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In vivo activation of yeast plasma membrane H^+-ATPase by ethanol: effect on the kinetic parameters and involvement of the carboxyl-terminus regulatory domain

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Abstract

The in vivo activation of *Saccharomyces cerevisiae* plasma membrane H^+-ATPase by ethanol was observed during ethanol-stressed cultivation or following the rapid incubation of cells with ethanol (6% (v/v)). Ethanol activated both the basal and the glucose-activated forms of the enzyme being the H^+-ATPase fully activated by glucose (5% (w/v)) still additionally activable by ethanol. The kinetic parameters of ethanol-activated and non-activated H^+-ATPase were calculated based directly on Michaëlis–Menten equation (with MgATP concentrations in the range 0.16–8.18 mM and 7.5 mM of free Mg^{2+}); the rectangular hyperbolic function was solved using iterative procedures. Ethanol-induced stimulation of plasma membrane H^+-ATPase activity was associated to the increase of V_{max} whereas the K_m for MgATP increased. Results obtained with mutants constructed and used in previous studies envisaging the analysis of the molecular mechanisms underlying plasma membrane ATPase activation by glucose, external acidification and nitrogen starvation, suggested that the carboxyl-terminus (C-terminus) regulatory domain may also be involved in the in vivo activation by ethanol. © 1998 Elsevier Science B.V.

Keywords: Plasma membrane H^+-ATPase; Carboxyl-terminus; In vivo activation; Ethanol; (*Saccharomyces cerevisiae*)

1. Introduction

Yeast plasma membrane H^+-ATPase creates the electrochemical proton gradient that drives the uptake of nutrients by secondary transport and it is implicated in intracellular pH homeostasis. Therefore, this membrane enzyme plays an essential role in the physiology of yeast; it is rate-limiting and essential for growth [1] and influence tolerance to stress factors such as heat [2], ethanol [3] and weak acids [4]. This enzyme hydrolyses much of the ATP generated by the cell and accurate control mechanisms must operate to modulate its activity. Like glucose [5], several types of environmental stresses have been reported to stimulate in vivo the activity of yeast plasma membrane H^+-ATPase. Examples of these stresses are: subcritical inhibitory concentrations of ethanol [6,7], supraoptimal temperatures below 40°C [8], organic acids at low pH [4,9–12] and deprivation of nitrogen source [13]. The H^+-ATPase activation

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under stress constitutes a response that presumably helps the cell to counteract the stress-induced dissipation of the proton motive force across the plasma membrane and the decrease in the intracellular pH [8,10,12–14]. Several pieces of evidence indicate that it is PMA1 H^+-ATPase that is activated by ethanol, octanoic acid or supraoptimal temperatures by post-translational mechanisms and that the PMA2 H^+-ATPase activation is not involved [7,8,11]. However, if the molecular mechanism of H^+-ATPase activity stimulation by glucose metabolism is still not fully understood [15,16], the mechanism(s) underlying H^+-ATPase activation under the various stress conditions are much more obscure. Deletion analysis [17] and site-directed mutagenesis [1,15,18–20] have shown that the carboxyl-terminus (C-terminus) of yeast plasma membrane H^+-ATPase may act as an auto-inhibitory domain being its effect counteracted by modification of the enzyme triggered by glucose metabolism [15,16]. The glucose-activated H^+-ATPase has a higher affinity for MgATP, a higher V_{max}, a more alkaline optimum pH and a greater sensitivity to orthovanadate than the non-activated enzyme. On the other hand, activation during nitrogen starvation or growth in medium with glucose plus high concentrations of ethanol (around 6% (v/v)) is mainly due to changes in the V_{max} whereas the K_m for MgATP, optimum pH and K_i for orthovanadate remain virtually constant [6,13]. Nevertheless, results obtained with different mutations of the H^+-ATPase *PMA1* gene indicate that the C-terminus regulatory domain involved in the activation by fermentable substrates is also involved in activation by nitrogen starvation or external acidification with succinic acid [13].

In the present work, we have examined thoroughly the modifications of the kinetic parameters of the plasma membrane H^+-ATPase of yeast cells due to growth in medium supplemented with ethanol (6% (v/v)), or due to cell incubation, for a short period of time (20 min), in aqueous solutions of glucose or sorbitol, supplemented or not with 6% (v/v) ethanol. The possible involvement of the C-terminus regulatory domain in the in vivo H^+-ATPase activation by ethanol was also investigated going over some of the mutants constructed and used in previous studies concerning activation by glucose, nitrogen starvation and medium acidification [13,17,19].

2. Materials and methods

2.1. Yeast strains and growth conditions

Saccharomyces cerevisiae YPH499 [21], used in previous studies concerning ethanol-induced plasma membrane H^+-ATPase activation [7], was examined in this work. Cells were batch cultured at 30°C with orbital agitation (150 rpm) in 250 ml Erlenmeyer flasks with 150 ml of liquid medium containing: 20 g l^{-1} glucose, 6.7 g l^{-1} Yeast Nitrogen Base (without amino acids) (Difco), 40 mg l^{-1} adenine, 20 mg l^{-1} uracil and a mixture of several amino acids [11] supplemented or not with 6% (v/v) of ethanol. Growth was monitored by measuring culture absorbance at 600 nm ($OD_{600\ nm}$). Genetically engineered strains with the constitutive promoter of the chromosomal wild-type PMA1 H^+-ATPase gene replaced by a galactose-dependent promoter and harbouring recombinant plasmids with wild-type or mutated H^+-ATPase gene or no H^+-ATPase gene were also used. These strains express, in glucose medium, wild type PMA1 H^+-ATPase (RS303) or no H^+-ATPase (RS357; control cells) [18] or mutant alleles $Gln^{908} \rightarrow$ stop (RS503) or $Ser^{911} \rightarrow$ Ala $Thr^{912} \rightarrow$ Ala (IB355) [17,19]. In order to express the plasmid-borne gene, cells grown in a medium with galactose (2% (w/v)), where both the wild type (chromosomal) H^+-ATPase and mutant (plasmid) H^+-ATPase were expressed, were transferred to a medium containing glucose as described previously [17,19]. Cell growth was monitored by optical absorbance measurements at 600 nm being the initial $OD_{600\ nm}$ in the glucose medium 0.10 ± 0.02 in order to express the plasmid-borne gene. The residual wild type H^+-ATPase from glucose grown cells of the inoculum was assessed using the control strain RS357 expressing no plasmid-borne H^+-ATPase gene.

2.2. Cell incubation conditions

Cells of the different yeast strains were grown in the media referred above in the presence or absence of ethanol 6% (v/v). Cells were harvested in the late exponential phase of growth by centrifugation and resuspended in sorbitol 2% (w/v) (at an $OD_{600\ nm}$ identical to that exhibited by the culture) and incu-

bated with orbital agitation (150 rpm) at 30°C for 30 min in order to reverse glucose activation [5]. Cells were then centrifuged and resuspended at an $OD_{600\ nm} \approx 24$ (≈ 12 mg dry biomass ml^{-1}) in aqueous solutions of sorbitol 2% (w/v) or glucose 2% (w/v) supplemented or not with 6% (v/v) of ethanol (pH = 5.9 ± 0.1). Samples of 2 ml were taken after 20 min of incubation at 30°C in the different solutions and rapidly frozen in liquid nitrogen after addition of Tris, EDTA and dithiothreitol to final concentrations of 100, 5 and 2 mM [9]. Samples were kept at −70°C until used for the preparation of plasma membrane suspensions.

2.3. H^+-ATPase assay and calculation of kinetic parameters

Crude and plasma membrane suspensions were obtained and plasma membrane H^+-ATPase activity was assayed as described by Monteiro et al. [7] using 2 mM of Na_2ATP and 10 mM of $MgSO_4$, corresponding to 1.6 mM of MgATP and 7.5 mM of free Mg^{2+} [22]. Kinetic parameters were calculated using MgATP concentrations varying between 0.16 and 8.18 mM and maintaining the concentration of free Mg^{2+} equal to 7.5 mM [22]. V_{max} and K_m were obtained from the rectangular hyperbolic function solved using iterative procedures (computer program: Solver from Microsoft Excel, Microsoft).

3. Results

3.1. Changes in the kinetic parameters of plasma membrane H^+-ATPase occur in yeast cells grown in medium supplemented with 6% (v/v) ethanol

Plasma membrane ATPase activity was assayed in crude membranes isolated from cells of YPH499 grown in medium supplemented or not with 6% (v/v) ethanol, at different MgATP concentrations in the range 0.16–8.18 mM, maintaining the concentration of free Mg^{2+} equal to 7.5 mM (Fig. 1). The kinetic parameters of ethanol-activated and basal H^+-ATPase were calculated based directly on the Michaëlis–Menten equation without its rearrangement into equations that can be presented graphically in a straight line. The nonlinear evaluation gives more reliable results, especially when errors are large, particularly because the correlation between V_{max} and K_m results in possible misinterpretations of simultaneous changes in both parameters [23]. The rectangular hyperbolic function was solved using iterative procedures (computer program: Solver from Microsoft Excel, Microsoft). Within the range of concentrations of MgATP used, the single Michaëlis–Menten kinetics fitted reasonably well the experimental results (Fig. 1). The fitting using an equation composed of the sum of two Michaelian terms [24] and a sigmoid relationship [25] was also attempted but some K_m values were of doubtful physiological and biochemical significance, as referred by Wach et al. [26], and these models were not considered. The values calculated for K_m and V_{max} for the plasma membrane H^+-ATPase of exponential cells grown in media with or without ethanol supplementation indicated that H^+-ATPase activation is due to the increase of V_{max}, that increased twofold, whereas the K_m for MgATP increased twice (Table 1 and Fig. 1). Similar changes in the kinetic parameters of plasma membrane H^+-ATPase of yeast cells grown in the absence or presence of ethanol stress were confirmed when plasma membrane fraction was further purified by acid precipitation using the method described by Monteiro et al. [7]. Under these conditions, the ki-

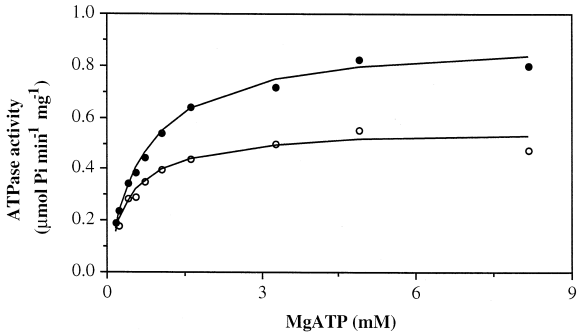

Fig. 1. Comparison of the H^+-ATPase activity in the plasma membrane of cells grown in media supplemented (●) or not (○) with ethanol 6% (v/v) as a function of MgATP concentration. Cells were harvested at the late exponential phase of growth ($OD_{600\ nm} = 1.9 \pm 0.1$). The lines through the points are the best fit of a Michaëlis–Menten relationship. This rectangular hyperbolic function was solved by iterative procedures using the computer program (Solver from Microsoft Excel, Microsoft). Results are average of at least 3 experiments.

Table 1
Comparison of the kinetic parameters of *S. cerevisiae* YPH499 plasma membrane H^+-ATPase

Cell treatment	K_m (mM)		V_{max} (μmol Pi min^{-1} mg^{-1})	
	0% (v/v)	6% (v/v)	0% (v/v)	6% (v/v)
Exponential cells	0.43 ± 0.08	0.72 ± 0.14	0.56 ± 0.06	0.91 ± 0.15
Deactivated cells	2.11 ± 0.21	1.10 ± 0.05	0.44 ± 0.02	0.33 ± 0.02
Cells incubated in				
Sorbitol	2.33 ± 0.15	2.23 ± 0.19	0.47 ± 0.05	0.41 ± 0.06
Sorbitol + ethanol	2.74 ± 0.09	3.00 ± 0.22	0.65 ± 0.06	0.46 ± 0.03
Glucose	0.60 ± 0.11	1.08 ± 0.17	1.31 ± 0.08	0.72 ± 0,21
Glucose + ethanol	0.74 ± 0.09	1.48 ± 0.21	1.52 ± 0.11	1.08 ± 0.25

Cells were grown in media supplemented or not with ethanol 6% (v/v) and harvested in the late exponential phase of growth ($OD_{600\ nm}$ = 1.8 ± 0.1) (exponential cells) and then incubated, with agitation at 30°C during 30 min, in an aqueous solution of sorbitol 2% (w/v) (to deactivate the enzyme-deactivated cells). The deactivated cells were incubated during an additional period of 20 min with agitation at 30°C, in the following aqueous solutions: (a) sorbitol (2% w/v), (b) sorbitol (2% (w/v)) + ethanol (6% (v/v)), (c) glucose (2% (w/v)), and (d) glucose (2% (w/v)) + ethanol (6% (v/v)). V_{max} and K_m values were calculated using concentrations of MgATP in the range 0.16–8.18 mM, maintaining the concentration of free Mg^{2+} (7.5 mM) and solving, by iterative procedure, the rectangular hyperbolic function of a Michaëlis–Menten relationship. Values are average ± standard deviaton of at least 2 independent incubation experiments with at least three enzyme assays each.

netic parameters calculated for the non-activated and for the ethanol-activated ATPase were K_m = 3.3 mM, V_{max} = 3.11 μmol Pi min^{-1} mg^{-1} and K_m = 5.10 mM and V_{max} = 5.13 μmol Pi min^{-1} mg^{-1}, respectively. These results differ from changes detected in the kinetic parameters of glucose-induced activated plasma membrane H^+-ATPase that exhibits higher affinity for MgATP than the basal form [5]. However, when linear analysis of Michaëlis–Menten equation (Lineweaver–Burk plot) and a narrower range of MgATP concentrations (0.4–2.0 mM) were used, the K_m for MgATP of ethanol-grown cells plasma membrane H^+-ATPase was considered to remain virtually constant [6] like the one reported by Benito et al. [13] for activation by nitrogen starvation.

3.2. Rapid H^+-ATPase activation due to ethanol (6% v/v) supplementation of the cell suspension medium with either sorbitol or glucose

Activation of plasma membrane H^+-ATPase was also found to occur when yeast cells were incubated for a standardised short period of time (10–20 min) in aqueous solutions of either sorbitol or glucose (2% (w/v)) due to ethanol (6% (v/v)) supplementation (Table 1). These activations by ethanol were also associated to the increase of V_{max} while the K_m for MgATP increased (Table 1), confirming the modifications of the kinetic parameters of plasma membrane H^+-ATPase when activation was due to cell growth under ethanol stress. Interestingly, although the H^+-ATPase V_{max} was higher in cells grown under ethanol stress (6% (v/v)) compared with cells grown in the absence of ethanol, the plasma membrane H^+-ATPase V_{max} of the cells incubated with glucose reached higher values in cells grown in the absence of ethanol stress (Table 1). The in vivo activation by

Table 2
Additional activation of plasma membrane H^+-ATPase specific activity occurring in cells of *S. cerevisiae* YPH499 incubated in aqueous solutions of glucose supplemented or not with ethanol

Glucose % (w/v)	H^+-ATPase (μmol Pi min^{-1} mg^{-1})	
	No ethanol	Ethanol 6% (v/v)
0	0.04	0.09
2	0.16	0.37
5	0.37	0.60
10	0.35	0.74

Cells were harvested in the late exponential phase of growth in the absence of added ethanol and incubated during 30 min with agitation at 30°C in an aqueous solution of sorbitol 2% (w/v) and then incubated (20 min at 30°C with agitation) in media with increasing concentrations of glucose (0, 2, 5 or 10% (w/v)) supplemented or not with ethanol 6% (v/v). Results are of one representative cell incubation experiment.

ethanol (6% (v/v)) occurred in both the glucose-activated and the basal forms of plasma membrane ATPase. Furthermore, when the concentration of glucose in the incubation medium was increased from 2 to 5 and 10% (w/v), which are concentrations leading to maximal glucose-activation levels (Table 2), the fully glucose-activated ATPase was still activable by ethanol (Table 2).

3.3. Role of the H$^+$-ATPase C-terminus on ethanol-induced activation

We have investigated if the C-terminus of plasma membrane H$^+$-ATPase is also involved in its in vivo activation by ethanol. For this, cells expressing mutated H$^+$-ATPase lacking the last 11 amino acids in the C-terminus (RS503) or with a double mutation Ser^{911} → Ala and Thr^{912} → Ala (IB355) were used. When cultivated in medium with glucose, strain RS303 (expressing wild type H$^+$-ATPase) and strain RS503 (expressing mutant H$^+$-ATPase lacking the last 11 amino acids) reached an $OD_{600\ nm}$ at the stationary phase of growth of about 2.7 and 2.5, respectively (Fig. 2b), while strains RS357 (containing only residual chromosomal wild-type H$^+$-ATPase, amounting to about 25% of the RS303 level) and IB355 (the non-activable mutated H$^+$-ATPase) only reached $OD_{600\ nm}$ values of about 1.4 and 1.5, respectively (Fig. 2b). These results are consistent with the critical physiological role of plasma membrane H$^+$-ATPase activity since the growth curves of the 4 strains in medium with galactose (where wild type chromosomal gene was also expressed) were similar (Fig. 2a). Under the experimental conditions used in the present work, it was confirmed that plasmid-borne H$^+$-ATPase from strains RS503 and IB355 remains either in the activated or in the non-activated form, respectively, and thus have lost the ability to be activated by glucose (Fig. 3) while plasmid-encoded wild-type H$^+$-ATPase (strain RS303) or residual wild-type H$^+$-ATPase (strain RS357), expressed from the chromosomal gene during the previous growth in galactose, were activable by glucose (Fig. 3). Since a residual activity of wild-type ATPase was detected in cells of the control strain RS357 due to the expression of wild-type chromosomal gene during growth of the inoculum in galactose medium (Fig. 3), this residual value was withdrawn from the specific AT-

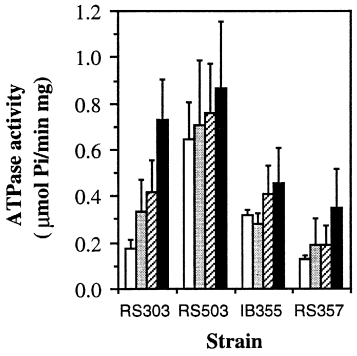

Fig. 3. Plasma membrane H$^+$-ATPase activities of late exponential cells grown in glucose medium to allow the expression of plasmid encoded wild-type (strain RS303) or mutated H$^+$-ATPase deleted of the last 11 amino acids in the C-terminus (strain RS503) or with Ser^{911} and Thr^{912} substituted by Ala and Ala (strain IB355) or no H$^+$-ATPase (strain RS357; control cells exhibiting residual values of H$^+$-ATPase resulting from cells present in the inoculum grown in galactose medium), incubated during 30 min in sorbitol 2% (w/v) to deactivate plasma membrane ATPase and subsequently incubated during an additional period of 20 min in the following aqueous solutions ($OD_{600\ nm} \approx 24$; pH = 5.9 ± 0.1): sorbitol 2% (w/v) (□), sorbitol 2% (w/v) + ethanol 6% (v/v) (dotted), glucose 2% (w/v) (hatched), glucose 2% (w/v) + ethanol 6% (v/v) (■). Values of plasma membrane H$^+$-ATPase in cells grown in glucose medium, before being washed and suspended in the various incubation media were (μmol Pi min^{-1} mg^{-1}): strain RS303, 0.51 ± 0.15; strain RS503, 0.68 ± 0.28; strain IB355, 0.62 ± 0.18; strain RS357, 0.14 ± 0.02. Results are the means of 3 independent incubation experiments with 4 assays each.

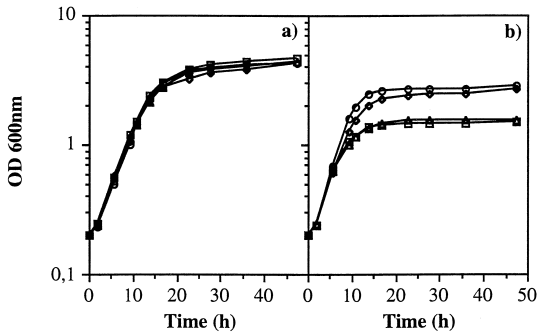

Fig. 2. Growth curves of strains RS357 (□), RS303 (○), RS503 (◇) and IB355 (△) in medium with (a) galactose 2% (w/v) or (b) glucose 2% (w/v) as the only carbon source. Inocula for both growth experiments were prepared by cell cultivation in galactose (2% (w/v)) medium. Results are representative of the various growth experiments carried out.

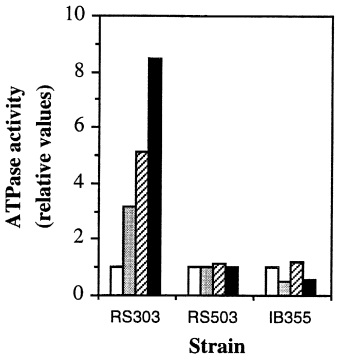

Fig. 4. Level of ATPase activation due to 20 min incubation in aqueous solutions of sorbitol 2% (w/v) (□), sorbitol 2% (w/v) + ethanol 6% (v/v) (dotted), glucose 2% (w/v) (hatched), glucose 2% (w/v) + ethanol 6% (v/v) (■) of cells of strains RS303, RS503, and IB355, only expressing plasmid encoded ATPases. Values were calculated based on data in Fig. 3, discounting the corresponding residual ATPase activity calculated for the control strain RS357. This residual activity was due to wild-type enzyme expressed from the chromosomal gene during previous growth of the inoculum in galactose medium; it was assumed that identical values of wild-type enzyme were expressed from the chromosome of strains RS303, RS503 and IB355 during their inocula growth in galactose medium.

Pase activity values calculated for strains RS303, RS503 and IB355 (Fig. 4). In Fig. 4, it can thus be solely compared the level of activation of plasmid-borne ATPases. These values clearly confirmed the distinct sensitivity of the wild-type and mutated ATPases to in vivo activation by glucose. In addition, they indicated that mutated H^+-ATPases have also lost the ability to be activated by ethanol (6% (v/v)), either in the absence or presence of glucose, although the in vivo activation of wild-type H^+-ATPase by ethanol was confirmed (Fig. 4).

4. Discussion

Results of the present work indicate that the C-terminus regulatory domain of yeast plasma membrane H^+-ATPase, involved in the in vivo activation by glucose, nitrogen starvation or external acidification [13,15,17,19], may also be involved in ethanol-induced activation. Activation induced by ethanol was observed either during ethanol-stressed cultivation or following the rapid incubation of cells with ethanol. Glucose- and ethanol-activations were observed in addition to each other. Ethanol activated both the basal and the glucose-activated forms of plasma membrane H^+-ATPase and this membrane enzyme, fully activated by glucose, was still additionally activated by ethanol. However, the incubation with glucose led to higher ATPase activity values in yeast cells grown in the absence of ethanol compared with those grown in the presence of 6% (v/v) of ethanol, which is a concentration that induces maximal stimulation in vivo. This observation can possibly be explained by the lower content of ATPase in the plasma membrane of cells cultivated under ethanol stress [7] and by the inhibition of ATPase activity by ethanol, detected in vitro [6]. Despite the experimental evidences suggesting that plasma membrane H^+-ATPase activation by glucose or ethanol might be dependent on the C-terminus regulatory domain, the two phenomena showed (at least) a critical difference. The affinity of the ethanol-activated form for MgATP was lower than the affinity of the non-activated form being the stimulation of H^+-ATPase activity due to the increase of V_{max}, while the affinity of glucose-activated ATPase is above the basal form affinity. Also contrasting with glucose-activation, plasma membrane ATPase activated by nitrogen starvation did not show a decreased K_m although the C-terminus regulatory domain was also proposed to be involved in this activation [13]. These observations are however in accordance with the existence of at least two regulatory sites at the C-terminus as proposed by Eraso and Portillo [15]. One site, defined by the amino acids Arg^{909} and Thr^{912}, is essential for the V_{max} change produced by glucose regulation of the ATPase [19] while the other site, defined by Ser^{899} and Glu^{901}, is necessary for the K_m change [15].

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