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In vivo activation of the yeast plasma membrane ATPase during nitrogen starvation

Identification of the regulatory domain that controls activation

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Yeast plasma membrane ATPase is activated during nitrogen starvation when a fermentable substrate is present. This activation is due to changes in the V_{max} and it is irreversible, independent of protein synthesis and apparently triggered by a decrease in the intracellular pH. It is shown that the ATPase regulatory domain implicated in the activation by fermentable carbon sources is also implicated in activation by nitrogen starvation and by external acidification.

Plasma membrane ATPase; Regulation of ATPase; Regulatory domain of ATPase; *Saccharomyces cerevisiae*

1. INTRODUCTION

Yeast plasma membrane ATPase is an extensively studied enzyme [1,2] implicated in the maintenance of the intracellular pH [3,4] and active transport of nutrients [5–8]. This enzyme is activated in vivo by fermentable carbon sources [9,10] through changes in several of its kinetic parameters [9]. In addition, plasma membrane ATPase is activated by acidification of the culture medium [11] and by the presence of high concentrations of ethanol [12]. In these later cases V_{max} is the parameter mainly affected. Deletion analysis [13] and site-directed mutagenesis [14] have shown that activation by fermentable substrates is controlled by a regulatory domain placed at the C-terminus of the enzyme. Information on the domain(s) that control the other activation processes is not yet available. The mechanism(s) of the activations are also still unknown. Although there are indications that this enzyme might be regulated through phosphorylation–dephosphorylation [15,16] there is no conclusive evidence that supports this possibility.

We report in this paper that yeast plasma membrane ATPase is activated during nitrogen starvation when a fermentable substrate is present. The characteristics of this activation are similar to those of the activation by acidification of the medium. The results obtained with different mutations of the ATPase gene indicate that the domain involved in activation by fermentable substrates is also involved in activation by nitrogen starvation and by external acidification.

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2. MATERIALS AND METHODS

Cycloheximide, MES, diethylstilbestrol, and ATP were from Sigma Chemical Co. (St. Louis, MO). ^{125}I-labeled protein A was from Amersham International (Amersham, UK). All other reagents were of analytical grade. Strain ATCC 42407 was grown aerobically with 2% glucose in minimal medium as previously described [17]. Strains expressing wild type ATPase (RS-303) or mutant alleles Ala→stop (RS-503); Ser^{911}→Ala, Thr^{912}→Ala (IB-355); Ser^{911}→Ala (IB-169); and Gln^{908}→Glu (IB-438) have been described previously [13,14]. These strains were grown in galactose [13,14]. To express the mutations the cells were transferred to a medium containing glucose as described in [18]. Cell growth was monitored by optical absorbance measurements at 640 nm. Conditions for nitrogen starvation were as in [17]. Crude extracts were obtained from the cellular homogenate as previously described [19]. Crude and purified plasma membrane preparations were obtained from washed cells (non-fermenting cells) by sucrose gradient centrifugation as previously described [19]. When indicated the cells were treated with glucose before homogenization (fermenting cells) as in [9]. ATPase was determined as in [19]. To determine the amount of ATPase the immunoassay described in [20] was used. Intracellular pH was determined using labeled benzoic acid as in [21]. Protein was determined after precipitation with 5% trichloroacetic acid by the method of Lowry et al. [22].

3. RESULTS AND DISCUSSION

3.1. *Effect of nitrogen starvation on the content and activity of ATPase*

When yeast growing on a glucose-containing medium was deprived of a nitrogen source, the amount of ATPase decreased. This decrease followed first order kinetics indicating a half-life for this protein of about 11 h (Fig. 1A). When ethanol was used as energy source,

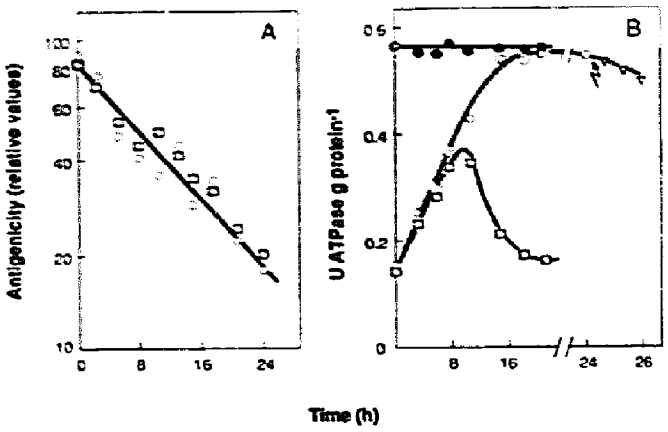

Fig. 1. Effect of nitrogen starvation on the content and activity of ATPase. Cells were harvested during exponential growth on glucose and transferred to five times the initial volume of the media specified below. (A) Ammonium-free medium containing 2% glucose (○); ammonium-free medium containing 10 μg ml⁻¹ cycloheximide and 2% glucose (□). After incubation at 30°C for different times, cells were harvested, washed and assayed for plasma membrane ATPase content using anti-ATPase antibodies and crude extracts. (B) Ammonium-free medium containing 2% glucose (○,●); ammonium-free medium containing 10 μg·ml⁻¹ cycloheximide and 2% glucose (□). After incubation at 30°C for the indicated times cells were washed (non-fermenting cells, basal activity of the enzyme) (○,□) or treated with glucose as described in [9] (fermenting cells, activated-form of the enzyme) (●) and assayed for plasma membrane ATPase activity at pH 6.5 and 2 mM ATP using crude plasma membrane preparations. Where indicated by the arrow NH_4Cl was added to 50 mM final concentration (↑).

nitrogen starvation was without detectable effect and the calculated half-life for ATPase was >30 h (data not shown). All these results confirm data previously reported [20].

Fermentable carbon sources reversibly convert a non-activated form (basal activity) of yeast plasma membrane ATPase into an activated form through the effect of an activating system [9]. We investigated the effect of nitrogen starvation on these two forms of the enzyme. We found that the activity of the activated form (fermenting cells) remained constant (Fig. 1B) in spite of the observed decrease in the amount of ATPase (Fig. 1A). In addition we found that the basal activity of the enzyme (non-fermenting cells) increased until reaching a plateau in about 15 h of starvation (Fig. 1B). After this period both forms of the enzyme showed similar activities (Fig. 1B). When instead of glucose ethanol was present, no changes in any of the two forms of the enzyme were observed (results not shown).

These results indicate that nitrogen starvation triggers an activation process of the yeast plasma membrane ATPase that affects both forms of the enzyme and that requires the presence of a fermentable substrate. The fact that the differences between the basal activity and the activated form of the enzyme decreased during nitrogen starvation (Fig. 1B) is consistent with the reported unstability of the ATPase activating system whose half-life under these conditions is ca. 5 h [20].

3.2. *Characteristics of the activation by nitrogen starvation*

The activation processes of the yeast plasma membrane ATPase previously reported differ in several characteristics. Activation by fermentation is a reversible process, independent of protein synthesis, that occurs through changes in the V_{max}, affinity for ATP, optimum pH, and K_i for orthovanadate of the enzyme [9]. However, activation by high concentrations of ethanol, although reversible, is dependent on protein synthesis and is due to changes in the V_{max} [12]. Also due to changes in the V_{max} is the activation by acidification of the external medium but in this case the process is irreversible and independent of protein synthesis [11].

We have investigated the characteristics of the activation by nitrogen starvation of the basal activity of ATPase and have found that it is an irreversible process since it remains for, at least, 2 h after a nitrogen source is restored to starved cells (Fig. 1B). We have also found that this activation is due to changes in V_{max} that increased up to 3.5-fold whereas the K_m for ATP, optimum pH, and K_i for orthovanadate remained virtually constant (Table 1). In addition this process seems to be independent of protein synthesis. This is inferred from

Table 1

Changes in the kinetic parameters of the ATPase occurred after nitrogen starvation in the presence of glucose

Nitrogen starvation (h)	K_m for ATP[a] (mM)	V_{max}[a] (U mg protein⁻¹)	Optimum pH[b]	K_i for orthovanadate[c] (μM)
0	6.3	2.0	5.6	4
16	5.1	6.8	5.7	4

Cells were harvested during exponential growth on glucose and transferred to five times the initial volume of ammonium-free medium containing 2% glucose. After incubation at 30°C for the indicated times cells were harvested, washed (non-fermenting cells) and purified plasma membrane preparations were obtained.

[a] ATPase activity was assayed at pH 6.5 and ATP ranging from 0.2 to 8 mM. The apparent K_m and V_{max} were extrapolated from double-reciprocal plots.

[b] ATPase activity was assayed at 2 mM ATP and pH ranging from 5.0 to 8.0.

[c] ATPase activity was assayed at pH 6.5 and 2 mM ATP in the absence or in the presence of vanadate ranging from 1 to 10 μM. Similar results were obtained with two different membrane preparations.

the fact that during 8 h, activation took place in the presence of cycloheximide. After this period the enzyme activity decreased until reaching its original value (Fig. 1B). These results indicate that ATPase activation resulting from nitrogen starvation shows similar characteristics to ATPase activation resulting from acidification of the culture medium [11] suggesting that a common mechanism could be implicated in both phenomena.

3.3. *Decay of the intracellular pH occurring during nitrogen starvation*

Activation of ATPase by acidification of the external medium is supposed to be triggered by a decrease of the internal pH produced by the passive movement of protons across the plasma membrane [11]. In the case of nitrogen starvation, in the presence of a fermentable substrate activation might be related to a decrease in the internal pH produced by catabolism. We investigated changes of the intracellular pH under these conditions and found that the ATPase activation taking place during 15 h of nitrogen starvation was accompanied by a decrease of the intracellular pH from 6.66±0.052 to 6.20±0.033 (data are mean values and stardard deviations of 4 experiments). These results support the idea that this activation is due to intracellular acidification.

3.4. *ATPase domain involved in activation by nitrogen starvation and acidification*

The C-terminus of ATPase is involved in regulation by fermentable substrates [13]. We have investigated if this regulatory domain is also involved in the activation by nitrogen starvation and acidification of the external medium. A mutant with a deletion of the last 14 amino acids in the C-terminus (RS-503) produces an ATPase that remains always in its activated form and, thus, has lost the ability to be activated by fermentation [13]. We used this mutant to see if the three activation processes are controlled by the same regulatory domain. The obtained results support this possibility since the enzyme of this mutant has also lost the ability to be activated by nitrogen starvation and acidification of the medium (Table II).

It has been shown that Thr^{912} placed in the ATPase regulatory domain, is involved in activation by fermentable carbon sources and that the effect of this amino acid is reinforced by Ser^{911}. It has been also shown that the role of Ser^{911} became apparent only after mutation of Thr^{912}, and that Gln^{908} has no effect on the activation. We have investigated the eventual role of these amino acids in the activation by nitrogen starvation and acidification and for this purpose we have used two single mutants, in Ser^{911} (IB-169) and Gln^{908} (IB-438), that produce ATPase's normally activated by fermentation. In addition we have used a double mutant in Ser^{911} and Thr^{912} (IB-355) that produces an ATPase that remains always in the non-activated-form and, thus, hast lost the ability to be activated by fermentation [13,14]. Our rationale was that if the three activation processes are controlled by the same amino acids, the ATPase's of the two single mutants would be normally activated by nitrogen starvation and acidification whereas the ATPase of the double mutant would have lost both activation processes. The results obtained confirm this prediction indicating that Gln^{908}, Ser^{911}, and Thr^{912} produce similar effects in the three studied activation processes (Table II).

Table II

ATPase activation of deletion and site directed mutants

Activation process	State of the cells	ATPase (U mg protein^{-1})				
		Wild-type strain	Mutants			
		RS-303	RS-503	IB-355	IB-169	IB-438
Fermentation[a]						
	Non-fermenting	0.13	0.67	0.20	0.18	0.10
	Fermenting	0.56	0.65	0.18	0.68	0.80
Nitrogen starvation[b]						
	0 (h)	0.13	0.65	0.21	0.18	0.17
	16 (h)	0.61	0.58	0.20	0.92	0.65
Acidification[c]						
	pH 6.0	0.10	0.50	0.10	ND	ND
	pH 3.5	0.23	0.54	0.10	ND	ND

Cells were harvested during logarithmic growth in galactose and diluted 50-fold in glucose medium in order to express the mutant enzymes as described in [18]. After incubation at 30°C for 24 h, cells were treated as follows. [a]Cells were washed (non-fermenting) or treated with glucose (fermenting) as described in [9] and then assayed for ATPase activity. [b]Cells were harvested and suspended in ammonium-free medium containing 2% glucose, incubated at 30°C for the indicated times washed and assayed for ATPase activity. [c]Cells were washed, suspended in the appropriate buffer, incubated at 30°C for 3 h as described in [11], washed and assayed for ATPase activity. In all cases purified plasma membrane preparations were used. Similar results were obtained in two different experiments.

4. FINAL REMARKS

Plasma membrane ATPase controls the intracellular pH in yeast cells [3,4] and, most likely, any condition that tends to acidify the cells would result in the activation of the enzyme. In the starvation conditions used in this work a decrease in the intracellular pH of 0.5 resulted in an activation of ATPase of up to 12-fold. This value has been calculated taking into account the simultaneous decrease of the enzyme amount. The activation produced by acidification occurs by a mechanism different from those of the activations produced by fermentable substrates [9] or by an excess of ethanol [12]. This is suggested by the different characteristics of these three activation processes. We have found that the ATPase regulatory domain that controls activation by fermentation also controls activation by acidification. It would be interesting to establish if this domain is also involved in activation by ethanol. Would this be the case it would suggest that, independently of the mechanism involved, a unique domain of ATPase is responsible for the modulation of the activity of this enzyme.

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