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Analysis of the regulatory domain of yeast plasma membrane H^+-ATPase by directed mutagenesis and intragenic suppression

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The yeast plasma membrane H^+-ATPase is activated in vivo by glucose metabolism, and previous deletion analysis has shown the C-terminus of the enzyme to be involved in this regulation. Site-directed mutagenesis demonstrates that Arg^{909} and Thr^{912} at the C-terminus are important for the increase in V_{max} of the ATPase induced by glucose. Other changes in kinetic parameters induced by glucose are largely independent of these amino acids. Arg^{909} and Thr^{912} form a potential phosphorylation site for calmodulin-dependent multiprotein kinase. A double mutation of Ser^{911} and Thr^{912} to Ala results in no cell growth in glucose medium and greatly reduced activation of the ATPase by glucose. Growth and activity are restored by a third mutation (Ala^{547}→Val) at the catalytic domain, providing genetic evidence for domain interaction.

H^+-ATPase; Plasma membrane; Domain interaction; Site-directed mutagenesis; *Saccharomyces cerevisiae*

1. INTRODUCTION

The activity of the plasma membrane H^+-ATPase of the yeast *Saccharomyces cerevisiae* is rate-limiting for growth [1] and it is regulated by glucose metabolism [2]. The mechanism of this regulation is unknown but deletion of the last 11 amino acids of the ATPase produces an enzyme with high activity independently of glucose metabolism [3]. We have mutagenized individual amino acids within this regulatory domain and identified one arginine and one threonine important for regulation. In addition, we have obtained intragenic suppression of mutations at the carboxyl terminus by a mutation in the catalytic domain.

2. MATERIALS AND METHODS

2.1. *Oligonucleotide-directed mutagenesis and expression of mutant ATPases*

Site-directed mutagenesis of the 2.2 kb *Xba*I fragment of the ATPase gene and sequencing was performed in M13mp19 as previously described [4]. The sequences of mutagenic oligonucleotides and the nomenclature of ATPase alleles and yeast strains are indicated in Table I. The strains expressing in glucose medium either wild-type or no ATPase (RS-303 and RS-357, respectively) have been described previously [4]. Strain RS-249 expressing allele *pma1-200* has also been described [5]. Mutation Ala^{547}→Val from this strain was introduced in the Ser^{911}→Ala, Thr^{912}→Ala double mutant by exchange of the *Bst*XI fragments of the corresponding ATPase genes. The presence of the three mutations in the same gene was checked by sequencing at the plasmid level. The mutant ATPase genes were subcloned on yeast centromeric plasmid pSB32 and introduced into strain RS-72 as described [4]. RS-72 is a genetically engineered strain with the expression of the chromosomal wild-type ATPase gene under galactose control [6]. Transformants were selected and propagated on galactose medium, where both wild-type (chromosomal) and mutant (plasmid) ATPases are expressed. In order to express only the mutant ATPases, galactose-grown cells were diluted 50-fold in glucose medium and the cells were harvested after 24 h. Cultures expressing active ATPase reach an absorbance at 660 nm of about 1.5, while cultures expressing either inactive ATPase or no ATPase only reach about 0.3 [4,6]. In the latter case the cells contain, in addition to the mutant ATPase expressed from the plasmid-borne gene, residual wild-type ATPase (about 10% of normal levels) expressed from the chromosomal gene during the previous growth on galactose [4,6].

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2.2. *Biochemical methods*

Yeast plasma membranes were purified from either glucose-starved or glucose-metabolizing cells as described [2]. Specific ATP hydrolysis by the plasma membrane H^+-ATPase was measured as the activity resistant to azide, molybdate and nitrate, but sensitive to diethylstilbestrol [7]. Inhibition by the latter drug was always greater than 70%. In the case of K_m determinations ATP and Mg concentrations were changed to obtain an excess of 5 mM Mg over ATP and the effective ATP concentration was taken as the average between starting and final concentrations (ATP consumption amounted to less than 20%). SDS-PAGE and Western blot were as described [3,4].

3. RESULTS

There are three potential phosphorylation sites in the regulatory carboxyl terminus of yeast ATPase:

.....(908) QRVSTQHEKET (918)

Ser^{911} and Thr^{912} and the nearby Gln^{908} and Arg^{909} are fully conserved in different fungal ATPases but Thr^{918}

Table I

Nomenclature of strains and mutant ATPases and sequences of mutagenic oligonucleotides

Yeast strain	ATPase allele expressed on glucose	Mutagenic oligonucleotide (5′→3′, changes underlined)
RS-303	*PMA1* (wild type)	-
RS-357	none	-
IB-169	*pma1-224* (Ser^{911}→Ala)	AAAGAGTCGCTACTCAA
IB-170	*pma1-225* (Thr^{912}→Ala)	GAGTCTCTGCTCAACAC
IB-355	*pma1-226* (*Ser*^{911}→Ala and Thr^{912}→Ala)	AGAGTCGCTGCTCAACA
IB-356	*pma1-227* (Thr^{918}→Ala)	AAAAGGAAGTCTAATCC
RS-249	*pma1-200* (Ala^{547}→Val)	-
IB-380	*pma1-229* (Ser^{911}→Ala, Thr^{912}→Ala and Ala^{547}→Val)	-
IB-436	*pma1-206* (*Arg*^{909}→Ile)	CTATGCAAATAGTCTCTA
IB-437	*pma1-207* (Arg^{909}→Glu)	CTATGCAAGAAGTCTCTA
IB-438	*pma1-208* (Gln^{908}→Glu)	CTGCTATGGAAAGAGTC
IB-439	*pma1-209* (Gln^{908}→Glu and Arg^{909}→Glu)	GGCTGCTATGGAAGAAGTCTCTACT

Table II

Growth rate on glucose medium and catalytic activity of purified plasma membranes from glucose-starved (GS) and from glucose-metabolizing (GM) cells in different ATPase mutants[a]

ATPase allele expressed on glucose	Growth rate[b]	ATP hydrolysis[c]	
		GS	GM
Wild type	0.23	0.15	1.3
None	<0.04	0.08	0.2
Ser^{911}→Ala	0.22	0.16	1.3
Thr^{912}→Ala	0.14	0.15	0.4
Thr^{918}→Ala	0.23	0.16	1.3
Ser^{911}→Ala and Thr^{912}→Ala	<0.04	0.15	0.3
Gln^{908}→Glu	0.23	0.14	1.4
Arg^{909}→Ile	0.19	0.14	0.5
Arg^{909}→Glu	0.19	0.16	0.5
Gln^{908}→Glu and Arg^{909}→Glu	0.19	0.14	0.5
Ala^{547}→Val	0.20[d]	0.75	1.0
Ser^{911}→Ala, Thr^{912}→Ala and Ala^{547}→Val	0.20[d]	0.75	1.0

[a] Results of a typical experiment are shown. Values are the averages of duplicates differing by less than 10%. Similar results (within 20%) were obtained in three different experiments

[b] The specific growth rate (h^{-1}) is defined as ln2/doubling time. Temperature was 30°C

[c] ATPase activity ($\mu mol \cdot min^{-1} \cdot mg\ protein^{-1}$) was assayed at pH 6.5 with 2 mM ATP

[d] Measured at 25°C because of thermosensitivity conferred by the Ala^{547}→Val mutation

Table III

Kinetic properties of mutant ATPases in purified plasma membranes from glucose-starved (GS) and glucose-metabolizing (GM) cells

ATPase allele	K_m[a]		V_{max}[a]		I_{50} vanadate[b]	
	GS	GM	GS	GM	GS	GM
Wild type	4.1	0.8	0.7	2.9	>5	0.5
Arg^{909}→Glu	4.2	1.3	0.7	1.1	>5	0.5
Thr^{912}→Ala	4.0	1.4	0.7	0.9	>5	0.5

[a] ATPase activity was assayed at pH 6.5 with ATP concentrations from 0.4 to 6 mM. The apparent K_m (mM) and V_{max} ($\mu mol \cdot min^{-1} \cdot mg\ protein^{-1}$) were extrapolated from double reciprocal plots fitted by using a standard least-squares method (linear regression coefficients, $r = 0.97$–0.99). Similar values (within 10%) were obtained with two different membrane preparations

[b] Concentration of vanadate (μM, tested from 0.25 to 5 μM) which gives 50% inhibition with 2 mM ATP and pH 6.5. Similar values (within 10%) were obtained with two different membrane preparations

is not [8]. Site-directed mutagenesis of these residues indicates that Arg^{909} and Thr^{912} are important for regulation because ATPases mutated at these residues show reduced activation by glucose metabolism and cells exhibited reduced growth rate on glucose medium (Table II). On the other hand, mutations of Gln^{908}, Ser^{911} and Thr^{918} influence neither glucose activation nor cell growth on glucose medium.

A double mutation of Ser^{911} and Thr^{912} results in slightly less glucose-activation than in the Thr^{912} single mutant and, more significatively, the cells are unable to grow on glucose medium (duplication time greater than 16 h vs 3 h in wild type and 5 h in the Thr^{912} single mutant). Therefore Ser^{911} is also involved in glucose regulation, although its role is only apparent after mutation of Thr^{912}. Mutations of Gln^{908} show no effect even in conjunction with mutations of Arg^{909} (Table II).

The kinetic properties of two mutant enzymes are shown in Table III. Arg^{909} and Thr^{912} seem important for the glucose-induced increase in V_{max} of the ATPase. On the other hand, other kinetic parameters influenced by glucose such as K_m and vanadate sensitivity (Table III) and pH dependence (Fig. 1), are not dependent on these residues because glucose produces the same quantitative changes in the $Arg^{909} \rightarrow$Ala mutants as in wild type.

We have previously identified a mutation with the ATP binding domain, $Ala^{547} \rightarrow$Val, which results in high ATPase activity, independent of glucose metabolism [5]. As indicated in the last line of Table II, this mutation is able to suppress the defect of growth and ATPase activity of the double mutation $Ser^{911} \rightarrow$Ala, $Thr^{912} \rightarrow$Ala.

All the mutant ATPases described were produced at similar levels as wild-type as determined by Western blot analysis with a specific antibody (data not shown). In addition, the ATPase activity of membranes from glucose-starved cells is very similar in all the mutants of Table II (from 0.14 to 0.16), except for the last two, which express constitutively activated ATPase. This fact also indicates that similar levels of enzyme are present in all cases.

4. DISCUSSION

The yeast ATPase is a phosphoprotein [9] and treatment with wheat germ acid phosphatase inhibits its activity [10]. All these results are consistent with a regulatory mechanism involving glucose-dependent phosphorylation of the regulatory carboxyl terminus of the ATPase. The two amino acids identified in the present work as important for glucose regulation, Arg^{909} and Thr^{912}, define a potential phosphorylation site (RXXS/T) for the calmodulin-dependent multiprotein kinase [11], an enzyme present in yeast [12]. Glucose seems to increase the intracellular calcium level in yeast [13] and increasing intracellular calcium activates the plasma membrane H^+-ATPase in *Neurospora* [14].

There is controversial evidence for the role of a different protein kinase, the cAMP-dependent protein kinase (protein kinase A), in glucose activation of the ATPase. Glucose increases cAMP and stimulates protein phosphorylation in yeast and the proteins encoded by the genes *CDC25, IRA, RAS, CDC35* (adenylate cyclase), *BCY* (regulatory subunit of protein kinase A) and *TPK* (catalytic subunit of protein kinase A) are involved in this phenomenon [15]. *CDC25* [16] and *CDC35* [17] are required for glucose regulation of the ATPase and exogenous cAMP can activate the enzyme in strains with defective cyclase [17]. However, glucose regulation of the ATPase still occurs in mutants with constitutive activation of protein kinase A [18] and in *cdc25* mutants where glucose does not increases cAMP [19] (R. Serrano, unpublished). As the carboxyl terminus of yeast plasma membrane H^+-ATPase does not contain potential phosphorylation sites for cAMP-dependent protein kinase [8,11], the participation of this kinase in ATPase regulation must be indirect.

The fact that a mutation within the ATP binding domain ($Ala^{547} \rightarrow$Val) makes the ATPase independent of glucose metabolism [5,8] and suppresses inactivating mutations at the carboxyl-terminus (present work), provides genetic evidence for a regulatory mechanism based on modulation of an inhibitory interaction of the carboxyl-terminus with the active site. The carboxyl-

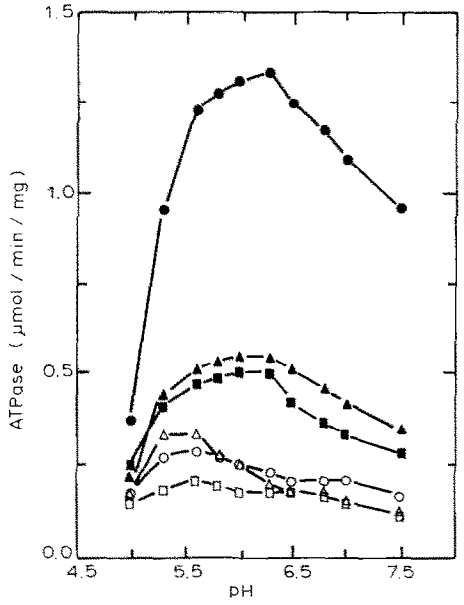

Fig. 1. Effect of pH on ATPase activity. Yeast cells expressing either the wild-type ATPase (circles), the $Arg^{909} \rightarrow$Glu mutant (triangles) or the $Thr^{912} \rightarrow$Ala mutant (squares) were homogenized after incubation in the absence (glucose-starved cells, open symbols) or presence (glucose-metabolizing cells,closed symbols) of glucose. The ATPase activity of purified plasma membranes was determined with 2 mM ATP at the indicated pH.

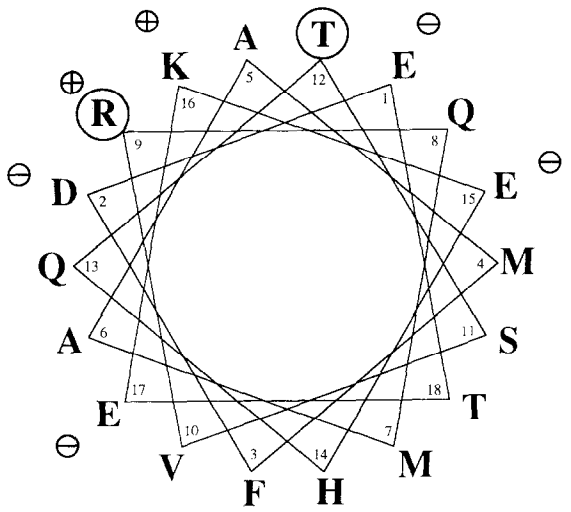

Fig. 2. Helical wheel model [23] of the carboxyl-terminus of yeast ATPase. Position 1 corresponds to amino acid 901 [8]. Arg^{909} and Thr^{912} are encircled.

terminus of yeast ATPase is predicted to fold into an amphipatic α-helix (Fig. 2) with Arg^{909} and Thr^{912} facing the same side. This face could interact with a complementary surface at the active site and phosphorylation of Thr^{912} could disrupt such complementarity.

The regulatory device of yeast ATPase is clearly more complicated than the potential phosphorylation site defined by Arg^{909} and Thr^{912}. Although these amino acids are involved in the increase of V_{max} of the ATPase determined by glucose metabolism, other changes induced by glucose such as a decrease in K_m, increase in pH optimum and increase in vanadate sensitivity are largely independent of mutations on Arg^{909} and Thr^{912}. As these changes are also dependent on the carboxyl-terminus [3], other unidentified amino acids within this region may be involved. The possibility cannot be discarded that Ser^{911} could mediate the glucose-induced changes in kinetic parameters still observed after mutation of either Arg^{909} or Thr^{912}. However, this is not easily compatible with the lack of detectable effects of the $Ser^{911} \rightarrow$Ala mutation.

Future studies should identify additional amino acids at both the C-terminus and the active site involved in the regulation of yeast ATPase. In addition, biochemical experiments should provide evidence for domain interaction and phosphorylation. These biochemical approaches have been succesfully applied to the Ca^{2+}-ATPase of animal plasma membranes, where a carboxyl-terminal inhibitory domain also mediates the regulation of the enzyme by calmodulin [20] and protein kinases [21]. The higher plant plasma membrane H^+-ATPase seems to utilize a similar regulatory mechanism because it is activated by proteolytic removal of a carboxyl terminal fragment [22]. Therefore, the existence of an inhibitory domain at the carboxyl-terminus may be a common regulatory mechanism in the family of cation-pumping ATPases with phosphorylated intermediates [8]. One important difference between the model presented for the plasma membrane Ca^{2+}-ATPase and our hypothesis for regulation of plasma membrane H^+-ATPase refers to the site of interaction of the inhibitory domain. In the former case the inhibitory domain at the C-terminus is proposed to interact with the binding site for the transported cation (calcium in that case) [20] while we propose an interaction with the ATP-binding site. It is hoped that although detailed structural information (X-ray crystallography) is not yet available [8], a combination of biochemical and genetic approaches will uncover the molecular details of this important regulatory device of cation-pumping ATPases.

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