = 400,000) by this method [92].

## Mechanism

Little is known about the mechanism of the vacuolar ATPase. The kinetics of activity as a function of substrate concentration are fit well by the Michaelis-Menten equation and thus do not indicate interaction between subunits [27, 80]. (It should be noted that the kinetics of the mitochondrial ATPase are also fit well by the Michaelis-Menten equation, even though there is strong evidence for cooperativity between subunits [68, 104, 105].) In the case of the enzyme from *S. carlsbergensis* a phosphorylated intermediate of the type seen in the plasma membrane ATPases was looked for but not found [85]. While the presence of a small DCCD-binding protein suggests proton transport and ATP hydrolysis occur on different subunits, further structural information is clearly required.

## Function

Just as the plasma membrane ATPase generates an electrochemical gradient across the plasma membrane, the vacuolar ATPase is postulated to generate a gradient across the vacuolar membrane. Measurements of the internal pH of the *N. crassa* vacuole, observed in vivo with NMR, showed it to be an acidic compartment, pH 6.1, while the surrounding cytosol was neutral, pH 7.1 [81]. Less is known about the electrical gradient, but, in a few instances, measurements with microelectrodes

have shown a slightly positive value (25–40 mV) for the interior of the vacuole relative to the cytosol (C.L. Slayman, *personal communication*). The total electrochemical potential thus appears to be 100–110 mV. Under some growth conditions small molecules such as arginine and ornthine accumulate in vacuoles at concentrations 1000-fold higher than in the cytosol [47, 163, 164]. Their uptake is presumably driven by the difference of electrochemical potential [50]. If all the small molecules were in free solution, 150 mV would not be sufficient to achieve a 1000-fold gradient. This apparent discrepancy is probably explained by the binding of many of these positively charged amino acids to polyphosphate, which is present at very high levels in the vacuoles [43, 45].

Experiments with vacuolar membrane vesicles have demonstrated the generation of an electrochemical gradient in vitro. Proton transport was assayed by measuring fluorescence changes of 9-aminoacridine and quinacrine, compounds that accumulate in acidic compartments. By this assay, proton pumping activity had the same substrate specificity, substrate affinity, and inhibitor sensitivity as ATPase activity [28, 80, 84, 99]. The magnitude of the electrochemical potential was measured with vesicles from *S. cerevisiae* by the technique of flow dialysis [80]. These vesicles showed a pH gradient of 1.7 units and an electrical gradient of 75 mV, giving a total gradient equivalent to 177 mV.

Thus, the size of the gradient across the vacuolar membrane appears to be about half that across the plasma membrane. For the vacuolar pump to be most efficient it should pump at least two protons for each ATP hydrolyzed. The stoichiometry of the pump has not yet been measured in vivo or in vitro.

In experiments with plant vacuolar membrane vesicles some of the data have been interpreted to indicate an obligatory uptake of $Cl^-$ with protons [11, 41]. Clearly, the $Cl^-/H^+$ stoichiometry cannot always be 1:1, because these same experiments also show proton pumping is electrogenic. The question of obligatory coupling to $Cl^-$ transport has not yet been addressed with fungal vacuolar membranes. All published experiments have included $Cl^-$ when assaying proton transport [28, 80, 84, 99]. For *N. crassa* we have found that proton pumping, as measured by quinacrine fluorescence, does occur but is significantly lower in the absence of $Cl^-$. The simplest explanation may be that the $Cl^-$ is the most permeable of the anions tested and that chloride permits the build-up of a larger pH gradient by partially neutralizing the electrical gradient generated by the proton pump.

Vacuolar membrane vesicles have been used to demonstrate ATP-dependent cation uptake and amino acid uptake [99, 100, 101*b*, 170]. As described by kinetic differences, there appear to be a large number of amino acid transport systems (seven in *S. cerevisiae*); all of these are different from the amino acid transport systems in the plasma membrane [120, 121]. Transport into vacuoles is completely blocked by protonophores, indicating uptake is not directly driven by ATP but rather is driven by the difference of electrochemical potential. Presumably, the carriers are proton antiport systems. The basic organization of active transport across the vacuolar membrane is the same as in the plasma membrane. An ATP-driven proton pump generates a gradient used by a series of proton-coupled nutrient carriers. The relative direction of proton and nutrient movement, and the structure and kinetic properties of the proteins, however, are different for the two membranes.

## Conclusions

The three $H^+$-ATPases we have described by no means represent all the $H^+$-ATPases in the eucaryotic cell. There have been reports on $H^+$-ATPases on virtually every membrane, including those of coated vesicles [60, 167], Golgi [40], lysosomes [128], and a variety of intracellular storage vesicles [63, 98, 112].

In the fungi the mitochondrial, plasma membrane, and vacuolar membrane ATPases are particularly amenable to study, because they are major components of their respective membranes. This has permitted good progress to be made in biochemically characterizing these enzymes. The application of unique electrophysiological and genetic methodology will continue to make the fungi an excellent source of information on these enzymes.

A major goal is to see if the three ATPases represent different evolutionary answers as to how to move a proton across a membrane. Are the different ATPases really different in their underlying mechanisms, or do their structural and kinetic differences represent minor variations of a fundamental mode of ion transport, common to all cells?

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