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MINI-REVIEW

Molecular Properties of the Fungal Plasma-Membrane [H^+]-ATPase

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Abstract

The fungal plasma membrane contains a proton-translocating ATPase that is closely related, both structurally and functionally, to the [Na^+, K^+]-, [H^+, K^+]-, and [Ca^{2+}]-ATPases of animal cells, the plasma-membrane [H^+]-ATPase of higher plants, and several bacterial cation-transporting ATPases. This review summarizes currently available information on the molecular genetics, protein structure, and reaction cycle of the fungal enzyme. Recent efforts to dissect structure–function relationships are also discussed.

Key Words: ATPase; [H^+]-ATPase; proton transport; *Neurospora crassa*; *Saccharomyces cerevisiae*; *Schizosaccharomyces pombe*.

Introduction

The [H^+]-ATPase of the fungal plasma membrane pumps protons out of the cell, energizing the membrane for the uptake of sugars, amino acids, and inorganic ions. During recent years, the fungal ATPase has become a well-studied example of a primary ion pump. Gene cloning has established that it belongs to the E_1E_2 or P-group of cation-translocation ATPases, found ubiquitously in the plasma membranes of animal and plant cells and in some bacteria as well. Purification and reconstitution of the ATPase polypeptide has led to studies of catalysis and transport, and electrophysiological analysis has provided significant information about the charge transfer step of the reaction cycle. In the sections that follow, each of these topics will

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Table I. Fungal Plasma-Membrane [H^+]-ATPases

Species	Gene		Protein	
	Name	Map location	Residues	MW
Neurospora crassa	*pma1*	I (left)	920	99,886
Saccharomyces cerevisiae	*pma1*	VII	918	99,530
	pma2	XVI (left)	947	102,157
Schizosaccharomyces pombe	*pma1*	I	919	99,769

be reviewed, with special emphasis on molecular properties and structure–function relationships.

The pma Gene Family

The genes encoding the plasma-membrane [H^+]-ATPases of *Saccharomyces cerevisiae, Schizosaccharomyces pombe*, and *Neurospora crassa* have been defined by both classical and molecular techniques (Table I). Initially, Ulaszewski *et al.* (1983) isolated several allelic mutants of *S. cerevisiae* that were resistant to the antifungal compound Dio-9 and that exhibited qualitative changes in plasma-membrane ATPase activity, and they named the gene *pma1*. Additional *pma1* mutants have been selected in *S. cerevisiae* using a different drug, hygromycin B (McCusker *et al.*, 1987), and in *S. pombe* using Dio-9 (Ulaszewski *et al.*, 1986). In each case, the mechanism of resistance appears to be indirect, with a reduction in ATPase activity leading to a decrease in drug uptake. Consistent with this notion, *pma1* mutants are cross-resistant to an array of growth inhibitors that are positively charged but functionally unrelated (decamethylene-diguanidine, ethidium bromide, *N,N′-p*-xylylidene-bis-aminoguandidine, miconazole, and L-lysine). The *pma1* gene of *S. cerevisiae* maps to chromosome VII, very near *leu1* (Ulaszewski *et al.*, 1987a), and the corresponding gene of *S. pombe* maps to chromosome I, about 5 centimorgans from *cyh1* (Ulaszewski *et al.*, 1986).

Concurrently with the mutational studies, the *pma1* genes of *S. cerevisiae* and *N. crassa* have been cloned by immunoscreening of λgt11 expression libraries (Serrano *et al.*, 1986a; Hager *et al.*, 1986; Addison, 1986), and the *pma1* gene of *S. pombe* has been isolated by hybridization with the *S. cerevisiae* gene (Ghislain *et al.*, 1987). As expected, tight linkage of the *S. cerevisiae* gene to *leu1* was confirmed by both mitotic and meiotic mapping using a *ura3* marker integrated into *pma1* (Serrano *et al.*, 1986a). At the same time, the *pma1* gene of *N. crassa* was mapped to the left arm of chromosome I, very

Table II. Homologies among the Fungal [H^+]-ATPases[a]

N. crassa 1	*S. cerevisiae 1*	*S. cerevisiae 2*	*S. pombe 1*	
100	75.2	75.3	73.5	*N. crassa 1*
	100	88.5	73.8	*S. cerevisiae 1*
		100	71.0	*S. cerevisiae 2*
			100	*S. pombe 1*

[a]The numbers refer to the *pma1* and *pma2* ATPases for which deduced amino acid sequences are available, as described in the text. In each case, the sequences were aligned in pairwise combinations by the algorithm of Needleman and Wunsch (1970), and the percent of identical amino acid residues was calculated.

close to the mating type locus, by restriction fragment length polymorphism (B. J. Bowman and R. L. Metzenberg, personal communication).

The *pma1* genes of *N. crassa, S. cerevisiae*, and *S. pombe* encode closely related proteins of 920, 918, 919 amino acid residues (Tables I and II), with marked sequence homology to other P-type ATPases. There is little doubt that the *pma1* gene product is the primary plasma-membrane ATPase of vegetatively growing cells, given the pronounced phenotype of *pma1* mutants, the fact that transformation of such strains with the cloned wild-type gene restores normal drug sensitivity (Ulaszewski *et al.*, 1987a), and the demonstration that disruption of *pma1* in *S. cerevisiae* is lethal (Serrano *et al.*, 1986a). Additional evidence has been provided by the isolation and sequencing of a number of tryptic peptides from the purified *Neurospora* enzyme, which agree perfectly with the overall amino acid sequence deduced from the *pma1* gene (Hager *et al.*, 1986; Aaronson *et al.*, 1988; Mandala and Slayman, 1988; Rao *et al.*, 1988).

At the same time, however, it is now apparent that the *pma1* enzyme is not the only cation-transporting ATPase of the fungal cell. Schlesser *et al.*, (1988) have cloned and sequenced a closely related gene *(pma2)* from *S. cerevisiae* (Table I), and preliminary evidence exists for as many as three other *pma* genes in that organism (H. Rudolf *et al.*, cited in Schlesser *et al.*, 1988). In *Neurospora* as well, there appears to be at least a second *pma* gene (S. Mandala, unpublished results). Because little is known about the expression of these additional genes, and because the corresponding ATPases have not yet been identified directly, the remainder of this review will focus on the well-characterized *pma1* enzyme.

The *pma1* Protein

As one might expect in view of its important role in nutrient uptake, the *pma1* ATPase is a major constituent of the fungal membrane, comprising 5

to 10% of total plasma membrane protein (Bowman *et al.*, 1981). It can be solubilized by detergents such as lysolecithin or deoxycholate and purified to near-homogeneity by centrifugation through a sucrose or glycerol gradient (Dufour and Goffeau, 1978; Bowman *et al.*, 1981). The resulting preparation contains ATPase oligomers (Dufour and Goffeau, 1980; Chadwick *et al.*, 1987) which, upon the addition of phospholipid, dissociate and become incorporated into the lipid vesicles. Maximal specific activities range from 20 to 90 μmol ATP hydrolyzed per mg protein per minute (e.g., Dufour and Goffeau, 1978; Bowman and Slayman, 1981; Smith and Scarborough, 1984; Dufour *et al.*, 1988).

For measurements of ATP-dependent proton translocation, methods have been worked out to reconstitute the purified protein under carefully controlled conditions by freeze–thawing (Dufour *et al.*, 1982, 1988), sonication (Goormaghtigh *et al.*, 1986), or cholate dialysis (Villalobo *et al.*, 1981; Dufour *et al.*, 1988). The addition of ATP to such proteoliposomes leads to the generation of a pH gradient (acid inside) and a membrane potential (positive inside), which can be detected with the use of fluorescent probes or distribution indicators. As expected, maximal pH gradients are seen in the presence of permeant anions (e.g., chloride or thiocyanate) and maximal potentials are seen in the absence of such anions (Perlin *et al.*, 1984). At least in the case of the *Neurospora*, ATPase, monomers of the 100-kDa polypeptide can carry out efficient ATP-dependent proton translocation (Goodmaghtigh *et al.*, 1986), and there is no evidence in any of the fungal enzymes for additional subunits.

With this background information in hand, attention has focussed recently on the molecular structure of the ATPase. The hydropathy profiles of the *Neurospora, S. cerevisiae*, and *S. pombe* polypeptides are illustrated in Fig. 1, as determined by the alogorithm of Engelman *et al.* (1986). In each case, the extremely hydrophilic N-terminus is followed by four well-defined hydrophobic segments whose length (20 to 30 amino acids) and free energy of transfer to water (>20 kcal/mol) suggest that they span the membrane. The central one-third of the molecule is relatively hyrophilic, and because it contains residues involved in ATP binding and phosphorylation (see below), must be located in the cytoplasm. The topology of the C-terminal portion is much less certain, with four to six predicted membrane-spanning segments. In this regard, it is significant that antibodies directed against both the N- and C-termini of the *Neurospora* ATPase bind to inside-out membrane vesicles but not to intact cells (Mandala and Slayman, 1989). Thus, both termini are exposed at the cytoplasmic surface of the membrane, as shown in the model of Fig. 2, and there must be an even number of membrane-spanning segments on either side of the central cytoplasmic domain.

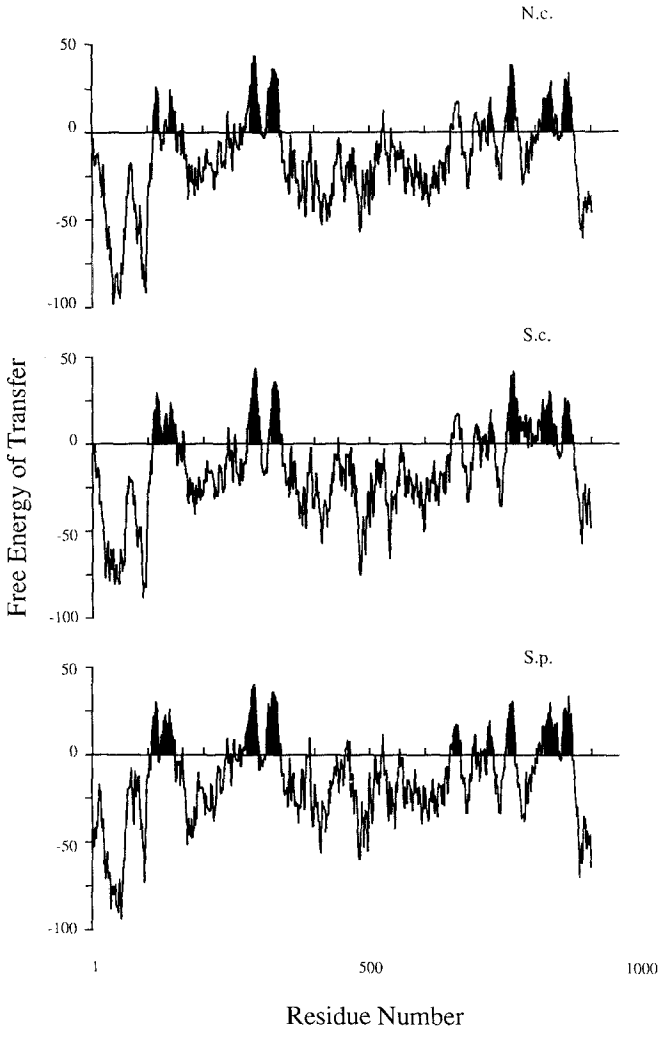

Fig. 1. Hydropathy plots for three fungal ATPases, generated by the algorithm of Engelman *et al.* (1986). The shaded areas represent membrane-spanning segments proposed by Hager *et al.* (1986) for *Neurospora crassa* (N.c.), Serrano *et al.* (1986) for *Saccharomyces cerevisiae* (S.c.), and Ghislain *et al.* (1987) for *Schizosaccharomyces pombe* (S.p.).

The fact that the 100-kDa polypeptide loops back and forth through the membrane at least eight times lends considerable interest to the study of its biogenesis. In *Neurospora*, mRNA transcribed from the cloned *pma1* gene has been used to direct the synthesis of the ATPase *in vitro* (Aaronson *et al.*, 1988). Under these conditions, the ATPase is inserted into the

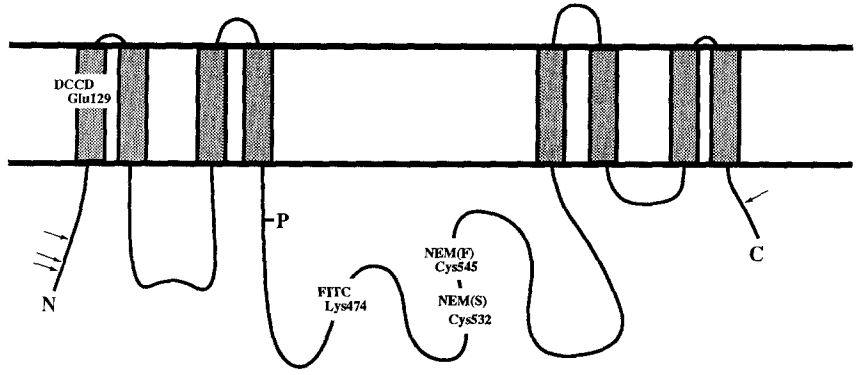

Fig. 2. Model for the topology of the *Neurospora* ATPase in the membrane, based on Hager *et al.* (1986) and Mandala and Slayman (1989). The arrows refer to sites of tryptic cleavage (Mandala and Slayman, 1988), and P refers to the site at which the enzymes is phosphorylated by ATP. Amino acid residues known to react with group-specific reagents are also indicated (see Table III).

membrane co-translationally, although its conformation (as judged by partial trypsinolysis) is not identical to that of the fully mature enzyme (A. Chang, unpublished results). *In vivo*, the *Neurospora* ATPase is presumably synthesized in the rough endoplasmic reticulum and undergoes only minimal post-translational modification, with cleavage of the N-terminal methionine residue and acetylation of the penultimate alanine (Aarsonson *et al.*, 1988). Thus, its entry into the lipid bilayer does not require an N-terminal leader, but rather must depend upon one or more internal sequences; preliminary experiments in which truncated ATPase mRNA was injected into *Xenopus* oocytes have suggested that the first membrane-spanning hairpin may function as such an internal signal (Money *et al.*, 1988). In a further study of biogenesis, Perona and Serrano (1988) have obtained presumptive evidence for expression of the yeast ATPase in mammalian fibroblasts, based on the elevation of intracellular pH.

Reaction Cycle

As an E_1E_2 or P-ATPase, the fungal enzyme alternates between two major conformations and splits ATP by way of a transient phosphorylated intermediate. It resembles its mammalian counterparts in being sensitive to micromolar concentrations of vanadate (Bowman and Slayman, 1979); this inorganic oxyanion can assume a stable trigonal bipyramidal form and is believed to act as a transition-state analogue for phosphate hydrolysis, trapping the protein in the E_2 conformation (Cantley *et al.*, 1978; Smith *et al.*, 1980).

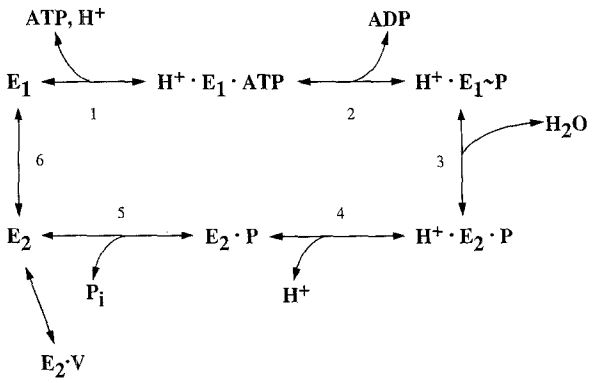

Fig. 3. Simplified model for the reaction cycle of the fungal ATPase, based on Amory *et al.* (1982) and Slayman and Sanders (1985). V denotes inorganic orthovanadate.

Although much remains to be learned about the reaction cycle of the fungal ATPase, a simplified version can be pieced together from existing data:

(i) The first step must involve the binding of ATP to the catalytic site of the enzyme. The diagram of Fig. 3 depicts a single nucleotide site, but the true mechanism may be more complex. Kinetic analysis has revealed that the rate of hydrolysis is a sigmoid function of [MgATP], consistent with a single site that exists in multiple conformational states or with two or more sites that interact cooperatively (Bowman, 1983; Koland and Hammes, 1986). As reviewed recently by Slayman and Zuckier (1989), the $K_{1/2}$ for ATP hydrolysis is quite high (1–3 mM), impeding the characterization of the nucleotide site(s) by direct binding measurements. Recently, however, Ronjat *et al.* (1987) have reported that TbFTP (a complex of terbium with the fluorescent ATP analogue, formycin triphosphate) binds to the *S. pombe* enzyme with a dissociation constant of 1 μM. Interestingly, TbATP acts as a true competitive inhibitor with a K_i of 4.5 μM; the relationship between the nucleotide binding site defined in this way and the site (or sites) deduced from measurements of ATP hydrolysis remains to be worked out.

(ii) The phosphorylation step is assayed by the reaction of the enzyme with [γ-^{32}P]-ATP, producing an acid-stable, alkali-labile phosphoenzyme (Dame and Scarborough, 1980; Amory *et al.*, 1980; Foury *et al.*, 1981; Malpartida and Serrano, 1981). The intermediate has been identified chemically as a β-aspartyl phosphate (Dame and Scarborough, 1981; Amory and Goffeau, 1982), and has been confirmed to turn over rapidly enough to account for the rate of ATP hydrolysis (Dame and Scarborough, 1980; Smith and Hammes, 1988).

(iii) The E_1–E_2 transition has been studied in two independent ways. During ^{18}O exchange experiments on the *S. pombe* ATPase, Amory *et al.*

(1982) observed that ATP stimulates the exchange rate between P_i and water and increases the extent of phosphorylation by P_i; on the basis of these results, they proposed that the enzyme exists predominantly in one form (E_1) in the absence of nucleotides, but that the steady-state level of another form (E_2) rises during ATP hydrolysis. Direct evidence for a structural difference between E_1 and E_2 has come from limited trypsinolysis of the *Neurospora* enzyme. As noted by Addison and Scarborough (1982) and Brooker and Slayman (1983b), the rate and pattern of trypsinolysis vary markedly depending upon the ligands that are present; and Mandala and Slayman (1988) have recently mapped the sites of tryptic cleavage by immunoblotting with N- and C-terminal specific antibodies and by direct sequencing of proteolytic products. In the absence of ligands (presumably the E_1 conformation; see above), trypsin cleaves at three sites very near the N-terminus: Lys-24, Lys-36, and Arg-73. In the presence of vanadate and Mg^{2+} (E_2 conformation), the rate of trypsinolysis at Arg-73 is greatly reduced, and a new cleavage site (Arg-900) is exposed. Thus, the N-terminus becomes less accessible to trypsin, and the C-terminus more accessible, as the enzyme shifts from the E_1 to the E_2 conformation. Consistent with this idea, C-terminal specific antibodies bind more tightly to the *Neurospora* ATPase in the presence of vanadate (Mandala and Slayman, 1989).

(iv) The transport step involves the outward movement of one proton for each ATP hydrolyzed. This aspect of the reaction cycle has been thoroughly documented for the *Neurospora* enzyme, where information on transport stoichiometry has come from thermodynamic calculations (reviewed by Slayman and Zuckier, 1989), from the analysis of pump current as a function of cytoplasmic H^+ concentration (Sanders *et al.*, 1981; Slayman and Sanders, 1984), and from measurements of proton pumping and ATP hydrolysis by isolated plasma-membrane vesicles (Perlin *et al.*, 1986). By analogy with the mammalian P-ATPases, protons are assumed to bind to the E_1 form of the fungal enzyme at the inner surface of the membrane and to be released from the E_2 form at the outer surface (Fig. 3). Current–voltage studies of the *Neurospora* pump during changes of intracellular and extracellular pH have made it possible to estimate pK's for the proton site at both surfaces; the resulting values are $pK_i = 5.44$ (compared with a cytoplasmic pH of 7.2) and $pK_o = 2.93$ (compared with a standard extracellular pH of 5.8; Slayman and Sanders, 1985). Thus, under normal physiological conditions, the binding site is mostly unloaded at both surfaces, but the pump is primed to accelerate markedly upon acidification of the cytoplasm.

(v) For the fungal [H^+]-ATPase, as for the mammalian [Na^+, K^+]- and [Ca^{2+}]-ATPases, there is evidence that charge transfer across the membrane occurs during the transition between $E_1 \sim P$ and $E_2 \cdot P$. The forward rate constant for this step has been estimated from steady-state current–voltage

analysis of the *Neurospora* pump (3000 to 60,000 sec^{-1}; Slayman and Zuckier, 1989) and from ^{18}O exchange measurements on the *S. pombe* pump (9200 sec^{-1}; Amory *et al.*, 1982). The resulting values are considerably larger than those for the mammalian [Na^+, K^+]- and [Ca^{2+}]-ATPases ($< 300\ sec^{-1}$), and may point to a fundamental difference in the mechanism of proton movement through the protein.

Structure–Function Relationships

Deduced amino acid sequences are now available for an array of eukaryotic and bacterial P-ATPases, and have been aligned to identify the parts of the polypeptide that have been conserved in evolution (see reviews by Serrano, 1988; Taylor and Green, 1989; Nakamoto *et al.*, 1989; Rao *et al.*, 1989). While there is noticeable homology in one of the predicted membrane-spanning segments (segment IV) and in the hydrophilic loop that connects segments II and III, most of the conserved stretches of sequence are concentrated in the large central cytoplasmic domain. This region appears to have at least two important functions. (i) Because it is labeled in an ATP-protectable way by group-specific reagents and ATP affinity analogues (see below), and because it contains two consensus sequences found in a range of otherwise unrelated nucleotide-binding proteins (Walker *et al.*, 1982), it is likely to encompass part or all of the nucleotide site. (ii) In addition, it contains the aspartyl residue with which ATP reacts to form the covalent phosphorylated intermediate; not surprisingly, this residue is part of a short stretch of strongly conserved amino acids (Cys Ser Asp Lys Thr Gly Thr Leu Thr in all of the eukaryotic enzymes), embedded within a longer, ca. 40-amino acid, region that shows appreciable homology throughout the P-type group (Rao *et al.*, 1989).

Further knowledge of structure–function relationships has come from experiments with group-specific reagents, which provide a useful way to identify amino acid residues that participate in the reaction cycle. In the case of the fungal plasma-membrane [H^+]-ATPase, the current state of information is summarized in Table III. Three residues (Lys-474, Cys-532, and the phenylglyoxal- and butanedione-sensitive arginine) are protected from reacting with their respective inhibitors when MgATP or MgADP is bound to the enzyme, and thus may be located in or near the catalytic site (Brooker and Slayman, 1982, 1983a; Di Pietro and Goffeau, 1985; Kasher *et al.*, 1986; Davenport and Slayman, 1988; Pardo and Slayman, 1988, 1989). Of these residues, Lys-474 is part of a short stretch (Lys Gly Ala Pro/Ser) that is conserved in all of the eukaryotic P-ATPases; and Cys-532, although not itself conserved, lies just before a long, clearly homologous region consisting

Table III. Use of Group-Specific Reagents to Define Functionally Important Residues of the *Neurospora* ATPase

Residue	Inhibitor	Protection by ATP	Reference
Lys-474	Fluorescein isothiocyanate (FITC)	Yes	Pardo and Slayman, 1988
Cys-532	*N*-ethylmaleimide (NEM)	Yes	Pardo and Slayman, 1989
Cys-545	*N*-ethylmaleimide (NEM)	No	Pardo and Slayman, 1989
Arg	Phenylglyoxal, butanedione	Yes	Di Pietro and Goffeau, 1985: Kasher *et al.*, 1986
Glu-129	*N,N'*-dicyclohexylcarbodiimide (DCCD)	No	Sussman *et al.*, 1987
Glu/Asp	*N*-ethoxycarbonyl-2-ethoxy-1,2,- dihydroquinoline (EEDQ)	No[a]	Addison and Scarborough, 1986

[a]Protection is seen in the presence of MgATP + vanadate (Addison and Scarborough, 1986).

of an Asp Pro Pro Arg motif followed by Gly . . . Thr Gly Asp. Although three-dimensional crystal structures are not yet available for any of the P-ATPases, there have been recent attempts to model the nucleotide site based on sequence and secondary structure comparisons with other ATP-binding proteins such as adenylate kinase and phosphofructokinase (Serrano, 1988; Taylor and Green, 1989; reviewed in Rao *et al.*, 1989).

An additional residue of interest is Glu-129. In studies on the *Neurospora* enzyme, it has been shown to be the principal binding site for *N,N'*-dicyclohexylcarbodiimide (DCCD), a well-known inhibitor of proton translocation through the F_0F_1-ATPases of mitochondria, chloroplasts, and bacteria (Sussman and Slayman, 1983; Sussman *et al.*, 1987). Like the DCCD-reactive residue of the F_0F_1 enzymes, Glu-129 resides in the middle of a hydrophobic segment that is predicted to span the membrane. It is conserved, not only in the other fungal [H^+]-ATPases, but also in P-ATPases from *Arabidopsis thaliana* (a higher plant) and *Leishmania donovani* (a protozoan parasite), both of which are believed to function as proton pumps (Harper *et al.*, 1989; Meade *et al.*, 1987). By contrast, none of the other P-ATPases has a charged residue in the first predicted membrane-spanning segment (reviewed in Nakamoto *et al.*, 1989).

An independent approach to the study of structure–function relationships exploits mutants carrying alterations in the ATPase gene. In the case of the yeast enzyme, as described above, such mutants have been produced in two ways.

(i) A large number of strains have been isolated by resistance to Dio-9 (Ulaszewski *et al.*, 1983, 1986) and hygromycin B (McCusker *et al.*, 1987), and found to possess both quantitative and qualitative changes in ATPase activity (Perlin *et al.*, 1988). Among them, a particularly interesting Dio-9 resistant mutant of *S. pombe* has proved to contain a single amino acid

replacement (Gly-268 → Asp), leading to a marked decrease in the sensitivity of the purified enzyme to vanadate (Ulaszewki *et al.*, 1987b; Ghislain *et al.*, 1987). As further strains are characterized, and as the mutational alterations are mapped within the polypeptide, a great deal of useful information should be forthcoming about the role of particular amino acids in the reaction cycle.

(ii) In parallel, Serrano and his co-workers have employed recombinant DNA techniques to produce yeast strains carrying defined mutations within the *pma1* gene. Some of these strains have been used for biochemical and physiological studies of ATPase function: for example, several temperature-sensitive *pma1* mutants (Serrano *et al.*, 1986b; Cid and Serrano, 1988) and a strain in which the constitutive *pma1* promotor has been replaced by a galactose-dependent promoter (Cid *et al.*, 1987). Others are designed to carry single amino acid replacements in interesting regions of the polypeptide (Portillo and Serrano, 1988); such mutants will prove helpful in the dissection of structure–function relationships.

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