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Dissection of functional domains of the yeast proton-pumping ATPase by directed mutagenesis

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Cation-pumping ATPases characterized by a phosphorylated intermediate have been proposed to contain kinase, phosphatase and transduction domains. Evidence is provided for this model by mutagenesis of critical residues in the proposed domains. The Glu233-Gln mutation blocks the turnover of the intermediate and serves to define the phosphatase domain. Mutations in aspartate residues 534, 560 and 638 alter the nucleotide specificity of the enzyme. These amino acids are therefore part of the ATP binding site. Lys474 seems to be essential for activity in this kinase domain. Finally, mutations in Asp378, the amino acid forming the phosphorylated intermediate, indicate that the formation of a phosphorylated intermediate is not an obligatory step in ATP hydrolysis but is required for coupling this process with proton pumping.

Key words: yeast/H^+-ATPase/mutagenesis/domains

Introduction

A family of cation-pumping ATPases has been identified in the plasma and endoplasmic membranes of eukaryotic cells and in bacterial membranes (Serrano, 1988). These enzymes all include, as the major catalytic component, a 70-140 kd polypeptide which forms a phosphorylated intermediate and which contains regions with sequence similarity. They differ in the nature of the pumped cation: either H^+ (Serrano, 1985), K^+ (Wieczorek and Altendorf, 1979; Furst and Solioz, 1986), Na^+ and K^+ (Jorgensen, 1982), H^+ and K^+ (Sachs *et al.*, 1982) or Ca^{2+} (Hasselbach, 1981; Carafoli and Zurini, 1982). Ion gradients generated by these enzymes play important energetic and regulatory roles in cellular physiology, participating in active nutrient uptake and in the control of signalling concentrations of H^+, Ca^{2+} and K^+ during cell activation.

Chemical modifications, proteolytic digestions, sequence similarities and structure predictions have been combined to suggest a working model for the structure and function of cation-pumping ATPases (Brandl *et al.*, 1986; Serrano, 1988). Basically, these enzymes are considered to consist of four functional domains (Figure 1). The transmembrane stretches and their cytoplasmic extensions would represent the cation transport domain. Conserved regions g, h and i would delineate an ATP-binding domain with protein kinase activity. Region i was originally proposed to constitute a 'hinge domain' (Brandl *et al.*, 1986) but we have later proposed to form part of the ATP-binding or kinase domain (Serrano, 1988). Region f contains the phosphorylated intermediate while the adjacent region e is the only transmembrane segment conserved in different ATPases of the family. Both regions together could constitute a transduction domain linking ATP hydrolysis with cation transport. Finally, conserved regions b, c and d could form part of a phosphatase domain catalysing the hydrolysis of the phosphorylated intermediate. This domain was originally designated as 'transduction domain' (Brandl *et al.*, 1986) and we have later proposed a phosphatase function (Serrano, 1988). The catalytic and transduction domains should contain amino acid residues essential for activity and conserved within the ATPase family. The functionally important residues in the transport domain should differ in enzymes pumping different cations.

We have tested this model by directed mutagenesis of potentially important residues within the proposed domains of the yeast plasma membrane ATPase (Figure 1). The mutant enzymes have been tested for physiological activity after introduction within plasmids into a yeast strain where the expression of the wild-type chromosomal ATPase gene can be turned off (Cid *et al.*, 1987a). This approach avoids the complications inherent to expression in heterologous systems, where post-translational modifications and protein-protein interactions may differ from the normal physiological context. Studies of the catalytic and transport properties of the mutant ATPases support the postulated domain model.

Results

Strategy for the mutational analysis of the yeast plasma membrane ATPase

Individual amino acids of the yeast ATPase were selected

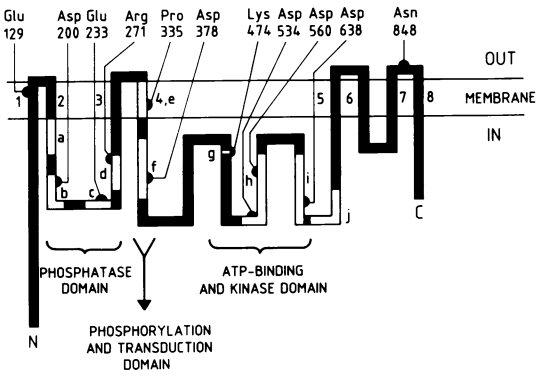

Fig. 1. Location of the mutagenized amino acids within the proposed structure of the yeast plasma membrane ATPase (Serrano, 1988). Regions 1-8 correspond to the hydrophobic stretches proposed to be transmembrane α-helices. Regions a-j correspond to sequences with significant similarity in all the ATPases with phosphorylated intermediate. The proposed functional domains of the enzyme are indicated.

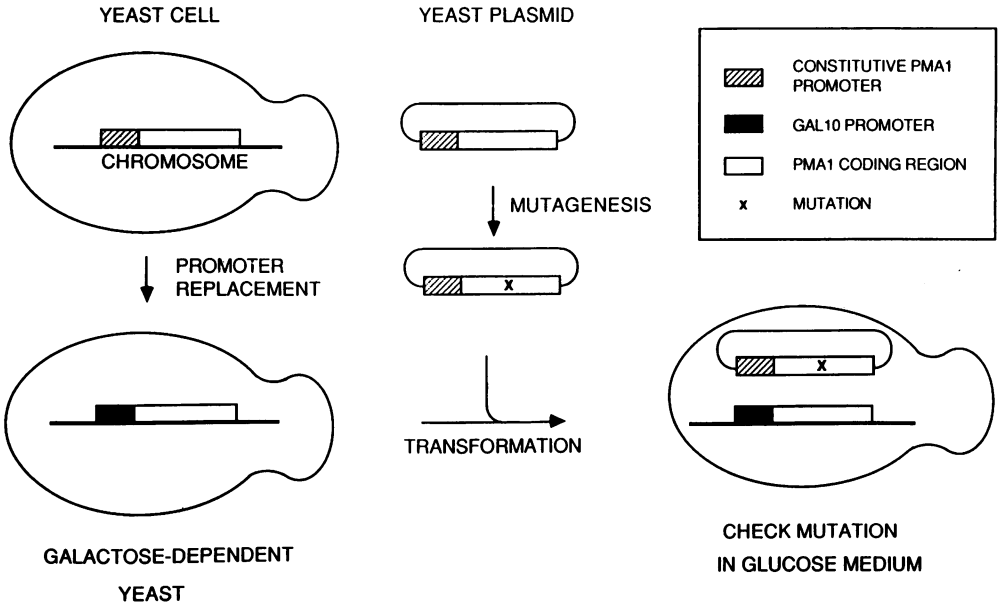

Fig. 2. Strategy for the mutational analysis of the yeast plasma membrane ATPase.

Table I. Growth, catalytic and transport properties of ATPase mutants

Yeast strain	ATPase allele expressed on glucose	Growth on glucose	ATP hydrolysis[a]	Proton transport[b]	Amount ATPase[c]
RS-303	PMA1 (wild type)	+	100	100	100
RS-357	None (only residual ATPase)	−	19	23	27
RS-369	pma1-210 (Glu129−Gln)	+	100	100	100
RS-352	pma1-211 (Glu129−Leu)	+	100	100	100
RS-353	pma1-212 (Asp200−Asn)	−	23	26	44
RS-354	pma1-213 (Glu233−Gln)	−	33	32	53
RS-355	pma1-214 (Arg271−Thr)	+	100	100	100
RS-356	pma1-215 (Pro335−Ala)	−	53	46	56
RS-304	pma1-216 (Asp378−Asn)	+	73	72	89
RS-306	pma1-217 (Asp378−Glu)	−	67	41	71
RS-368	pma1-218 (Asp378−Thr)	−	49	34	62
RS-400	pma1-219 (Lys474−Gln)	−	19	20	65
RS-365	pma1-220 (Asp534−Asn)	−	37	32	44
RS-366	pma1-221 (Asp560−Asn)	−	24	25	43
RS-367	pma1-222 (Asp638−Asn)	−	24	26	53
RS-401	pma1-223 (Asn848−Asp)	+	100	100	100

[a]Measured in purified plasma membranes. The value for wild-type enzyme (0.75 μmol ATP × min^{-1} × mg $protein^{-1}$) was taken as 100%.
[b]Measured in whole cells. The value for wild-type enzyme (7.0 nmol H^+ × min^{-1} × mg fresh weight $cells^{-1}$) was taken as 100%.
[c]Quantified by immunoassay in purified plasma membranes. The value for wild-type enzyme (0.1 mg ATPase × mg $protein^{-1}$) was taken as 100%.

for site-directed mutagenesis (Figure 1) because of their conservation in all the ATPases with a phosphorylated intermediate or on the basis of chemical modification studies (Serrano, 1988). Glu129 has been implicated as part of the proton transport pathway because in the closely related ATPase from *Neurospora crassa* it is modified by dicyclohexylcarbodiimide (Sussman *et al.*, 1987). Asp200 and Glu233 are fully conserved in the proposed phosphatase domain of all the ATPases and the Arg271 is only replaced by lysine in some enzymes of the family. Pro335 is in the middle of the only hydrophobic stretch conserved in the ATPase family. It has been proposed that the *cis−trans* isomerization of intramembrane prolines may be important for the conformational changes associated with transport (Brandl and Deber, 1986). Asp378 forms the phosphorylated intermediate while Lys474 has been implicated in the active site on the basis of chemical modification studies. Both these amino acids, as well as Asp534, Asp560 and Asp638, are fully conserved within the ATPase family. The last three aspartates are present in regions of homology with the ATP-binding site of phosphofructokinase and which chemical modification studies suggest to participate in ATP binding by the ATPases. Finally, Asn848 is a potential glycosylation site (sequence Asn-Trp-Thr) which could be important for the structure and biosynthesis of the enzyme. In most of the cases we made isosteric replacements (Asp by Asn, Glu by Gln) in order to prevent structural changes in the protein.

We previously constructed a yeast strain (RS-72) in which the constitutive promoter of the plasma membrane ATPase

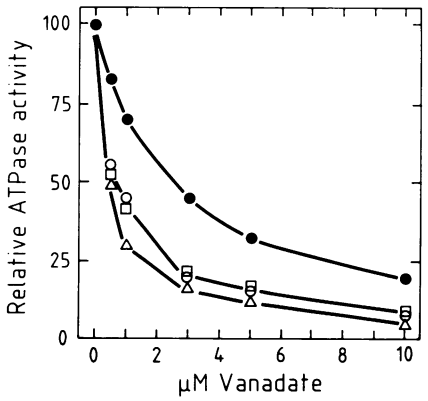

Fig. 3. Increased sensitivity to vanadate of mutant ATPases. ●, Wild type; ○, Asp378−Asn mutant; △, Asp378−Glu mutant; □, Asp378−Thr mutant.

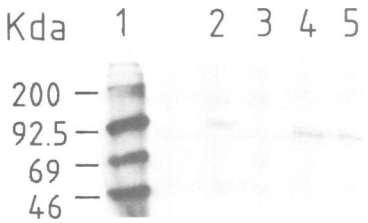

Fig. 4. Covalent binding of dicyclohexylcarbodiimide to mutant ATPases. **Lane 1**, molecular weight standards; **lane 2**, strain RS-303 (wild-type ATPase); **lane 3**, strain RS-357 (no ATPase expressed on glucose medium, only residual wild-type ATPase); **lane 4**, strain RS-369 (Glu129−Gln mutant); **lane 5**, strain RS-352 (Glu129−Leu mutant). Plasma membranes (50 μg) were incubated for 45 min at 30°C with 100 μM dicyclohexyl[^{14}C]carbodiimide (57 mCi/mmol, Amersham). The inhibition of activity was of 70% in all cases. After electrophoresis the gel was treated with Enlightning (New England Nuclear) and the autoradiograph exposed for 24 h.

gene was replaced by a galactose-dependent promoter (Cid *et al.*, 1987a). As the ATPase gene is essential (Serrano *et al.*, 1986a), this strain only grows normally on galactose medium. When transferred to glucose medium some residual growth occurs (two to three duplications) until the preformed ATPase is diluted to <20% of normal. This strain is ideal for '*in vivo*' testing of mutant ATPase genes because normal growth on glucose medium depends on the ATPase gene introduced in a plasmid (Figure 2). A temperature-sensitive mutant of the ATPase has already been obtained by this simple testing procedure (Serrano *et al.*, 1986b).

Capability of mutant ATPases to support yeast growth

The physiological activity of mutant ATPases was tested by their ability to support the growth of strain RS-72 on glucose medium (Table I). Glu129, Arg271 and Asn848 were not required for physiological activity because normal growth was observed in yeast expressing enzymes with amino acid changes at these positions. On the other hand, application of the same criterion indicated that Asp200, Glu233, Pro335, Lys474, Asp534, Asp560 and Asp638 are essential residues. Replacement of Asp378 by Asn resulted in a functional ATPase. On the other hand, replacement by Glu or Thr abolished the ability of the enzyme to support growth under the experimental conditions.

It must be pointed out that the growth test did not score mutant ATPases which supported very slow growth (growth rates lower than ~30% of wild type). This limitation results from the high frequency of reversion (10^{-4}) of strain RS-72 on glucose plates (Cid *et al.*, 1987a). Therefore, during extended incubation times the revertants overgrew the slow-growing ATPase mutants.

During growth on galactose medium both the wild-type and the mutant ATPase genes are expressed. Wild-type yeast and most of the strains expressing mutant ATPases exhibited a growth rate under these conditions of 0.17 h^{-1} (doubling time of 4.1 h). On the other hand, strains expressing the Asp378−Thr and Asp534−Asn mutations grew slower (growth rate 0.13−0.14 h^{-1}, doubling time 5.0−5.4 h). Apparently these two mutations have dominant deleterious effects.

ATPase and proton transport activities of mutants

Mutant ATPases which scored positive in our growth test had either normal rates of ATP hydrolysis and proton transport (Glu129−Gln, Glu129−Leu, Arg271−Thr and Asn848−Asp) or slightly decreased activities (Asp378−Asn, 72−73% of wild type). On the other hand, growth-negative mutants exhibited <50% of wild-type proton transport activity (Table I). ATPase activity paralleled proton transport except in the Asp378−Glu and Asp378−Thr mutants, where ATP hydrolysis was diminished proportionally less than proton transport.

It is apparent from the data of Table I that the activities of many mutants were similar (Lys474−Gln) or only slightly higher (Asp200−Asn, Asp560−Asn and Asp638−Asn) than in the yeast strain expressing no ATPase in glucose medium. The background activity corresponds to preformed ATPase synthesized on galactose and diluted during the residual growth of this strain on glucose (Cid *et al.*, 1987a). This residual wild-type activity therefore contributes to the values recorded with mutants unable to grow on glucose medium. Although subunit interactions in an oligomeric structure may complicate the assignment of activity to wild-type and mutant enzymes, as a first approximation simple additive contributions can be assumed. When this factor is taken into account, the estimated proton transport activities of all the growth-negative mutants were <25% of wild type. For the Asp378−Glu and Asp378−Thr mutants the relative activity of ATP hydrolysis was about three times higher than that of proton transport.

The '*in vivo*' assembly of the enzyme into the plasma membrane and its turnover may be affected by the mutations and then contribute to the observed changes in activity. We have estimated the amount of mutant ATPases in purified plasma membranes by quantitative immunoassay (Table I). The reduced ATPase activity of the Pro335−Ala, Asp378−Glu, Asp378−Thr and Asp534−Asn mutants is almost completely explained by decreased amounts of plasma membrane ATPase. This is most apparent for the later mutation, which after correcting for residual wild-type ATPase resulted in a 5-fold reduction in the amount of enzyme. For the other mutations the reduction was 2- to 3-fold. The reduction in activity of the Asp200−Asn, Glu233−Gln, Lys474−Gln, Asp560−Asn and Asp638−Asn mutants is only partially explained by a decrease in the amount of enzyme and a reduction in specific activity seems also to be involved. This is most apparent in the Lys474−Gln mutation, which exhibited activities not significantly different

from the control strain which expressed only residual wild-type ATPase. This mutation seems to reduce the specific activity of the enzyme to < 10% of wild type. The specific activities of the Asp200−Asn (20%), Glu233−Gln (50%), Asp560−Asn (25%) and Asp638−Asn (15%) mutants seem also to be affected (estimated percentage of wild type in brackets), but to a lesser extent.

Mutants expressing kinetic changes

The three mutations of Asp378, the amino acid forming the phosphorylated intermediate, increased the sensitivity of the enzyme to vanadate (Figure 3). For the wild-type enzyme 50% inhibition was obtained with 2.5 μM vanadate. The same degree of inhibition was obtained in the Asp378−Glu mutant with only 0.4 μM and in the Asp378−Asn and Asp378−Thr mutants with only 0.6 μM vanadate. No change in vanadate sensitivity was observed in all the other mutants.

Table II. Specificity of nucleotide hydrolysis by mutant plasma membrane ATPases[a]

ATPase expressed in glucose medium	Hydrolysis rate (μmol × min^{-1} × mg protein^{-1}			
	ATP	UTP	GTP	CTP
Wild type	0.75	0.13	0.15	0.13
None (only residual ATPase)	0.14	0.02	0.03	0.02
Glu129−Gln	0.75	0.13	0.15	0.13
Glu129−Leu	0.75	0.13	0.15	0.13
Asp200−Asn	0.17	0.03	0.05	0.03
Glu233−Gln	0.25	0.04	0.06	0.04
Arg271−Thr	0.75	0.15	0.17	0.15
Pro335−Ala	0.40	nd	nd	nd
Asp378−Asn	0.55	0.09	0.11	0.09
Asp378−Glu	0.50	0.08	0.10	0.08
Asp378−Thr	0.37	nd	nd	nd
Lys474−Gln	0.14	0.03	0.04	0.03
Asp534−Asn	0.28	0.19	0.28	0.12
Asp560−Asn	0.18	0.09	0.14	0.06
Asp638−Asn	0.18	0.09	0.18	0.07
Asn848−Asp	0.75	nd	nd	nd

nd; Not determined.
[a]For the denomination of yeast strains and ATPase alleles see Table I.

The hydrolysis of different nucleotides by mutant ATPases is shown in Table II. ATP is the best substrate for the wild-type enzyme, followed by GTP (20%) and UTP and CTP (17%). A similar pattern was observed in most of the mutants, independently of their level of ATPase activity. On the other hand, with the mutations Asp534−Asn, Asp560−Asn and Asp638−Asn the differences between nucleotides were greatly reduced and GTP was as good a substrate as ATP. As the strains expressing these mutations contain residual wild-type ATPase (see above), an estimate of the contribution of the mutant enzymes can be made by subtracting the activities of the control strain RS-357, which only contains the residual wild-type enzyme. Then the order of effectiveness for the three mutant ATPases was: GTP > UTP > ATP,CTP, suggesting a dramatic change in nucleotide specificity. The very low rate of hydrolysis of ADP and *p*-nitrophenylphosphate by yeast plasma membranes (0.05 and 0.001 μmol × min^{-1} × mg protein^{-1} respectively) cannot be attributed to the ATPase because these activities were not decreased in either the RS-357 control or in any of the mutants.

The inhibition of all the mutant ATPases by dicyclohexylcarbodiimide was normal, including mutants with altered Glu129, the amino acid which has been reported to be the target of the drug (Sussman *et al.*, 1987). The binding of [^{14}C]dicyclohexylcarbodiimide to the Glu129−Gln and Glu129−Leu mutants also seemed to be normal (Figure 4) and these mutations did not interfere with any of the tested activities of the enzyme.

The level of phosphorylated intermediate under steady-state conditions was normal in all the mutant ATPases supporting growth. Among the growth-negative mutants only Glu233−Gln and Asp378−Glu formed significant amounts of phosphorylated intermediate (Figure 5A). All these intermediates were sensitive to hydroxylamine. When the level of intermediate was correlated to the amount and activity of ATPase in the plasma membrane, it seemed that the Glu233−Gln mutant accumulated more phosphorylated intermediate than the wild-type enzyme. This was corroborated by the observation that the decay of intermediate after a chase with non-radioactive ATP was much slower in this mutant than in the wild type (Figure 5B). This result suggests a defect in the hydrolytic step of the catalytic cycle.

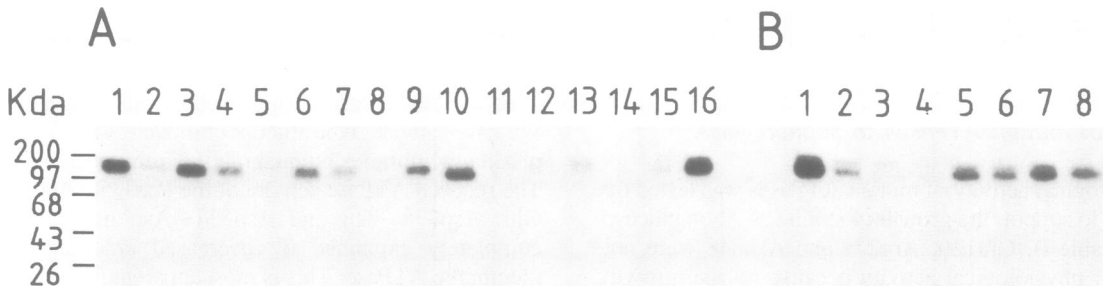

Fig. 5. Phosphorylated intermediate in mutant ATPases. (**A**) Steady-state level of intermediate. Plasma membranes (15 μg) were incubated with 0.2 mM [γ-^{32}P]ATP (2000 mCi/mmol) during 15 s at 0°C. After electrophoresis the gel was dried and the autoradiograph exposed for 15 h at −70°C with intensifying screen. The radioactive band corresponds to the ATPase polypeptide of 100 kd partially nicked by proteolysis. **Lanes: 1**, strain RS-303 (wild-type ATPase); **2**, strain RS-357 (no ATPase expressed on glucose medium, only residual wild-type ATPase); **3**, Asp378−Asn; **4**, Asp378−Glu; **5**, Asp378−Thr; **6**, Glu129−Leu; **7**, Glu129−Gln; **8**, Asp200−Asn; **9**, Glu233−Gln; **10**, Arg271−Thr; **11**, Pro335−Ala; **12**, Asp534−Asn; **13**, Asp560−Asn; **14**, Asp638−Asn; **15**, Lys474−Gln; **16**, Asn848−Asp. (**B**) Hydrolysis of the intermediate. After 15 s phosphorylation of wild type (**lanes 1−4**) and Glu233−Gln mutant (lanes 5−8), a 100-fold excess on unlabelled MgATP was added and samples taken after 0 (**lanes 1** and **5**), 10 (**lanes 2** and **6**), 20 (**lanes 3** and **7**) and 30 (**lanes 4** and **8**) s at 0°C.

Discussion

The major difficulty in the interpretation of mutational analysis of enzyme function is the possibility of major structural disruption (Knowles, 1987). Although we do not have direct structural information, the level of mutant ATPase in the yeast plasma membrane may reflect the structural integrity of the protein. Distorted conformations of plasma membrane proteins are retained in the endoplasmic reticulum and do not appear in the plasma membrane (Pfeffer and Rothman, 1987). On the other hand, catabolic proteinases selectively remove abnormal polypeptides from cells (Bond and Butler, 1987). Therefore mutations reducing the level of plasma membrane ATPase might be causing structural alteration.

The main effect of the Pro335−Ala and Asp534−Asn mutations is a 2- to 3-fold reduction in the amount of ATPase detected in the plasma membrane. The specific activity of the residual enzyme seems to be normal and therefore the mutated amino acids are unlikely to participate in catalysis, although they are conserved in all the ATPases with a phosphorylated intermediate. Only a structural role for these residues can be deduced from these experiments. Other mutations which reduce the level of plasma membrane ATPase 2- to 3-fold, such as Asp200−Asn, Glu233−Gln, Asp560−Asn, Asp638−Asn, additionally show a decrease in enzyme specific activity. Either these amino acids have both structural and functional roles or the structural alterations cause functional disturbances.

The most critical of the studied amino acids seems to be Lys474 because replacement by glutamine reduces enzyme activity to undetectable levels. Lys474 corresponds to the residue modified in animal ATPases by the active site inhibitor fluorescein 5′-isothiocyanate (Serrano, 1988). The drastic effect of this mutation would be expected if Lys474 were crucially involved in ATP binding or participated directly in catalysis.

In the proposed phosphatase domain (Figure 1) a conserved amino acid, Glu233, seems to fulfill the expectations for a component of the phosphatase machinery. The Glu233−Gln mutant accumulates phosphorylated intermediate during steady-state ATP hydrolysis and has a slower turnover of the intermediate. The specific activity and the level of enzyme are only moderately reduced by this mutation (50% of wild type). Mutation of another conserved amino acid in this domain, Asp200, results in a greater reduction of enzyme level and activity (20−30% of wild type) but the phosphorylated intermediate does not accumulate.

The ATP-binding site of the ATPase seems to be defined by the Asp534−Asn, Asp560−Asn and Asp638−Asn mutations, which result in altered nucleotide specificity. Asp560 and Asp638 are present in regions of sequence similarity with the ATP-binding site of phosphofructokinase and correspond to residues participating in binding (Serrano, 1988). The apparent affinity for ATP of these mutant ATPases has not been determined because the activities of the membranes containing these enzymes are only 1.3- to 2.0-fold greater than the expected residual wild-type ATPase (see Tables I and II). Although this difference is quite reproducible, it is not big enough to allow a simple estimation of the contribution of the mutant enzymes to the overall saturation kinetics. Therefore, although a decrease in affinity for ATP would be expected, with the present experimental limitations we can only demonstrate the participation of these conserved aspartate residues as specificity determinants.

Some mutations disprove any critical role for the corresponding amino acid. Arg271, although conserved, is not important for either ATPase level or activity, and the same is true for Asn848 which defines a potential glycosylation site. The two mutagenized residues in the transmembrane domain, Glu129 and Pro335, similarly prove unimportant. Although the Pro335−Ala mutation reduces the level of ATPase in the plasma membrane to 50%, the ATPase and proton transport activities of the mutant enzyme are normal, not supporting the prediction that transmembrane prolines may be critical for transport activity (Brandl and Deber, 1986). Glu129 provides an even more disappointing result, because chemical modification by dicyclohexylcarbodiimide in the closely related ATPase from *N.crassa* suggested its involvement in proton transport (Sussman *et al.*, 1987). Some other carboxylic group located in a hydrophobic region may be the target for the drug in the *Saccharomyces* ATPase.

A final point of discussion is the function of the phosphorylated intermediate. Replacement of Asp378 by Asn results in a functional enzyme with a normal phosphorylated intermediate. The amido group of the Asn is unlikely to have been hydrolysed because the mutant enzyme has slightly different properties to the wild type (greater sensitivity to vanadate and slightly less activity). Therefore a novel chemistry may be involved. One prediction would be an imido-ester bond (-C [=NH]-O-P) fulfilling the role of the normal intermediate (-C [=O]-O-P). On the other hand, a residue other than the mutated residue 378 may act as acceptor of phosphate and we are currently investigating this possibility. The same reservation applies to the Asp378−Glu mutation, which forms an apparently normal intermediate and has normal ATPase activity but proton pumping is defective, suggesting uncoupling of ATP hydrolysis and proton transport. Uncoupling is also apparent for the Asp378−Thr mutant, which does not form phosphorylated intermediate and has almost normal ATPase activity with impaired proton pumping. We cannot decide whether the small difference in proton pumping between these mutants and the control strain expressing residual wild-type ATPase is due to intrinsic transport activity of the mutant enzymes or to enhancement of activity of the residual wild-type ATPase. Therefore the degree of uncoupling between proton transport and ATP hydrolysis in these mutants may be greater than calculated by assuming additive contributions of wild-type and mutant enzymes. In any case, it seems that in this energy-transducing machinery the formation of the proper phosphorylated intermediate is critical for proton pumping. These results do not support the contention that the intermediate is a non-essential side reaction (Whittam and Chipperfield, 1975). On the other hand, the intermediate does not seem to be required for ATP hydrolysis, as demonstrated by the Asp378−Thr mutant. In this case the nucleotide is probably hydrolysed by the phosphatase activity of the enzyme.

As a general conclusion, our results support the dissection of cation-pumping ATPases into kinase, phosphatase and transduction domains and the participation of the phosphorylated intermediate in cation transport (Serrano, 1988).

Table III. Sequences of mutagenic oligonucleotides

Mutation	Oligonucleotide (5′−3′, changes underlined)
Glu129−Gln	TCGTCATGCAAGCTGCT
Glu129−Leu	CGTTATGTTGGCCGCTG
Asp200−Asn	CCCAGGTAACATTTTGC
Glu233−Gln	TACTGGTCAATCCTTGG
Arg271−Thr	CGTTGGTACCGCTGCTG
Pro335−Ala	TGGTGTCGCTGTCGGTT
Asp378−Asn	GTGTTCCAACAAAACCG
Asp378−Glu	GTGTTCCGAAAAAACCG
Asp378−Thr	GTGTTCCACCAAAACCG
Lys474−Gln	TTGTGTTCAAGGTGCTC
Asp534−Asn	CCATGTATGAACCCACC
Asp560−Asn	AACTGGTAACGCTGTTG
Asp638−Asn	TGTTAACAACGCTCCAT
Asn848−Asp	GGTCTGAAGACTGGACT

Materials and methods

Oligonucleotide-directed mutagenesis

A 5-kd *Hin*dIII fragment containing the yeast plasma membrane ATPase gene (Serrano *et al.*, 1986a) was inserted into a modified pUC18 plasmid (Norrander *et al.*, 1983) with a polycloning region containing only *Pst*I, *Sph*I and *Hin*dIII sites. The following fragments from the ATPase gene (Serrano *et al.*, 1986a) were subcloned into M13mp19 (Norrander *et al.*, 1983) for mutagenesis; (i) *Sal*I fragment of 0.5 kb (mutations of Glu129); (ii) *Eco*RI fragment of 0.8 kb (mutations of Asp200, Glu233, Arg271, Pro335 and Asp378); (iii) *Kpn*I fragment of 2.1 kb (mutations of Lys474 and Asp534); (iv) *Xba*I fragment of 2.2 kb (mutations of Asp534, Asp560, Asp638 and Asn848). Mutagenesis was performed by the method of Eckstein (Taylor *et al.*, 1985) with the Mutagenesis System of Amersham (code RPN.2322). The mutagenic oligonucleotides (Table III) were synthesized with an Applied Biosystems DNA Synthesizer. The ATPase gene fragments submitted to oligonucleotide-directed mutagenesis were sequenced by improved versions (Garoff and Ansorge, 1981; Biggin *et al.*, 1983) of the dideoxynucleotide method, utilizing mutagenic oligonucleotides as primers. The efficiency of mutagenesis ranged from 50 to 100% and the only sequence changes observed were those introduced by the mutagenic oligonucleotides. The ATPase gene fragments containing the mutations were liberated from the replicative form of M13mp19 and utilized to replace the corresponding fragment in the ATPase gene present in the modified pUC18 plasmid described above.

Transformation of yeast with the mutant ATPase genes and growth of the transformants

The 5-kb *Hin*dIII fragments containing the mutant ATPase genes were liberated from the modified pUC18 plasmid and inserted into the yeast plasmid pSB32 (Cid *et al.*, 1987a). This is an autonomous, single copy (centromeric) plasmid containing the LEU2 gene for selection of transformants. Yeast cells were made competent for plasmid uptake by treatment with lithium acetate and polyethylenglycol (Ito *et al.*, 1983). Control plasmids contained either a wild-type 5-kb *Hin*dIII fragment or no insert. The recipient strain was RS-72 (Cid *et al.*, 1987a) a derivative of BWG1-7A (Guarente *et al.*, 1982) where the constitutive promoter of the ATPase gene has been replaced by the galactokinase gene (GAL1) promoter. Transformants were selected in agar plates with 2% galactose, 0.7% yeast nitrogen base w/o amino acids (Difco), 0.2 mM adenine and 0.4 mM histidine. The different ATPase mutations carried on the plasmid by the transformed yeast strains are specified in Table I. Growth in liquid medium of the same composition results in a final absorbance at 660 nm of ~3. Under these conditions both the wild-type (chromosomal) ATPase and the mutant (plasmid) ATPase are expressed. In order to express only the mutant enzyme, the galactose grown cells were diluted 65-fold in medium of the same composition but with glucose instead of galactose. After 30 h at 30°C, cultures of the active mutant ATPases reached an absorbance of ~3 while those of inactive mutants only attained 0.4−0.5 (Cid *et al.*, 1987a). Growth tests were made by spotting drops of 5 μl containing ~10^4 galactose-grown cells on agar plates with either glucose or galactose medium. Growth was scored after 24 h.

Biochemical methods

Yeast plasma membranes were purified after treatment of the cells with glucose as described (Serrano, 1983). Nucleotide hydrolysis was measured with a concentration of 2 mM as described (Serrano, 1978). The assay medium included 0.2 mM ammonium molybdate, 5 mM sodium azide and 50 mM potassium nitrate to inhibit residual contamination by acid phosphatase, mitochondrial ATPase and vacuolar ATPase respectively. Binding of dicyclohexylcarbodiimide (Cid *et al.*, 1987b) and electrophoresis in the presence of sodium dodecyl sulfate (Laemmli, 1970) were as described. Proton efflux from the cells was measured after starvation at 4°C and glucose addition at 30°C as described (Serrano, 1980). The formation of the phosphorylated intermediate was as in Vara and Serrano (1983) and the electrophoresis of phosphoenzyme as in Niggli *et al.* (1979). Protein concentration was measured by the method of Bradford (1976), using the Bio-Rad protein assay reagent and bovine globulin as standard. The amount of ATPase in purified plasma membranes was quantified by immunoassay as described (Eraso *et al.*, 1987).

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