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Effect of extracellular acidification on the activity of plasma membrane ATPase and on the cytosolic and vacuolar pH of *Saccharomyces cerevisiae*

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Abstract

The rapid in vivo activation of *Saccharomyces cerevisiae* plasma membrane H^+-ATPase that has been attributed to medium acidification from pH 6.5 to pH 3.5 is not caused by the low pH itself but is induced by the weak organic acid (succinic) used as the acidulant. The activation induced by 50 mM succinic acid at pH 3.5 occurred in both the presence or absence of glucose. Activation at pH 3.5 was also induced by acetic acid and it was maximal at 50 mM concentration. To investigate the role of plasma membrane ATPase activation in pH homeostasis, the internal pH (cytosolic and vacuolar) of yeast cells incubated in media at pH 6.5 or at pH 3.5, acidified either with HCl or acetic acid, were compared by using in vivo ^{31}P-NMR. Despite plasma membrane ATPase activation by acetic acid, the decrease in cytosolic pH caused by external acidification was much more important when the permeant acetic acid was used instead of HCl as the acidulant. The supplementation of the incubation medium at pH 3.5 with glucose led to higher cytosolic pH values, consistent with the observed in vivo activation of plasma membrane ATPase by glucose. At the external pH value of 6.5 the vacuole was maintained at a mildly acidic pH (around 6) while the cytosol was at about neutral pH; however, when cytoplasmic pH decreased due to external acidification, vacuolar pH accompanied that decrease. Vacuolar pH reached 5.4–5.5 during incubation with HCl and dropped sharply to values below 4.4 in cells incubated with acetic acid. These results indicate that the vacuole also plays a role in homeostasis of the intracellular pH.

1. Introduction

The proton translocating ATPase in the plasma membrane of yeast creates the transmembrane electrochemical proton gradient and is also involved in the regulation of intracellular pH by pumping protons

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out of the cells [1–4]. Like glucose [2], several environmental stressors such as ethanol [5–7], octanoic acid [8,9], acid pH [3], deprivation of a nitrogen source [10] and supraoptimal temperatures [11] stimulate, in vivo, the activity of this membrane enzyme. This activation constitutes a response that presumably helps the cell to counteract the dissipation of the proton motive force across the plasma membrane and/or the decrease of internal pH taking place in cells exposed to liposoluble compounds, external acidification or supraoptimal temperatures [3,5–9,11–13]. The activation of plasma membrane ATPase in cells grown in the presence of inhibitory concentrations of ethanol, octanoic acid or at supraoptimal temperatures cannot be ascribed to increased synthesis of this membrane enzyme [6,9,11]. The most probable hypothesis is that ATPase activation results from a post-translational modification of PMA1 enzyme [6,9,11] as it was proposed for the activation by glucose or nitrogen starvation [10,14–17]. It is possible that ATPase activation is caused, at least partially, by the alteration in the plasma membrane lipid constituents of cells grown under inhibitory conditions as recently proposed for the decanoic acid-induced activation [18].

The rapid in vivo activation of yeast plasma membrane ATPase, reported by Eraso and Gancedo [3], in response to medium acidification from pH 6.5 to pH 3.5, was not confirmed by us when HCl was used as the acidulant. However, activation was observed when we used, like these authors, succinic acid as the acidulant in the cells suspending buffer. These results enabled us to compare the effects on ATPase activity of low pH created by organic acids or strong acids. The antimicrobial activity of short chain weak acids at low pH relies not only on the high concentration of protons around the cell but also on the anion trapping which leads to additional acidification of the cytosol.

In the present work we compare the effects of strong acids (HCl or H_2SO_4) and short chain weak acids (succinic and acetic), in the activity of yeast plasma membrane ATPase at an extracellular pH value of 3.5. To investigate the role of plasma membrane ATPase activity in pH homeostasis, the values of internal pH (cytosolic and vacuolar) of yeast cells exposed to low pH (3.5) created by HCl or acetic acid, in the absence or presence of glucose, were compared by using in vivo ^{31}P-NMR. This is a

powerful technique with the unique capability of measuring pH of different intracellular compartments, such as, the cytosol and the vacuole [20,21]. The role of plasma membrane H^+-ATPase and of the vacuole in pH homeostasis is discussed based on the results obtained.

2. Materials and methods

2.1. Strains

Saccharomyces cerevisiae YPH499 (Mat a, *ade2-101*[ochre] *leu2-D*1 *his3-D*200 *ura3-52 trp1-D*1 *lys2-801*[amber]) studied by Viegas et al. [9,11] and Monteiro et al. [6] was used in this work.

2.2. Growth media and culture conditions

Cells were batch cultured at 30°C with orbital agitation (150 rpm) in 250-ml Erlenmeyer flasks with 150 ml of liquid medium (pH 5.0) containing 30 g l^{-1} glucose, 6.7 g l^{-1} Yeast Nitrogen Base (w/o amino acids) (Difco), 40 mg l^{-1} L-adenine, 20 mg l^{-1} uracil and a mixture of amino acids [9]. Media were inoculated with cells (initial $A_{600} = 0.2 \pm 0.02$) pregrown until mid-exponential phase ($A_{600} = 1.6 \pm 0.1$) in the same growth medium. Growth was monitored by measuring culture A at 600 nm.

2.3. Assay of plasma membrane ATPase activity in cells subjected to external acidification

Yeast cells were harvested at $A_{600} = 1.7 \pm 0.1$ by centrifugation, washed twice with distilled water and resuspended at $A_{600} = 40$ (equivalent to 19 mg dry biomass/ml) in 0.1 M Mes adjusted to pH 6.5 with Tris (2.5 M) and incubated with agitation at 30°C for 30 min in order to reverse glucose-activation [2]. Cells were then washed, centrifuged and resuspended at the same optical density in the following media, supplemented or not with 0.1 M glucose: (1) 0.1 M Mes adjusted to pH 6.5 with Tris; (2) 0.1 M Mes, 50 mM succinic acid adjusted to pH 3.5 with Tris; (3) 0.1 M Mes, 41 mM Tris adjusted to pH 3.5 with HCl or H_2SO_4. Samples of 2 ml were taken at suitable times during 30 min of incubation at 30°C in the different media; after addition of Tris, EDTA and

dithiothreitol to the final concentrations of 100, 5 and 2 mM, each sample was rapidly frozen at $-70°C$ and kept at this temperature until used for the preparation of total plasma membrane suspensions. In other experiments, cells were incubated for only 10 min in 0.1 M Mes media, adjusted to pH 3.5 with Tris (2.5 M) and containing increasing concentrations of succinic acid, acetic acid or citric acid (range 25–100 mM), and 2-ml samples were taken and treated in a similar way.

Total membrane or plasma membrane suspensions for the assay of ATPase were obtained as described by Viegas et al. [11] or Monteiro et al. [6], respectively. Protein concentration in the membrane suspensions was determined by the method of Bradford, using bovine serum albumin fraction V (Sigma) as standard. Plasma membrane ATPase was assayed as described by Rosa and Sá-Correia [7] and the activity expressed as nmol of Pi released per minute (mU) per mg of membrane protein used in the assay.

2.4. Determination of cytosolic and vacuolar pH by using in vivo ^{31}P-NMR in cells subjected to external acidification

For the NMR experiments, yeast cells were grown and harvested as described above for the ATPase assays. Cells were washed twice, centrifuged and resuspended in different media in order to obtain a $A_{600} = 100$. This high cell density was required due
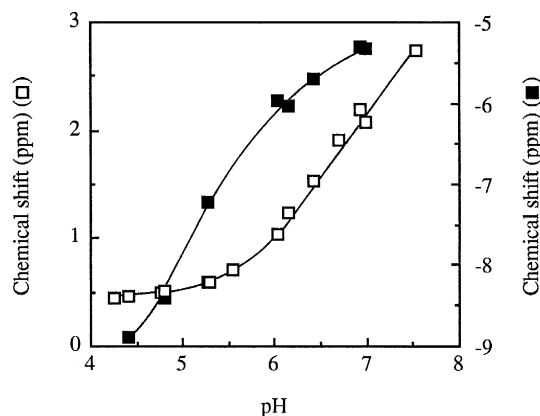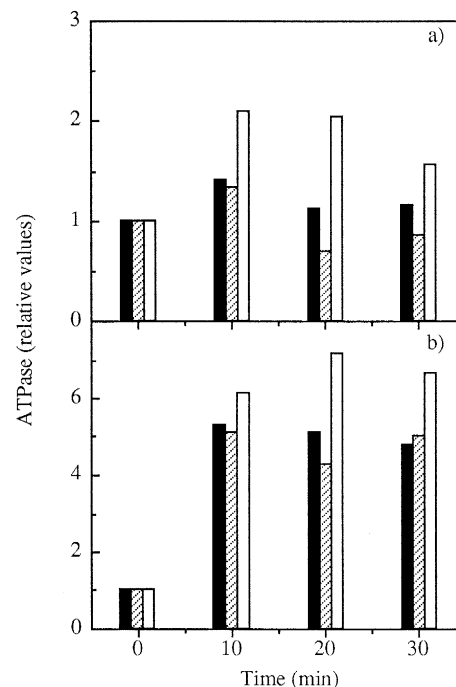

Fig. 2. Effect of external acidification using succinic acid or HCl on the plasma membrane ATPase activity of *S. cerevisiae* YPH499. Cells were harvested in the exponential phase of growth, washed twice with water and incubated at 30°C with agitation for 30 min in 0.1 M Mes-Tris (pH 6.5) to eliminate glucose activation and then resuspended at the same cell concentration and incubated for further period of 30 min in: 0.1 M Mes-Tris (pH 6.5) (■); 0.1 M Mes-Tris-HCl (pH 3.5) (hatched); 0.1 M Mes, 50 mM succinic acid-Tris (pH 3.5) (□); supplemented (b) or not (a) with 0.1 M of glucose. Results are representative of the many experiments carried out.

to the intrinsic insensitivity of ^{31}P-NMR. Different media with the following composition, supplemented or not with glucose (0.1 M), were used: (1) 0.1 M Mes adjusted to pH 6.5 with Tris; (2) 0.1 M Mes, 50 mM acetic acid adjusted to pH 3.5 with Tris; and (3) 0.1 M Mes, 41 mM Tris adjusted to pH 3.5 with HCl. Acquisition of NMR spectra was started immediately after cells resuspension. Spectra were acquired consecutively over about 15 min.

To follow the time course of the strong change in intracellular pH upon addition of acetic acid at an external pH of 3.5, cells were washed in 0.1 M Mes, 41 mM Tris adjusted to pH 3.5 with HCl, and resuspended in the same medium. Following the acquisition of the initial spectrum, 83 ml of glacial acetic acid were added to the NMR tube and spectra were acquired afterwards consecutively.
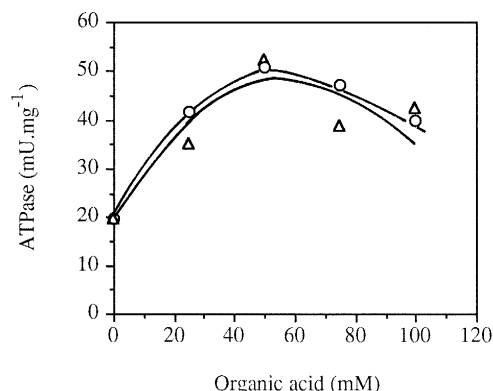

Fig. 1. Titration curves of *S. cerevisiae* YPH499 cell free extracts at 30°C. The chemical shifts of the ressonances due to the terminal group of polyphosphate (■) and inorganic phosphate (□) are plotted.

Fig. 3. Effects of increasing concentrations of succinic acid ($\bigcirc$), or acetic acid ($\triangle$) on the activity of plasma membrane ATPase after 10 min incubation of yeast cells in media 0.1 M Mes-Tris at pH 3.5 supplemented or not with these organic acids.

The cytosolic pH (pH_c) and the vacuolar pH (pH_v) of cell suspensions were measured by using ^{31}P-NMR. ^{31}P-NMR spectra were acquired at 202.46 MHz in a Bruker AMX500 spectrometer in a 10-mm quadruple nuclei probehead (^{31}P, ^{13}C, ^{15}N, ^{1}H). The following acquisition parameters were typically used: spectral width 20 kHz; pulse width 18 μs (corresponding to a 70° flip angle); data size 16K; repetition delay 0.6 s; number of scans 128. Deuterium oxide (5% v/v) was added to provide a lock signal. During the experiments, cell suspensions were mixed by using a simple gas-lift apparatus [22]. The temperature of the sample was kept at 30°C. Chemical shifts were referenced with respect to external 85% H_3PO_4. Calibration curves of the chemical shifts of the peaks assigned to inorganic phosphate and the terminal phosphate groups in polyphosphate were prepared using concentrated cell-free extracts (Fig. 1). Since the pool of inorganic phosphate in the vacuole was very small in these cells, and in many cases difficult to detect in the short acquisition times used here, the values of vacuolar pH were obtained from the chemical shifts of the resonance due to the terminal groups in polyphosphate. A parallel between the behaviour of the resonances due to vacuolar inorganic phosphate
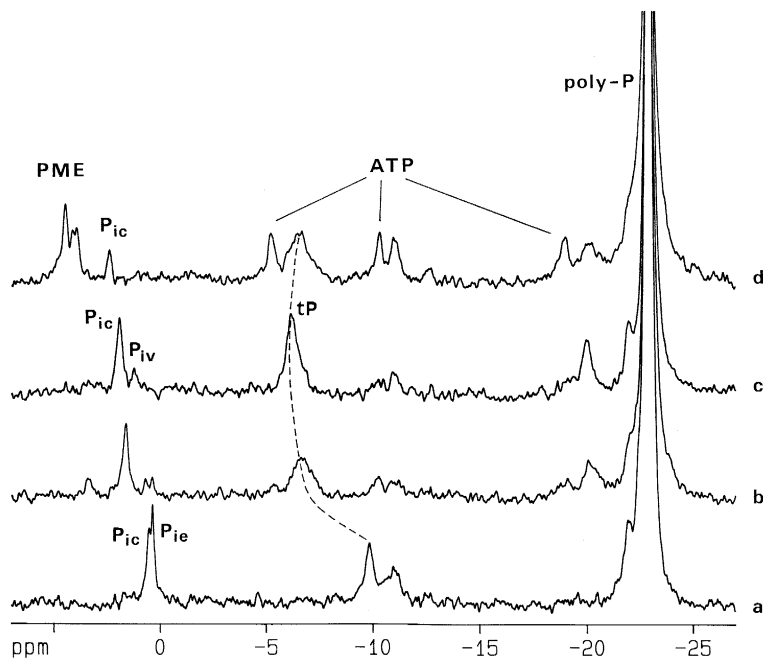

Fig. 4. ^{31}P-NMR spectra of yeast cells suspensions. Cells were washed and suspended in (a) 0.1 M Mes-50 mM acetic acid-Tris (pH 3.5); (b) 0.1 M Mes-Tris-HCl (pH 3.5); (c) 0.1 M Mes-Tris (pH 6.5); (d) 0.1 M Mes-Tris (pH 6.5) supplemented with 100 mM glucose. For each sample, a series of consecutive spectra were started immediately after cell resuspension. The second spectrum in each series is shown. Acquisition time of each spectrum, 1.5 min. Temperature 30°C. Resonances assignment: P_{ic}, cytosolic inorganic phosphate; P_{iv}, vacuolar inorganic phosphate; PME, phosphomonoesters; tP, terminal groups in polyphosphate; poly-P, inner groups in polyphosphate; ATP, nucleoside triphosphates. The values of cytosolic and vacuolar pH values were determined from the chemical shifts of the resonances P_{ic} and tP, respectively, and by using suitable calibration curves (Fig. 1).

and the terminal groups of polyphosphate as a function of pH has been previously reported for *S. cerevisiae* cells [20,23]. Below pH 4.5 the resonances due to the terminal groups in polyphosphate were no longer detected in cell extracts probably due to precipitation of polyphosphate, and therefore pH values lower than 4.5 could not be determined.

3. Results

3.1. Effects of external acidification on plasma membrane activity using strong or organic acids as the acidulant

In contrast with the conclusions of Eraso and Gancedo [3], who reported that medium acidification at pH 3.5 led to in vivo activation of the ATPase, we found that medium acidification with HCl (or H_2SO_4) caused some inactivation of the ATPase (Fig. 2a and results not shown). A rapid in vivo ATPase activation occurred however at pH 3.5 in the presence of 50 mM succinic acid, used by Eraso and Gancedo [3] to acidify the incubation medium (Fig. 2a). With cells where the plasma membrane ATPase was activated by glucose at pH 6.5, a further level of activation occurred in medium acidified at pH 3.5 by succinic acid but not HCl (Fig. 2b). Therefore, both the glucose-activated and the basal forms of the plasma membrane ATPase can be activated by succinic acid at low pH. Like succinic acid, acetic acid also induced the activation of plasma membrane ATPase at pH 3.5 with 50 mM of each organic acid giving optimal activation under the experimental conditions used (Fig. 3). A similar activation was observed when plasma membranes prepared as reported by Monteiro et al. [6] were used instead of total membranes to assay plasma membrane ATPase (results not shown).

3.2. Effects of external acidification by acetic acid or HCl in yeast cytosolic and vacuolar pH

In order to investigate the role of plasma membrane ATPase activation on pH homeostasis, the internal pH (cytosolic, pH_c and vacuolar, pH_v) of yeast cells incubated in media at pH 6.5 or at pH 3.5, acidified either with HCl or acetic acid (50 mM)
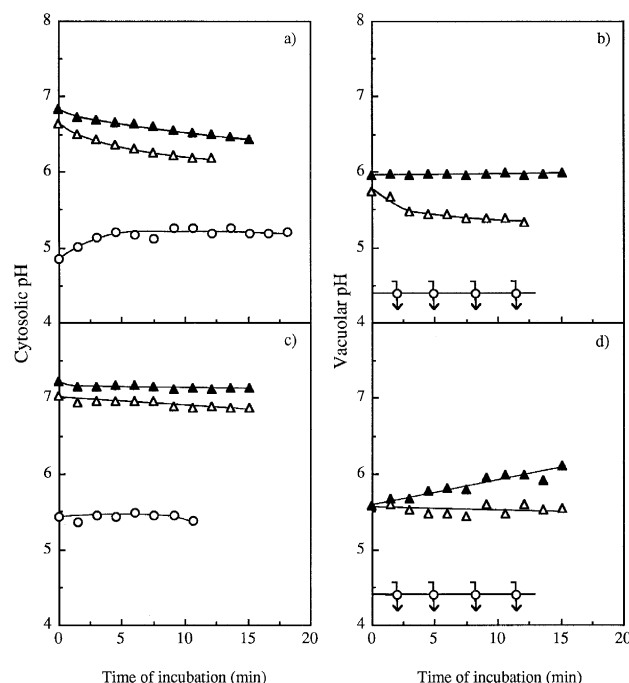

Fig. 5. Effects on the values of cytosolic (a, c) or vacuolar (b, d) pH determined by using in vivo ^{31}P-NMR of yeast cells incubation (30°C) in: 0.1 M Mes-Tris (pH 6.5) (▲), 0.1 M Mes-Tris-HCl (pH 3.5) (△) or 0.1 M Mes-50 mM acetic acid-Tris (pH 3.5) (○) (a and b) and in these same media supplemented with 100 mM glucose (c and d). The values of vacuolar pH of cells incubated with 50 mM acetic acid at pH 3.5, either in presence or absence of glucose dropped below 4.4.

were compared by using in vivo ^{31}P-NMR. Fig. 4 represents examples of *S. cerevisiae* in vivo ^{31}P-NMR spectra obtained in the different medium conditions examined.

Despite plasma membrane ATPase activation by acetic acid, the reduction of pH_c by external acidification was much more important when acetic acid was used instead of HCl as the acidulant (Fig. 5a and c). However, pH_c of cells incubated in acid media (pH 3.5) exhibited higher values as the result of glucose supplementation (Fig. 5c), consistent with the in vivo activation of plasma membrane ATPase by glucose (Fig. 2b). After 10 min of incubation in glucose supplemented as compared with unsupplemented acid media, pH_c decreased to 6.9 instead of 6.2 when HCl was the acidulant and to 5.5 instead of 5.2 when acetic acid was used (Fig. 5a and c). Interestingly, as incubation with acetic acid at pH 3.5 proceeds, pH_c slightly increased accompanying

plasma membrane ATPase activation during incubation with acetic acid (Fig. 2, Fig. 5a, c; and results not shown). On the contrary, during incubation with HCl at pH 3.5, pH_c slightly decreased (Fig. 5a and c) which was associated with some loss of ATPase activity observed during cells incubation under these conditions (Fig. 2). In unstressed cells the vacuole was maintained at a mildly acid pH (around 6) while the cytoplasm was at about neutral pH (Fig. 5). However, when pH_c decreased due to external acidification pH_v accompanied pH_c decrease (Fig. 5). The pH_v was therefore more acid in cells incubated in acid medium without glucose as compared with cells incubated in glucose supplemented medium. Values of pH_v reached 5.5 during incubation with HCl and dropped down to values below 4.5 in cells incubated with acetic acid (Fig. 5 and Fig. 6). In order to attempt to follow the abrupt drop in internal pH, NMR experiments were also carried out with cells washed in acetic acid-free medium at pH 3.5, and a pulse of acetic acid was provided directly to the cell suspension in the NMR tube. Upon acetic acid addition, the values of cytosolic and vacuolar pH decreased from 6.5 and 5.7 to 5.2 and 4.7, respectively in 40 s (Fig. 6). The value of vacuolar pH continued to decrease further with time, at least up to 4 min of incubation with acid, accompanying the slight increase of cytosolic pH (Fig. 6).

4. Discussion

Microbial growth and survival are influenced both by the external concentration of hydrogen ions H^+
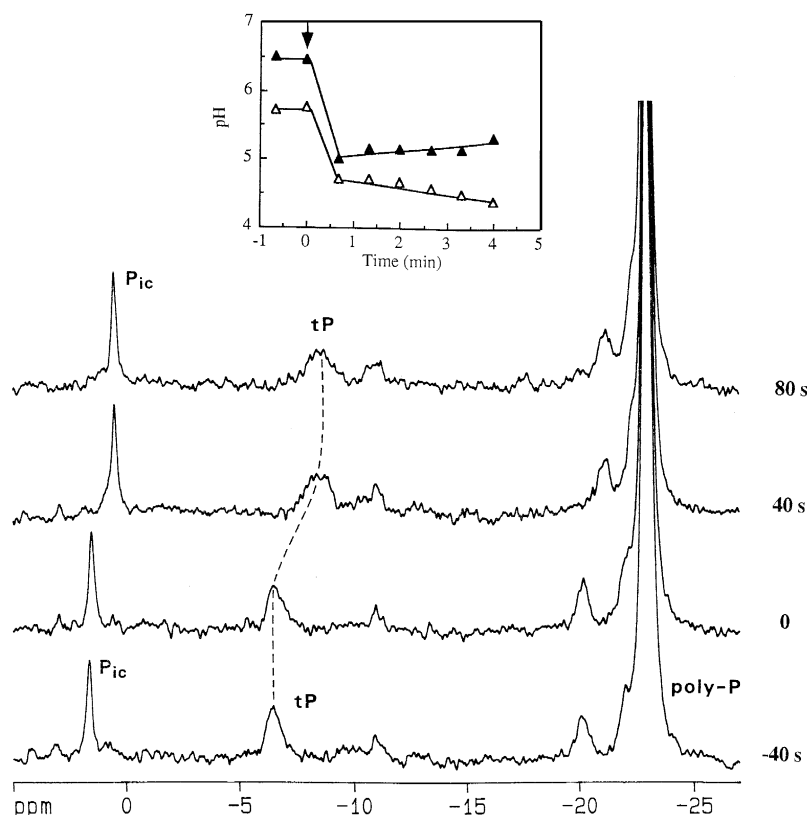

Fig. 6. In vivo ^{31}P-NMR spectra of a yeast cells suspension at pH 3.5. Cells were suspended in medium containing 0.1 M Mes-Tris-HCl (pH 3.5) and two spectra were acquired. A pulse of acetic acid (50 mM final concentration; final external pH 3.5) was provided at time zero as indicated by the arrow; spectra acquisition finished at the times indicated. The considerable shifts of resonances P_{ic} and tP denote strong acidification of the cytosol and vacuole, respectively, induced by addition of acetic acid. The corresponding pH values (cytosolic pH (▲), vacuolar pH (△)) are plotted in the inset. Acquisition time of each spectrum 40 s. Symbols as in Fig. 4.

and, at low pH, by the type and concentration of the acidifying acids [19]. Where the acidulant is a strong acid, its antimicrobial effect relies on the acid concentration providing a high concentration of protons around the cell. The antimicrobial activity of weak acids at low pH uses the effects of the undissociated acid that may reach up to 90% of the total concentration depending on pH and pKa. Undissociated forms of many weak acids are liposoluble and can stimulate the passive influx of H$^+$ into the cell by increasing the permeability of the plasma membrane [13]. The dissociation in the cytoplasm (with a pH near neutrality) of the permeant acid form, leads to an additional acidification of the cell interior. Since, in general, weak acids are largely ineffective at pH values above 5.5 at which they are present as the non-liposoluble anionic form, it is frequently difficult to separate the direct effect of the presence of the antimicrobial form and the stress caused by the low pH value itself, when external medium is acidified below the pKa value. The ability of the cells to grow or maintain viability at high external hydrogen ion concentration reflect their capacity to maintain control over their internal pH by excluding hydrogen ions. Many of *S. cerevisiae* mutants with mutations in the essential *PMA1* gene cannot grow either at low pH or in the presence of weak acids [24], suggesting that the lower plasma membrane ATPase activity of the *pma1* mutants reduced the ability to pump protons from the cell. A correlation was also established between yeast tolerance to ethanol and the activity of plasma membrane ATPase of cells grown in the presence of this toxic metabolite [5].

Results reported in the present work show that the rapid in vivo activation of yeast plasma membrane H$^+$-ATPase that have been attributed to medium acidification from pH 6.5 to 3.5 [3] is not caused by low pH itself but is induced by succinic acid that was used as the acidulant. A similar activation was observed when comparable concentrations of acetic acid were added to the medium acidified to pH 3.5 and maximal activation was observed at 50 mM for both succinic and acetic acid. Above the optimum value, the negative effect of the excessive acidification of the cytosol, below around pH 5, counteracted ATPase activation. Plasma membrane ATPase stability decrease sharply at pH below 5.5 [25]. Plasma membrane ATPase activation that has been analysed in the present work and that was reported, for the first time, by Eraso and Gancedo [3], was induced by rapid incubation of yeast cells in acid media with optimal concentrations of succinic or acetic acids. Succinic acid- and acetic acid-induced ATPase activations were rapid (10 min) and could hardly be attributed to the adaptive modification of the lipid environment of ATPase that is known to occur in yeast cells grown under stress as suggested by Alexandre et al. [18]. Besides the stimulatory effect of succinic acid on the basal form of ATPase previously observed by Eraso and Gancedo [3], the glucose-activated-ATPase was additionally activated by succinic acid as similarly reported for octanoic acid-induced activation [8]. The activation of ATPase by glucose is thought to result from a post-translational modification of the enzyme involving the carboxyl terminus of PMA1 ATPase, an autoinhibitory domain. Its effect on ATPase activity is counteracted by modification of the enzyme, triggered by glucose metabolism, presumably through phosphorylation–dephosphorylation [14–17]. The activation produced by acidification of the external medium (pH 3.5, with succinic acid) occurs by a mechanism different from that of the activation produced by glucose and other fermentable substrates as suggested by the different characteristics of these two activation processes. Activation by glucose is a reversible process that occurs through changes in V_{max}, affinity for ATP, optimum pH and K_i for orthovanadate of the enzyme [2]. The activation by acidification is also due to change in the V_{max} but without modification of optimum pH, the process being irreversible [3]. However, deletion analysis and site directed mutagenesis have shown that the ATPase regulatory domain implicated in the activation by glucose also controls activation by acidification [10]. Although the mechanism underlying plasma membrane ATPase activation in cells exposed to external acidification with short-chain-organic acid remains obscure at the present time, it is likely that this is triggered by a decrease in the intracellular pH [3,10]. Based on results obtained during the present work, maximal ATPase activity is observed in cells where the cytosolic pH decreases to values around 5–5.5. It is noteworthy that ATPase activation in cells grown or exposed to long-chain fatty acids, highly soluble in membrane phospholipid, such as octanoic or decanoic acids [8,9,18], can hardly be mainly attributed to cell

acidification. In fact, for the very low concentrations which induce activation, no significant decrease of internal pH was detected [8,26].

The vacuole is an acidic organelle that is involved in the degradation of intracellular macromolecules, in the compartmentalization of metabolites and ions and in pH homeostasis [27]. The role of vacuole in homeostasis of intracellular pH was previously suggested by the observation that some yeast mutants that lack a normal vacuole are pH-sensitive [27]. Results obtained in this present work also indicate that the vacuole plays an additional role in homeostasis of the intracellular pH. In fact, vacuolar pH was maintained at mildly acid pH (around 6) when the cytoplasm was at neutral pH in unstressed cells. However, when yeast cells were incubated at acid pH (pH 3.5), particularly when acetic acid was used as the acidulant, the vacuolar pH became strongly acid accompanying the decrease of the cytosolic pH. The pH and electrical potential differences across the vacuolar membrane may be regulated through the interactions of membrane potential-dependent cation channel, chloride transport systems and the vacuolar H^+-ATPase (for a review, see Ref. [27]). The observed regulation of ΔpH across vacuolar membrane may therefore be regulated by the modulation of the vacuolar membrane potential by the alteration of its ion conductivity or by the activation of vacuolar H^+-ATPase. This last hypothesis is, at the present time, under investigation.

Acknowledgements

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