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In vitro modulation of alkaline phosphatase activity of *Saccharomyces cerevisiae* grown in low or high phosphate medium

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Our objective was to characterize the modulation of the activity of *Saccharomyces cerevisiae* alkaline phosphatases (ALPs) by classic inhibitors of ALP activity, cholesterol and steroid hormones, in order to identify catalytic similarities between yeast and mammalian ALPs. *S. cerevisiae* expresses two ALPs, coded for by the *PHO8* and *PHO13* genes. The product of the *PHO8* gene is repressible by Pi in the medium. ALP activity from yeast (grown in low or high phosphate medium) homogenates was determined with p-nitrophenylphosphate as substrate, pH 10.4 (lPiALP or hPiALP, respectively). Activation of hPiALP was observed with 5 mM L-amino acids (L-homoarginine - 186%, L-leucine - 155% and L-phenylalanine - 168%) and with 1 mM levamisole (122%; percentage values, in comparison to control, of recovered activity). EDTA (5 mM) and vanadate (1 mM) distinctly inhibited hPiALP (2 and 20%, respectively). L-homoarginine (5 mM) had a lower activating effect on lPiALP (166%) and was the strongest hPiALP activator. Corticosterone (5 mM) inhibited hPiALP to 90%, but no effect was observed in low phosphate medium. Cholesterol, ß-estradiol and progesterone also had different effects on lPiALP and hPiALP. A concentration-dependent activation of lPiALP minus hPiALP was evident with all three compounds, most especially with ß-estradiol and cholesterol. These results do not allow us to identify similarities of the behavior of *S. cerevisiae* ALPs and any of the mammalian ALPs but allow us to raise the hypothesis of differential regulation of *S. cerevisiae* ALPs by L-homoarginine, ß-estradiol and cholesterol and of using these compounds to discriminate between *S. cerevisiae* lPiALP and hPiALP.

Key words: *Saccharomyces cerevisiae*; Alkaline phosphatase inhibitors; *PHO8* and *PHO13*; Cholesterol; Steroid hormones

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INTRODUCTION

Alkaline phosphatase (ALP; orthophosphoric-monoester phosphohydrolase, alkaline optimum) represents a family of several isoenzymes and isoforms whose expression depends on the species being considered and on physiological/pathological conditions (1-3). ALP substrates (either natural or artificial) possess a phosphate residue (Pi) in a terminal position. Pi released from an ALP substrate molecule is either released into the reaction medium (ALP functioning as phosphohydrolase) or transferred to an appropriate acceptor (ALP functioning as phosphotransferase) (1-8).

The extent of natural ALP substrates and *in vivo* specific ALP functions are still unknown. ALP participates, along with other enzymes, in Pi homeostasis (in a cyclic pathway of polyphosphate synthesis and degradation), a process including phosphorous acquisition and metabolic integration of Pi in *Saccharomyces cerevisiae*, and eutopic or ectopic bone mineralization in mammals (1-3,5-18).

ALP has also been associated with various fundamen-

tal cellular processes (differentiation, proliferation and cellular signaling, meiosis, mitosis, apoptosis, cell migration, immunological defense, and DNA synthesis regulation) in several species (1-3,12,15-17).

In *S. cerevisiae*, ALPs are the product of two structural genes: *PHO8* and *PHO13*, both located on chromosome IV. PHO8 ALP (E.C. 3.1.3.1) is an Mg^{2+}/Zn^{2+}-dependent dimeric protein similar to an ALP found in *Escherichia coli* and in mammalian cells, is very unspecific in terms of substrates and is located in the cell vacuole. The product of the *PHO13* gene (E.C. 3.1.3.41) is a monomeric protein, is highly specific for p-nitrophenylphosphate (p-NPP) and histidinylphosphate (not attacking other substrates at a significant rate), and, like PHO8 ALP, is also highly dependent on Mg^{2+}. The nonspecific enzyme is repressible by Pi in the medium, whereas the specific ALP is synthesized constitutively regardless of the Pi concentration in the medium. The repressible ALP, however, shows a significant level of activity even under repressible conditions. It has been reported that repressible ALP activity, due to transcription of the *PHO8* gene, is two to three times higher in low than in high Pi medium (5-7,11,19-22). Both *S. cerevisiae* ALPs have protein and/or peptide phosphatase activity (5-7).

The four ALP isoenzyme activities expressed in mammals, i.e., tissue-nonspecific, intestinal, placental, or germ-cell ALP, with different distributions among species, can be distinguished by their differential inhibition by several compounds such as L-amino acids or levamisole. Unlike mammalian ALPs, bacterial ALPs are not stereospecifically inhibited by L-amino acids through a non-competitive mechanism (1-3,17,23-25).

Since we found no data about the modulation of *S. cerevisiae* ALP activity, in the present study we characterized the modulation of *S. cerevisiae* ALP activity by L-amino acids, cholesterol and steroid hormones. This characterization will show the putatively different catalytic behavior of PHO8 and PHO13 ALPs and possibly permit inferences about ALP functions in yeast. Our group has a large experience in comparative modulation of mammalian ALPs and has carried out extensive research on ALP function (3,13-17,26-28). Additionally, by also examining the modulation of *S. cerevisiae* ALP activity by levamisole, EDTA and vanadate, we set out to identify catalytic similarities between yeast and mammalian ALPs in order to find out if *S. cerevisiae* ALPs could be used as a study model of mammalian ALPs.

MATERIAL AND METHODS

p-NPP (Sigma 104D, St. Louis, MO, USA), p-nitrophenol (p-NPL; Sigma 104-8), bovine serum albumin (BSA) (A-4503), L-amino acids, levamisole (L-9756), orthovanadate (S-6508), cholesterol (C-8667), and steroid hormones were purchased from Sigma (Sigma Alcobendas, Madrid, Spain). Yeast growth medium was purchased from Difco™ (YM Broth - 271120, yeast malt, Voigt Global Distribution Inc., Lawrence, KS, USA). All other reagents were of the highest quality and purity available.

Yeast and yeast growth conditions

S. cerevisiae strain 3507 (wild type) from the Portuguese Yeast Cell Culture (Gulbenkian Institute of Science, Lisbon, Portugal) was kindly provided by ESBUC, College of Biotechnology, Portuguese Catholic University, Porto, Portugal. The strain was grown in 250-mL Schott flasks with 150 mL medium under slow rotary agitation at 30°C on yeast malt medium supplemented with K_2HPO_4 at two different final concentrations: 3000 mg/L (high phosphate medium) and 30 mg/L (low phosphate medium) (11); the flasks were covered with gauze.

Yeast sample collection

Yeast samples were collected from the exponential phase. Yeast cells were separated by centrifugation at 6000 *g* for 10 min at 4°C and washed once with 5 mL phosphate-buffered saline (PBS), pH 7.4, also at 6000 *g* for 10 min at 4°C. Then, the yeast pellet was resuspended in 1 mL PBS and stored at -80°C for no longer than one month.

Alkaline phosphatase activity modulation

On the day of the experiment, yeast samples were homogenized with a Heidolph homogenizer in PBS + 0.5% Triton X-100 (4:1) (13,14,20,22) in a final volume of 1.25 mL and kept on ice. The final concentration of Triton X-100 in the ALP sample incubation medium was lower than its critical micellar concentration. The reaction mixtures (final volume of 500 µL) contained 80 mM Tris, pH 10.4, 0.4 mM $MgCl_2$, 20 µL yeast sample from high or low phosphate growth medium, 0.3760 mg of p-NPP and 50 µL of the compound solution to be tested. The enzymatic reaction was started by adding the ALP substrate. Incubation took place at 37°C for 15 min, was stopped by the addition of 2 mL 20 mM NaOH (ice-cold) and then the reaction mixture was placed on ice for 10 min. At the end of this period, a centrifugation was performed at 10,000 *g* for 16 min at 20°C. The supernatant was collected and the p-NPL released by ALP activity from yeast homogenates (grown in low or high phosphate medium, lPiALP or hPiALP, respectively), in the presence or absence of the compounds to be tested, was quantified by absorbance measurements at 410 nm (Spectronic Genesys 5, Milton Roy, Rochester,

NY, USA). For both lPiALP and hPiALP there was linearity with incubation time from 5 to 20 min. A p-NPL calibration curve was performed in order to calculate ALP activity. L-amino acids with Sigma numbers given within parentheses (1 and 5 mM: L-phenylalanine (P-2126), L-leucine (L-8912), and L-homoarginine (H-1007)), EDTA (1 and 5 mM), vanadate (0.1 and 1 mM), and levamisole (0.1 and 1 mM) were dissolved in water. Cholesterol (C-8667), progesterone (P-0130), corticosterone (C-2505), and ß-estradiol (E-8875) (0.01-5 mM) were dissolved in ethanol. Controls for drugs were run in the presence of a corresponding volume of solvent. All assays for each compound at the different concentrations tested were always carried out using the same homogenate batch (either lPiALP or hPiALP homogenates). The effect of each compound tested against ALP activity was determined as a percentage of the corresponding control ALP activity (representing the recovered activity). The buffer pH value used in the present study was chosen to ensure that only ALP activity would be measured since the ALP molecules were not isolated but their activity was measured in yeast homogenates. All assays were performed in triplicate (differences between replicates were always lower than 5%).

Protein determination

Protein concentration was determined as described by Bradford (29), with BSA as standard. For each yeast growth condition (low or high phosphate medium), the protein concentration of the homogenates used was similar.

Statistical analysis

Data are reported as means ± SD and the significance of differences between means was assessed by the paired or unpaired Student *t*-test.

RESULTS

ALP activity was about 30% lower in yeast homogenates obtained from *S. cerevisiae* grown in high phosphate medium than in yeast homogenates grown in low phosphate medium (results not shown).

L-homoarginine, L-leucine and L-phenylalanine significantly activated hPiALP and lPiALP in a concentration-dependent manner (Figures 1 and 2). For each of the tested concentrations (hPiALP), the results obtained with each L-amino acid were significantly different (P = 0.002-0.03), except for L-phenylalanine versus L-leucine (5 mM) and L-homoarginine versus the other two L-amino acids (1 mM; statistical results not shown in Figure 1). L-homoarginine (5 mM) had a significantly lower activating effect on lPiALP than on hPiALP (statistical result not shown, comparison of Figures 1 and 2).

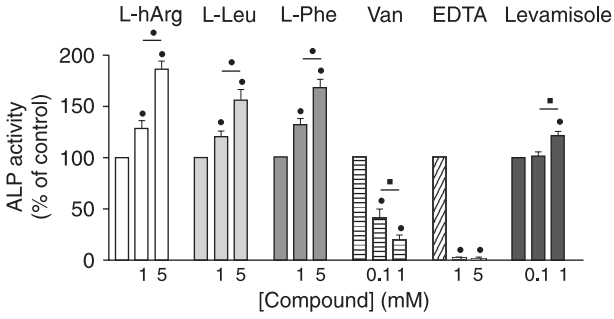

Figure 1. Effect of L-amino acids, EDTA, orthovanadate, and levamisole on alkaline phosphatase (ALP) activity from *Saccharomyces cerevisiae* homogenates. Yeast was grown in high phosphate medium (3000 mg Pi/L YM medium, N = 5). Data are reported as arithmetic means ± SD and as percentage of the corresponding control activity (= 100% (173.3 ± 48.83 nmol p-nitrophenol released min^{-1} mL $homogenate^{-1}$); first column of each set of results), determined as described in Material and Methods. Assays were performed in triplicate. Symbols at the top of the columns indicate comparison to the respective control of each compound. Symbols at the top of the horizontal bars indicate the comparison of the two concentrations. Circles indicate $P \leq 0.005$ and squares indicate $P \leq 0.05$. Leu = L-leucine; Phe = L-phenylalanine; hArg = L-homoarginine; Van = orthovanadate.

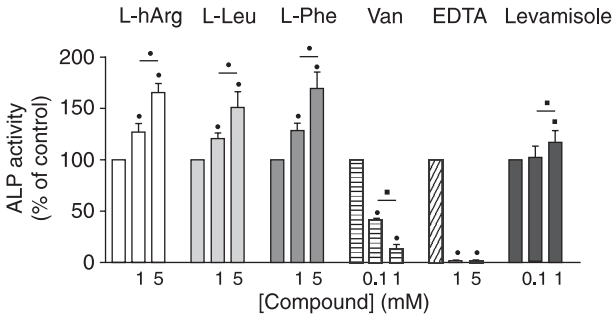

Figure 2. Effect of L-amino acids, EDTA, orthovanadate, and levamisole on alkaline phosphatase (ALP) activity from *Saccharomyces cerevisiae* homogenates. Yeast was grown in low phosphate medium (30 mg Pi/L YM medium, N = 7). Data are reported as arithmetic means ± SD and as percentage of the corresponding control activity (= 100% (235.8 ± 56.33 nmol p-nitrophenol released min^{-1} mL $homogenate^{-1}$); first column of each set of results), determined as described in Material and Methods. Assays were performed in triplicate. Symbols at the top of the columns indicate comparison to the respective control of each compound. Symbols at the top of the horizontal bars indicate the comparison of the two concentrations. Circles indicate $P \leq 0.005$ and squares indicate $P \leq 0.05$. Leu = L-leucine; Phe = L-phenylalanine; hArg = L-homoarginine; Van = orthovanadate.

hPiALP and lPiALP were significantly inhibited by both EDTA and vanadate, although with different potencies when comparing both compounds, and in an apparently concentration-independent way for EDTA (Figures 1 and 2). These inhibitory effects were significantly different in each growth condition (statistical results not shown in Figures 1 and 2; $P < 0.005$; low and high EDTA concentrations compared to low and high vanadate concentrations, respectively).

Levamisole (1 mM) significantly activated hPiALP and lPiALP (Figures 1 and 2). Levamisole effect was significantly different from that of the L-amino acids in each growth condition ($P < 0.005$; low and high levamisole concentrations compared to low and high L-amino acid concentrations, respectively, except for lPiALP in levamisole versus L-leucine at the lower concentrations, where $P < 0.05$; statistical results not shown in Figures 1 and 2).

Regarding the steroid compounds tested (cholesterol, ß-estradiol, progesterone, and corticosterone), only 5 mM corticosterone had an effect on hPiALP, i.e., significant inhibition (statistical result not shown; $P \leq 0.005$). This effect was only significantly different from that of 5 mM progesterone (statistical result not shown; $P = 0.002$). The inhibition by 5 mM corticosterone observed in high phosphate medium was not observed when yeast was grown in low phosphate medium (result not shown). Cholesterol, ß-estradiol and progesterone had different effects on lPiALP and hPiALP activities (statistical results not shown for lPiALP minus hPiALP: significantly different for 5 mM for the first two compounds and for 1 mM for the last one; $P \leq 0.05$). Among these three steroid compounds, ß-estradiol presented the strongest concentration-dependent activation of lPiALP minus hPiALP and closely followed by cholesterol (results not shown). Progesterone and corticosterone (1 mM) had significantly different ($P = 0.007$) effects on lPiALP (statistical result not shown) and the same tendency was observed at 5 mM for the same hormones ($P = 0.059$). Progesterone (1 mM) activated lPiALP (statistical result not shown, $P < 0.05$).

DISCUSSION

In the present study, the yeast *S. cerevisiae* was grown either in a low or high phosphate medium (30 or 3000 mg/L) (11). We observed that these distinct growth conditions induced a 30% difference in total ALP activity in the corresponding *S. cerevisiae* homogenates. This level of reduction of ALP activity induced by Pi concentration in the growth medium agrees with data from Refs. 11 and 21.

Mammalian ALPs can be differentially inhibited by L-amino acids, levamisole and EDTA. L-phenylalanine and L-leucine strongly inhibit intestinal, placental and germ cell ALPs (tissue-specific ALPs). Germ cell ALP is more sensitive to inhibition by L-leucine and EDTA than placental ALP. L-homoarginine strongly inhibits tissue-nonspecific ALP and levamisole inhibits placental and tissue-nonspecific isoenzymes. Levamisole, L-phenylalanine and L-homoarginine, which permit discrimination between tissue-nonspecific and tissue-specific ALPs, have been used for the clinical quantitation and identification of ALP isoenzymes for almost a century (2,24,25,30). Vanadate is an ALP inhibitor (12,17,31). Corticosterone (5 mM) inhibits rat renal ALP, but not rat liver ALP, and progesterone has no effect on rat renal or liver ALP (13).

To our knowledge, none of the compounds used in this study, with the exception of EDTA, have been tested before in ALPs from *S. cerevisiae*; EDTA has been described to also inhibit *Serratia marcescens* ALP (5,7,32). The assay conditions used in the present study (ALP sample/homogenate preparation, p-NPP concentration, buffer (type, concentration and pH value) as well as magnesium concentration) were the same as those we have been using with mammalian ALP homogenate samples (3,13,14,26,27) in order to permit comparison of the results. The concentration ranges for L-amino acids, levamisole, EDTA, vanadate, progesterone, and corticosterone chosen to be tested upon *S. cerevisiae* ALPs in the present study are the same as those tested on mammalian ALPs either in our laboratory or by other authors (7,12, 16,24,25).

For L-amino acids, instead of their reported inhibition, we observed activation of ALP activity in both *S. cerevisiae* homogenates. L-homoarginine (5 mM) was both the strongest activator of hPiALP and the better discriminator between lPiALP and hPiALP. EDTA and vanadate strongly inhibited lPiALP and hPiALP, each compound with the same potency in both types of ALP samples. ALP inhibition by EDTA was not a surprise since ALP needs zinc and magnesium to perform catalysis and EDTA was tested in a concentration range higher than the Mg^{2+} concentration in the incubation medium. The similar effect of levamisole on either lPiALP or hPiALP was different from that described for tissue-nonspecific ALP. Progesterone and corticosterone acted on hPiALP as described by our group for mammalian (rat) tissue-nonspecific ALP (13). ß-estradiol, progesterone and cholesterol acted similarly on hPiALP and lPiALP minus hPiALP, i.e., they had no significant effect on the former ALP activity and induced a concentration-dependent activation of the latter ALP activity (most especially ß-estradiol and cholesterol).

Under special conditions regarding substrate and magnesium, human ALP activation by L-phenylalanine (in a concentration range similar to that used in the present

study) has been reported (23). Human ALPs show different susceptibilities to inactivation or activation by 0.01 mM-0.1 M EDTA (30) and calf intestinal ALP is activated by low EDTA concentrations, 20-400 µM, and irreversibly inhibited by higher concentrations, 1 mM and above (31). Levamisole inhibition of rat liver ALP decreases with increasing levamisole concentration (Martins MJ, unpublished results).

S. cerevisiae is a genetically well-defined organism, easy to manipulate, that grows very fast, its growth and cell division being easily controlled by incubation conditions (33). Thus, *S. cerevisiae* ALP (either in living cells or in yeast homogenates or extracts) would be most useful as a model to study the modulation of mammalian ALPs. Unfortunately our results did not identify similarities between the behavior of *S. cerevisiae* lPiALP or hPiALP and any of the four mammalian ALPs.

However, our results allow us to raise the hypothesis of differential regulation of *S. cerevisiae* ALPs by L-homoarginine, cholesterol and ß-estradiol, all found in yeast metabolism.

In *S. cerevisiae*, ß-estradiol can be obtained from estrones present in the incubation medium and dehydrogenated by an *S. cerevisiae* peroxisomal multifunctional enzyme that is homologous to a mammalian protein. *S. cerevisiae* possesses a high-affinity estrogen-binding protein (34-36). The effect of this steroid compound on ALP should be further studied. Estrogen has been suggested to cause shortening of the unbudded period of *S. cerevisiae* old cells by stimulating energy metabolism (37). The *PHO8* gene of *S. cerevisiae* also codes for the yeast fructose-2,6-bisphosphate 6-phosphatase (5,38). On the other hand, ALP activity has been associated with proliferation and differentiation in several species (1-3,12). A truncated form of PHO8 ALP (lacking 62 amino acids from the N-terminus) obtained from a cell-free extract of *S. cerevisiae* has the ability to convert farnesyl diphosphate into E,E-farnesol (8), suggesting, in association with our results, that this enzyme activity might be involved in cholesterol synthesis regulation.

Modulation of *S. cerevisiae* PHO8 ALP may allow regulation of intracellular levels of nickel, since this enzyme is located in the vacuole where the metal is accumulated (11).

ALP may remove Pi residues from membrane phosphoproteins and from nucleotides such as ATP and AMP and may most probably be involved in transmembrane transport system regulation by modifying the phosphorylation level of transporters or of molecules that regulate them. Supporting this idea is the fact that ALP is an ubiquitous enzyme from humans to *Dictyostelium discoideum* (1,15-17,20-22), located in membranes.

ALP is associated with lipid intestinal transport in mammals, is involved in the modulation of organic cation transport in rat isolated hepatocytes, Caco-2 and RBE4 cells, as well as in insulin internalization in RBE4 cells (1-3,15-17,39,40). It also seems to modulate taurocholate uptake in rat isolated hepatocytes (3).

Although in the present study only the acute effects of ALP modulatory compounds on *S. cerevisiae* homogenates were investigated, the observed differential modulation of *S. cerevisiae* ALPs might be used to test the hypothesis of yeast transport system regulation by ALP activity since both PHO8 and PHO13 ALPs dephosphorylate phosphoproteins and/or phosphopeptides (5-7).

The present results regarding *in vitro* lPiALP and hPiALP modulation did not allow the identification of similarities in the behavior of *S. cerevisiae* ALPs and of any of the mammalian ALPs. However, they allow us to raise the hypothesis of differential regulation of *S. cerevisiae* ALPs by L-homoarginine, ß-estradiol and cholesterol and, consequently, to propose the use of these compounds to discriminate between *S. cerevisiae* lPiALP and hPiALP.

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