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In vivo inactivation of the yeast plasma membrane ATPase in the absence of exogenous catabolism

Lourdes Amigo [1], Eulalia Moreno and Rosario Lagunas

Instituto de Investigaciones Biomédicas del CSIC, Madrid (Spain)

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Yeast plasma membrane ATPase is inactivated up to 80% in the absence of catabolism of exogenous nutrients (exogenous catabolism). This inactivation, that is not accompanied by a decrease in the cellular content of ATPase, is due to an irreversible decrease of the V_{max} and does not require protein synthesis. The inactivated enzyme maintains the ability to be regulated by fermentable sugars but shows important alterations in the characteristics of this regulation. Upon addition of glucose, the V_{max} of the inactivated enzyme increases as well as its K_i for vanadate but, in contrast to the normal enzyme, its affinity for ATP or its pH optimum do not increase. It is concluded that in the absence of exogenous catabolism an irreversible modification of the yeast plasma membrane ATPase takes place that affects several of its kinetic properties.

Introduction

Yeast plasma membrane ATPase is implicated in the maintenance of the intracellular pH and active transport of nutrients [1–8]. This enzyme is activated by several mechanisms triggered by different metabolic conditions: fermentation [9–10], acidification of the culture medium [11], nitrogen starvation [12] and high ethanol concentration [13]. Based on the observation that plasma membrane ATPase activity decreased at the start of the stationary phase of growth it was suggested that this enzyme might be also inactivated under certain physiological conditions [14]. We have investigated this possibility and have found that in the absence of catabolism of exogenous nutrients (exogenous catabolism) a progressive and irreversible inactivation of the enzyme takes place. We have also found that the inactivated enzyme shows significant alterations of its regulatory characteristics.

Materials and Methods

Materials. Cycloheximide, Mes, diethylstilbestrol and ATP were from Sigma (St. Louis, MO, USA). ^{125}I-labelled protein A, inulin-[^{14}C]carboxylic acid, tritiated water and the cyclic AMP[^{3}H] assay system were from Amersham International (Amersham, UK). [7-^{14}C]benzoic acid was from NEN Du Pont (France). All other reagents were of analytical grade.

Yeast strain and growth conditions. Strain ATCC 42407 (MATa GAL MAL suc) of *Saccharomyces cerevisiae* was grown aerobically with 2% glucose in minimal medium at 30°C in a rotatory shaker as described in Ref. 15. Cell growth was monitored by optical absorbance measurements at 640 nm or by measuring total protein content [16].

Inactivation conditions. Cells were harvested during exponential growth (about 0.7 mg (dry weight)/ml), washed and transferred to fresh medium without carbon source or containing 2% ethanol or 2% galactose. The cellular density was about 0.2 mg (dry weight)/ml and the suspension was incubated as above.

Analytical procedures. At the times indicated in each experiment aliquots of the cell suspensions were centrifuged, washed, and suspended in water (non-fermenting cells) or 2% glucose (fermenting cells) to a cellular density of about 2 mg (dry weight)/ml. After incubation at 30°C for 8 min in a rotatory shaker, the cells were rapidly harvested by filtration, frozen in liquid nitrogen and stored at −70°C until use. Crude extracts and crude membrane preparations were obtained as previously described [17]. Purified plasma membrane preparations were obtained by sucrose gradient centrifugation [17]. Plasma membrane ATPase activity was determined at the pH and ATP concentration indicated in each experiment and in the presence

Correspondence to: R. Lagunas, Instituto de Investigaciones Biomédicas, Arturo Duperier 4, 28029 Madrid, Spain.

[1] Present address: Instituto de Fermentaciones Industriales del CSIC, Juan de la Cierva 3, 28006 Madrid, Spain.

of molybdate and azide to inhibit acid phosphatase and mitochondrial ATPase respectively as described in Ref. 17. The cellular content of ATPase was determined in crude extracts using anti-ATPase antibodies [18] by the immunoassay described in Ref. 19. The intracellular pH was determined using labelled benzoic acid as in Ref. 20 and the intracellular volume using labelled inuline and tritiated water as in Ref. 21. cAMP was determined in cellular extracts obtained as in Ref. 22 using the assay system kit from Amersham following the instructions supplied by the manufacturers. Protein was determined after precipitation with 5% trichloracetic acid by the method of Lowry et al. [23] using bovine serum albumin as standard.

Results

ATPase inactivation upon disappearance of exogenous catabolism

Tuduri et al. [14] reported that the activity of the yeast plasma membrane ATPase decreased by a factor 2–3 during the late exponential growth on glucose and suggested that this inactivation could be triggered by the growth arrest observed during adaptation to ethanol [14]. We checked this possibility by transfering cells growing on glucose to fresh medium with ethanol as carbon source. We observed that, upon this transfer, ATPase activity progressively decreased to a value lower than 20% of the original one (Fig. 1, non-fermenting cells). This low activity, that was attained in about 18 h, was maintained until cells were adapted to grow on ethanol and when growth began ATPase activity increased until a plateau (Fig. 1). Similar results were obtained when the medium contained galactose or no added carbon source (results not shown).

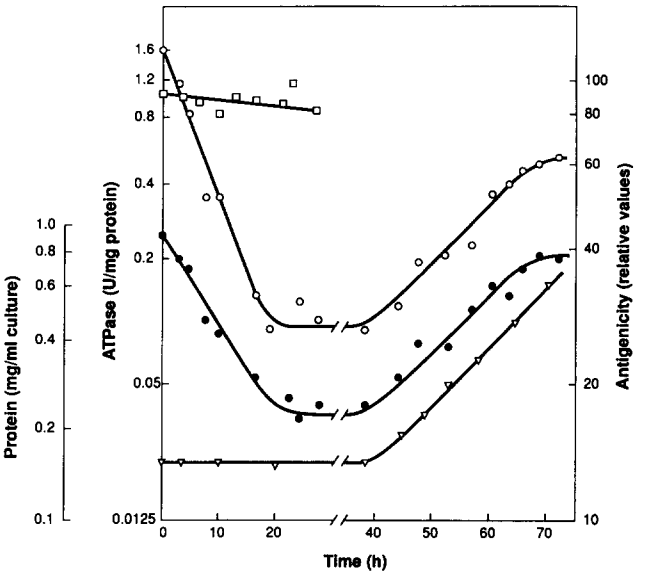

Fig. 1. Changes in the content and activity of the plasma membrane ATPase observed in the shift from glucose to ethanol containing medium. Cells were harvested during exponential growth on glucose and transferred to 3-times the initial volume of fresh medium containing 2% ethanol as carbon source. After incubation at 30°C for the indicated times, three aliquots of the cell suspension were centrifuged and washed. The cells of one of these aliquots (□) were assayed for plasma membrane ATPase content using antibodies (see Materials and Methods). The cells of other aliquot were suspended in water, incubated for 8 min at 30°C (●, non-fermenting cells) and assayed for ATPase activity (see Materials and Methods). The cells of the other aliquot were suspended in 2% glucose, incubated for 8 min at 30°C (○, fermenting cells) and assayed for ATPase activity (see Materials and Methods). In all cases ATPase activity was assayed in crude membrane preparations at pH 6.5, 2 mM ATP, and 5 mM $MgSO_4$. Cell growth (▽) was measured by determining protein content in the culture. Similar results were obtained in two different experiments.

Characteristics of the inactivation

The observed ATPase inactivation followed first-order kinetics (Fig. 1) was insensitive to the presence of cycloheximide (results not shown) and was due to an irreversible process. This is suggested by the fact that recovery of the activity was observed upon addition of glucose to inactivated cells but it was not observed when cycloheximide was present (Fig. 2). Among the kinetic parameters tested, only the V_{max} of the enzyme was affected during the inactivation process, whereas K_m for ATP, optimum pH and K_i for vanadate remained virtually constant (Table I). The possibility that inactivation could be due to extensive degradation of the protein was ruled out by measuring the cellular content of ATPase using antibodies that specifically recognize this protein (Fig. 3). It was found that the amount of ATPase present in the cells remained constant ($t_{1/2} > 20$ h) in conditions in which ATPase activity decreased rapidly ($t_{1/2} = 7$ h) (Fig. 1). These results strongly indicate that inactivation is not due to degradation but to some kind of structural modification of the protein.

Regulation by fermentable sugars of the inactivated ATPase

ATPase is regulated in vivo by fermentable sugars [9,10]. To establish if the inactivated enzyme is similarly regulated the effect of glucose on ATPase at different stages of inactivation was tested (Fig. 1, fermenting cells). The results showed that the inactivated enzyme maintained the capability to be regulated by this sugar although the regulating factor (ratio between activity of fermenting and non-fermenting cells) decreased from a value of approx. 6.5 at the start of the inactivation process to a value of approx. 3.5 at the end of this process (Fig. 1).

Glucose changes several kinetic parameters of ATPase: it increases the V_{max} by about 3-fold, it reduces the K_m for ATP (from 1.5–3 to 0.3–0.6 mM), it increases the pH optimum (from 5.6 to 6–7) and it

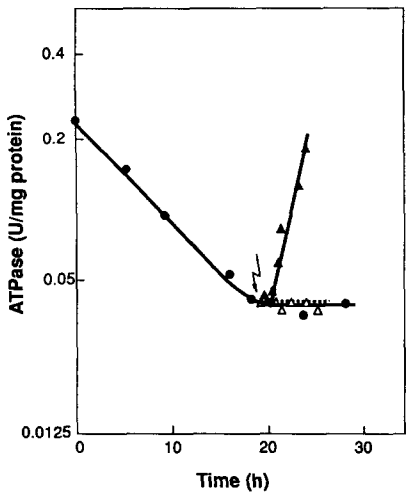

Fig. 2. Irreversibility of the inactivation process. Cells were harvested during exponential growth on glucose and transferred to 3-times the initial volume of fresh medium containing 2% ethanol. After incubation at 30°C for the indicated times samples of the suspensions (●) were centrifuged and the cells washed, suspended in water, incubated for 8 min at 30°C (non-fermenting cells) and assayed for ATPase activity (see Materials and Methods). When indicated by the arrow two aliquots of the suspension were centrifuged and the cells suspended in fresh medium containing 2% glucose in the absence (▲) and in the presence (△) of 10 μg cycloheximide. After incubation at 30°C for the indicated times samples of these suspensions were centrifuged and the cells suspended in water, incubated at 30°C for 8 min (non-fermenting cells) and assayed for ATPase activity. In all cases ATPase activity was assayed in crude membrane preparations at pH 6.5, 5 mM $MgSO_4$ and 2 mM ATP.

reduces the K_i for vanadate (from 10–20 to 2–5 μM) [9]. We have investigated if glucose produces a similar response in the inactivated enzyme and to this purpose cells whose ATPase was inactivated to different extent (no inactivation, 40% and 70% inactivation) were used. These cells were obtained by incubation in the conditions described in Fig. 1 for 0, 6 and 12 h, respectively. It was found that the V_{max} and the K_i for vanadate of the inactivated enzyme showed a similar response to the one described above for the non-inactivated enzyme (results not shown). However, important differences were observed in the case of the optimum pH and the K_m for ATP, whereas in normal cells glucose produced the complete conversion of the ATPase form with optimum pH 5.7 and K_m of approx. 3 mM to the form with optimum pH 6.6 and K_m of approx. 0.4 mM (Figs. 4A and 5A) in inactivated cells only a partial conversion took place. This is indicated by the fact that in these cells two peaks with optimum pH values of 5.7 and 6.7 (Fig. 4B,C) as well as a biphasic kinetics that suggests K_m values of approx. 3 and 0.4 mM (Fig. 5B, C) were clearly detected.

It can be observed that in the experiments shown in Figs. 4 and 5 certain differences in ATPase activity were found. This is due to the fact that two different purified plasma membrane preparations were used.

TABLE I

Changes in the kinetic parameters of ATPase occurring in the shift from glucose to ethanol-containing medium

Cells were harvested during exponential growth on glucose and transferred to 3-fold the initial volume of fresh medium containing 2% ethanol. After incubation at 30°C for the indicated times the cells were washed and suspended in water, incubated for another 8 min at 30°C, harvested and frozen in liquid nitrogen. ATPase activity was assayed in crude membrane preparations in the following conditions: [a] pH 5.7, 3 mM $MgSO_4$ and ATP-Mg ranging from 0.2 to 8 mM. The apparent K_m and V_{max} were extrapolated from double-reciprocal plots; [b] 2 mM ATP, 5 mM $MgSO_4$ and pH ranging from 5.2 to 8; [c] pH 5.7, 5 mM $MgSO_4$ and 2 mM ATP in the presence and in the absence of vanadate ranging from 1 to 25 μM. Similar results were obtained with two different membrane preparations.

Time (h)	V_{max} [a] (U/mg protein)	K_m for ATP [a] (mM)	Optimum pH [b]	K_i for orthovanadate (μM) [c]
0	0.71	2.9	5.7	17
12	0.25	2.5	5.7	18

It has been recently shown that regulation of ATPase by glucose is mediated by a phosphorylation at a specific site(s) of the enzyme catalyzed by a still unidentified kinase(s) [24]. According to this finding the incomplete effect of glucose on the inactivated cells could be due either to changes in the ATPase molecule itself or to a decrease in the activity of the kinase(s) responsible for its phosphorylation. To distinguish between these two possibilities we increased by twofold the time that this supposed kinase could be acting on

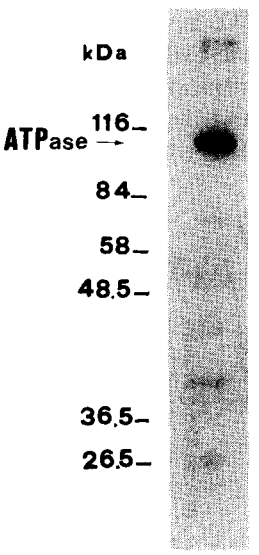

Fig. 3. Specificity of the anti-ATPase antibodies. An aliquot containing 40 μg protein of a crude extract obtained from exponentially growing cells was fractionated in 8% SDS-PAGE. The separated proteins were blotted to a nitrocellulose membrane and hybridized to the anti-ATPase antibodies [18]. An autoradiography of the antigene-antibody complex revealed with ^{125}I-labeled protein A [19] is shown.

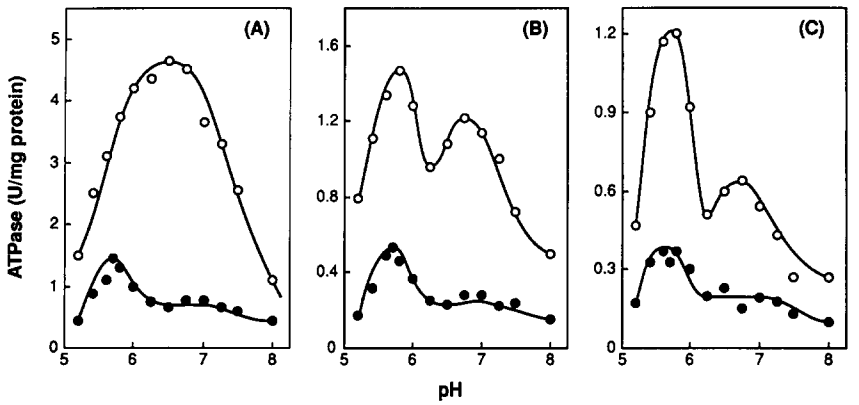

Fig. 4. Effect of fermentation on the optimum pH of ATPase at different stages of inactivation. Cells were harvested during exponential growth on glucose and transferred to 3-times the initial volume of fresh medium containing 2% ethanol. After incubation at 30°C for 0 h (A), 6 h (B) and 12 h (C) cells were harvested washed and suspended in water (•, non-fermenting cells) or 2% glucose (○, fermenting cells). After incubation at 30°C for 8 min the cells were harvested, frozen in liquid nitrogen and purified plasma membrane fraction were obtained. ATPase activity of these preparations was assayed at the indicated pH values, 5 mM $MgSO_4$ and 2 mM ATP. Similar results were obtained in two different experiments.

ATPase. This was achieved by increasing from 8 to 16 min the period in the presence of glucose (see Methods). The results obtained seem to rule out the last possibility since they were similar to those shown in Fig. 4. Therefore, the different proportion of the two ATPase forms observed at the different stages of inactivation (Fig. 4 and 5) are probably due to a structural modification of ATPase that progressively decreases the number of molecules able to be converted to the high K_m and high optimum pH form.

Cellular level of cAMP during the inactivation process

It has been suggested that cAMP can modulate ATPase activity by acting as a positive effector of the enzyme [25,26]. We have investigated the eventual correlation between cAMP levels and ATPase activity by measuring the intracellular concentration of this nucleotide. We found that the cellular content of cAMP dropped from 0.45 ± 0.04 to 0.21 ± 0.02 mM (mean values and standard deviation of four experiments) in the shift from glucose to ethanol containing medium and that this concentration remained constant during, at least, 10 h of incubation. As shown in Fig. 1 an ATPase inactivation of approx. 60% took place during this period. These results indicate that during the inactivation process there is no correlation between ATPase activity and cAMP level, since a strong inactivation of the enzyme was accompanied by a constant concentration of the nucleotide.

Intracellular pH occurring during inactivation

Intracellular acidification also controls plasma membrane ATPase in yeast cells by increasing the V_{max} and the affinity for ATP of the enzyme [11,12]. Interestingly, this activation shows two important similarities with the inactivation described in this work: both processes are irreversible and occur very slowly [12]. Taking this fact into account we have investigated the

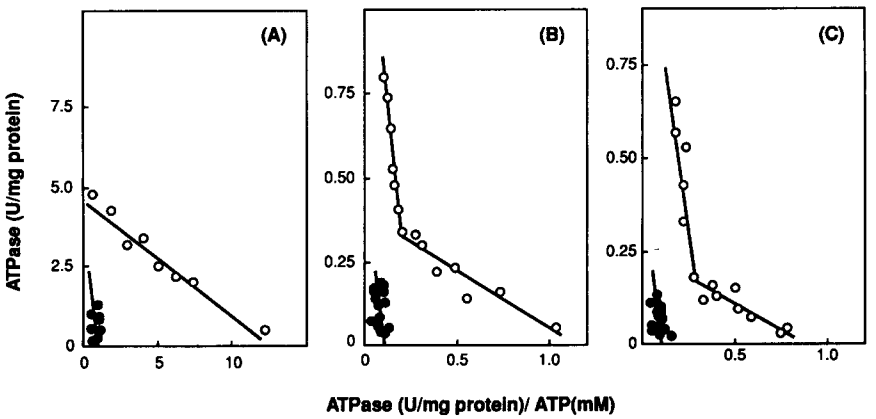

Fig. 5. Effect of fermentation on affinity of ATPase for ATP at different stages of inactivation. Cells were treated as in Fig. 3 and ATPase activity was assayed in purified plasma membrane preparations at pH 5.7, 3 mM $MgSO_4$ and ATP-Mg concentrations ranging from 0.2 to 8 mM. (•) Non-fermenting cells; (○) fermenting cells. Similar results were obtained in two different experiments.

eventual changes in the intracellular pH occurred during the inactivation process. Our rationale was that inactivation could be due to a cellular alkalinization. However, the results ruled out this possibility because the intracellular pH remained virtually constant during the inactivation process. We found intracellular pH values of 6.74 ± 0.07 and 6.54 ± 0.07, respectively, in glucose growing cells and in cells suspended for 18 h in an ethanol-containing medium (data are mean values and standard deviation of four experiments).

Discussion

Yeast plasma membrane ATPase is involved in the maintenance of intracellular pH and active transport of nutrients by pumping out protons produced in catabolism and passively diffused from the medium [3–8]. *S. cerevisiae* catabolizes glucose, fructose and mannose without previous adaptation [27]. However, catabolism of any other carbon source requires derepression and/or induction of the appropriate enzymes [27,28]. During adaptation of fermenting cells to a new carbon source or during carbon starvation, when no exogenous catabolism occurs, the consequent decrease in proton production is accompanied by a decrease in ATPase activity. This work shows that this decrease is achieved in two steps: a rapid one, reversible, that occurs in a few minutes upon disappearance of fermentation [9] and a slow one, irreversible, that requires hours to reach a plateau. The results indicate that both steps involve modifications of the enzyme that, as a consequence, exists in at least three different forms. (i) An activated form, present during fermentation [9], that in our experimental conditions shows a V_{max} of about 1.6 U/mg protein, a high optimum pH, and a high affinity for ATP and for vanadate (see above). (ii) A basal form, appearing inmediately upon disappearance of fermentation, that shows a V_{max} of about 0.25 U/mg protein, a low optimum pH, and a low affinity for ATP and for vanadate and that is readily converted into the activated form upon reappearance of fermentation [9]. (iii) An inactivated form, that slowly appears in the absence of exogenous catabolism, and that differs from the basal form in the V_{max}, that is only about 0.04 U/mg protein, as well as in the response to fermentation: whereas fermentation increases all mentioned parameters of the basal form, it only increases the V_{max} and the inhibition by vanadate of the inactivated one. The results also indicate that, when no exogenous catabolism occurs, the two later forms of ATPase coexists into the cells in proportions that change until the complete disappearance of the basal form.

The rapid and reversible activation of ATPase by fermentation appears to be mediated by phosphorylation-dephosphorylation of specific site(s) [24] that might be influenced by several factors (for a review, see Ref. 2). Since the enzyme also contains multiple constitutive phosphorylated Ser and Thr residues [24], it can be speculated that dephosphorylation of some of these residues could be involved in the irreversible inactivation described in this work. However, a different type of modification can be also envisaged as responsible for the inactivation. Yeast plasma membrane ATPase is active without any accessory subunit but it is possible that in vivo this enzyme exists in association with other membrane proteins or small peptides that regulate its activity. Actually other members of the same family of ATPases do show additional subunits [2]. Recently, a small proteolipid has been sequenced that is firmly bound to yeast ATPase [29]. It has been speculated that this proteolipid might be a yeast analogue to phospholamban [29], a small peptide that regulates the sarcoplasmic reticulum Ca^{2+}-ATPase [30]. Changes in this proteolipid could be responsible for changes in ATPase activity. In addition several lines of evidence indicate that ATPase could exist in an oligomeric state with a tendency to form higher-order structures [2]. This tendency, that seems to increase in the absence of exogenous catabolism [2], could affect the activity of the enzyme. It has been also found that yeast plasma membrane ATPase is strongly associated with the major glycoprotein of the plasma membrane [31] and the possibility that lipid changes could mediate the regulation of ATPase has been also considered [2].

It is worth remarking that the inactivation process described in this work occurs in nutritional conditions that are common to yeast during growth in its natural habitats, i.e., adaptation to a new carbon source and carbon source starvation. Therefore, this phenomenon is likely quite relevant to this organism.

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