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FURTHER CHARACTERIZATION OF A SPECIFIC *p*-NITROPHENYLPHOSPHATASE FROM BAKER'S YEAST

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SUMMARY

1. The properties of a specific Mg^{2+}-dependent *p*-nitrophenylphosphatase from baker's yeast (*Saccharomyces cerevisiae*) have been further studied.

2. The enzyme was found to be inactive on acetylphosphate, carbamylphosphate, ATP, *p*-nitrophenylsulfate and bis(*p*-nitrophenylphosphate).

3. P_i and ATP were competitive inhibitors, whereas PP_i and AMP were without effect.

4. Besides Mg^{2+}, the enzymatic activity was also stimulated by Co^{2+}, Zn^{2+}, Ni^{2+} and Mn^{2+}, but to a lesser extent. The activation by Mg^{2+} was strongly antagonized by Ca^{2+}, Zn^{2+} and Mn^{2+}. Na^+ and K^+ did not show any specific effects.

5. No phosphotransferase activity could be demonstrated when Tris, ammediol, ethanolamine, glucose and glycerol were used as acceptors.

6. The enzyme was inhibited by fluoride, arsenate and *p*-chloromercuribenzoate. Ouabain had no effect.

INTRODUCTION

In preceding papers[1,2] we have described the partial purification and characterization of a specific, Mg^{2+}-dependent *p*-nitrophenylphosphatase (*p*-nitrophenylphosphate phosphohydrolase) from baker's yeast (*Saccharomyces cerevisiae*), which is distinct from the nonspecific alkaline phosphatase (orthophosphoric phosphomonoester phosphohydrolase EC 3.1.3.1).

This enzyme was only recently reported to be present in a wild-type strain of *S. cerevisiae*[3] and in commercial baker's yeast[1,2]. However, no mention of it has been made before in different studies on yeast alkaline phosphatase[4–6].

p-Nitrophenylphosphatase activities have also been described in mammalian cells, which are associated with a Na^+-, K^+- and Mg^{2+}-dependent ATPase activity[7]. Their properties, however, have been studied with enzymes bound to cellular structures such as microsomes, blood cells or plasma membranes[8–13], and no purification procedures have been reported.

This paper deals with the further characterization of the catalytic properties of yeast *p*-nitrophenylphosphatase.

MATERIALS AND METHODS

Yeast

The yeast was the same commercial preparation (Lesaffre) which was used previously and was stored in the same conditions[1,2].

Enzyme preparations

Partially purified preparations of *p*-nitrophenylphosphatase were essentially obtained as described previously[2]. One major modification was introduced for the extraction and solubilization of the enzyme which was obtained by plasmolyzing the cells with toluene as described by Röschlau and Hess[14] for the purification of yeast pyruvate kinase. This method proved to be more reproducible than the extraction by autolysis of freeze-thawed cells which we used before.

Enzyme assay

Unless otherwise indicated the composition of the assay medium was 1 mM *p*-nitrophenylphosphate (disodium salt) in 50 mM Tris–HCl (pH 7.7) and 20 mM $MgCl_2$.

The assays for *p*-nitrophenylphosphatase activity were performed as described previously[2]. One enzyme unit is defined as the amount required to release 1 μmole of *p*-nitrophenol per minute.

Analytical methods

Inorganic phosphate and phosphotransferase activity were determined as described previously[2]. In the assays of ATPase activity, P_i was analyzed by the method of Lowry and Lopez[15]. The activity on acetylphosphate was assayed as described by Stadtman[16].

Chemicals

p-Nitrophenylphosphate (disodium salt) was purchased from Fluka; *p*-nitrophenylsulfate, bis(*p*-nitrophenylphosphate), *p*-nitrophenylphosphate (di-Tris salt), ATP (disodium salt), carbamylphosphate, and acetylphosphate from Sigma; ouabain from Merck.

RESULTS

Substrate specificity and inhibition studies

In order to further characterize the specificity of the active center two substrate analogues, *p*-nitrophenylsulfate and bis(*p*-nitrophenylphosphate), were tested as eventual substrates and for their effect on the hydrolysis of *p*-nitrophenylphosphate. The results showed that the enzyme was unable to catalyze the hydrolysis of these compounds. The relative rates of hydrolysis were, respectively, 0.3 and 0% compared to *p*-nitrophenylphosphate (Table I). Moreover, they did not affect the hydrolysis of *p*-nitrophenylphosphate when they were introduced into the assay medium up to a

TABLE I

SUBSTRATE SPECIFICITY OF YEAST *p*-NITROPHENYLPHOSPHATASE

The substrates were assayed at a concentration of 1 mM in 50 mM Tris–HCl buffer (pH 7.7) and 20 mM $MgCl_2$ at 37 °C.

Substrate	*Relative activity*
p-Nitrophenylphosphate	100
p-Nitrophenylsulfate	0.3
bis(*p*-Nitrophenylphosphate)	0
Acetylphosphate	0
Carbamylphosphate	0
ATP	0

concentration of 1 mM. Considering the fact that the affinity of the enzyme for *p*-nitrophenylphosphate is reflected by a K_m value of about 20–30 μM over a wide range of pH (Fig. 3), it may be concluded that the enzyme, has essentially no affinity for these analogues, confirming the narrow specificity of the active center for *p*-nitrophenylphosphate.

Acetylphosphate and carbamylphosphate were not hydrolyzed by the enzyme and did not affect the hydrolysis of *p*-nitrophenylphosphate at a concentration of 1 mM.

Other phosphate compounds showed variable effects towards the hydrolysis of *p*-nitrophenylphosphate. The enzymatic activity was decreased by P_i and ATP, whereas PP_i and AMP were without effect. The inhibition by P_i and ATP was of competitive type (Figs 1 and 2), the values of K_i determined by plotting $1/v$ *vs*$[I]$, were, respectively, 1.5 mM and 0.6 mM. The enzyme was, however, unable to catalyse the hydrolysis of ATP between pH 6.5 and 9.4 with or without Mg^{2+}. Similar results

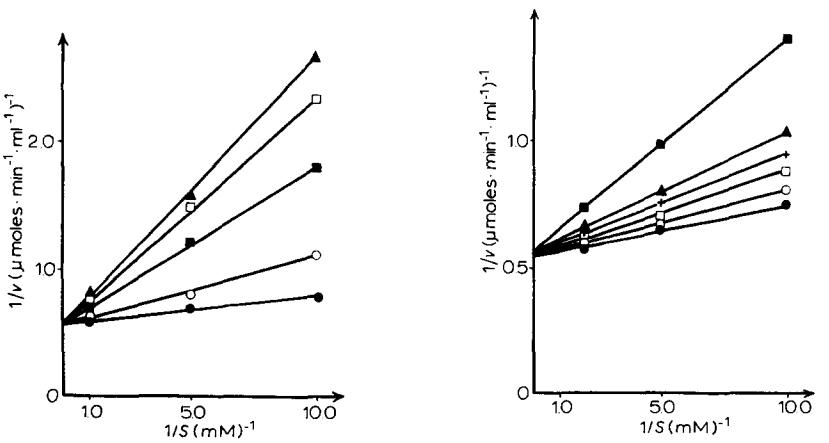

Fig. 1. Competitive inhibition of *p*-nitrophenylphosphate hydrolysis by P_i. Assay medium, 50 mM Tris–HCl (pH 7.7) and 20 mM Mg^{2+} at 37 °C with different concentrations of *p*-nitrophenylphosphate. ●, no P_i; ○, 1 mM; ■, 5 mM; □, 8 mM; ▲, 10 mM.

Fig. 2. Competitive inhibition of *p*-nitrophenylphosphate hydrolysis by ATP. The assay medium was the same as in Fig. 1. ●, no ATP; ○, 0.25 mM; □, 0.5 mM; +, 0.75 mM; ▲, 1 mM; ■, 2.5 mM.

have been reported by Fujita *et al.*[9] for the *p*-nitrophenylphosphatase of rabbit brain microsomes where ATP was a noncompetitive inhibitor but was not split by the enzyme.

Dependence of kinetic parameters on pH

The effect of pH on the kinetic parameters of the reaction was studied over the range 5.2–9.6 in different buffer systems. The determination of these parameters below pH 5.2 was not possible because the enzyme was inactivated during the assays. The values of K_m and V were determined by the method of Lineweaver and Burk, and pK_m, log V and log V/K_m were plotted *vs* pH as indicated by Dixon and Webb[17] (Fig. 3). The shape of the curves indicate that at least two ionizing groups are

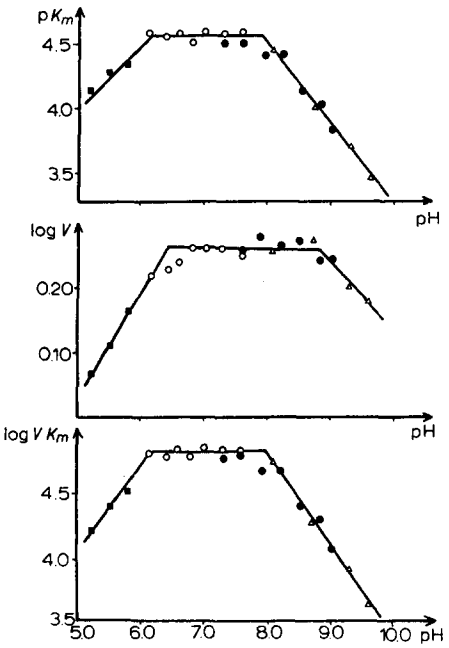

Fig. 3. Dependence of pK_m, log V and log V/K_m on pH at 37 °C. K_m and V were determined by the method of Lineweaver and Burk in the following buffer systems: 50 mM maleic acid–NaOH (■), 50 mM imidazole–HCl (○), 50 mM Tris–HCl (●) and 50 mM ammediol–HCl (△). K_m = mole·l^{-1}, V = μmoles·min^{-1}·ml^{-1}.

involved in the catalysis. In the acid pH range, bends at about pH 6.2 are observed in the three graphs, which may indicate the pK of an ionizing group in the free enzyme and in the enzyme–substrate complex. The pK of the free substrate (pK'_2 = 5.18) (ref. 18) is too far removed from this value to be accounted for by the bend of the log V/K_m curve. The alkaline bends occur around pH 8.0 for both the pK_m and log V/K_m plots, indicating the pH of an ionizing group of the free enzyme; and pH 8.8 for the log V plot indicating the pK of a group of the enzyme–substrate complex. These values suggest that imidazole, thiol or α-amino groups may be involved in the catalysis. However it should be noted that the slopes of the straight portions of the graphs did not show integer values.

Effect of divalent metal ions

The effect of divalent metal ions was compared to that of Mg^{2+}. Mg^{2+}-free enzyme solutions were obtained by gel filtration through Sephadex G-25 equilibrated with 0.1 M Tris–HCl (pH 7.4). The preparations were inactive when no Mg^{2+} was present in the assays. Divalent cations such as Co^{2+}, Ni^{2+}, Mn^{2+} and Zn^{2+} also stimulated the activity. However, the activation observed was lower than that produced by Mg^{2+} and some of these metal ions were inhibitors at high concentration. Other cations such as Ca^{2+}, Cu^{2+}, Fe^{2+}, Ba^{2+} and Sr^{2+} were essentially without effect (Table II).

TABLE II

EFFECT OF DIVALENT METAL IONS ON THE ACTIVITY OF YEAST *p*-NITROPHENYLPHOSPHATASE

The enzyme was assayed in the presence of increasing concentrations of the different metal ions. The activity in presence of 10 mM Mg^{2+} was taken as 100. Assay medium: 1 mM *p*-nitrophenylphosphate in 50 mM Tris–HCl (pH 7.7) at 37 °C.

Metal ion concn (mM)	Mg^{2+}	Co^{2+}	Zn^{2+}	Ni^{2+}	Ca^{2+}	Mn^{2+}	Cu^{2+}	Fe^{2+}	Ba^{2+}	Sr^{2+}
0.01	3.6	8.6	11.5	1.3	0	8	0	0	0	0
0.1	17.2	47	20	2.3	0	12.5	0	0	0	0
1.0	73.0	51	6.3	18.5	0	12	3	—	0	0
10.0	100	10.5	—	51	4.3	0	5	—	0	0

When added to an assay medium containing Mg^{2+}, these metal ions antagonized the activation by Mg^{2+} (Table III). It was observed in particular that Zn^{2+}, Mn^{2+} and Ca^{2+} were strong inhibitors although two of these ions were able to stimulate the activity in the absence of Mg^{2+}. The inhibition was only partly overcome by increasing the concentration of Mg^{2+}. The other divalent cations tested inhibited the activity to a lesser extent and the inhibition was abolished by high concentrations of Mg^{2+}.

TABLE III

EFFECT OF DIVALENT METAL IONS ON THE ACTIVATION BY Mg^{2+}

The effect of 1 mM concentrations of divalent metal ions was studied at two concentrations of Mg^{2+} in the assay medium (1 mM *p*-nitrophenylphosphate in 50 mM Tris–HCl (pH 7.7) at 37 °C). A value of 100 was taken for the activity in the presence of Mg^{2+} alone.

Mg^{2+} concn in assays (mM)	*Metal ion (1 mM)*								
	None	Ba^{2+}	Sr^{2+}	Ca^{2+}	Zn^{2+}	Mn^{2+}	Ni^{2+}	Co^{2+}	Cu^{2+}
0.25	100	84	68	0	0	16	42	79	35
20	100	93	99	10	7	25	105	90	100

Effect of Na^+ and K^+

Na^+ and K^+ are known to exert specific effects on animal *p*-nitrophenylphosphatases which are in general K^+ stimulated and Na^+ inhibited[7]. The activity of yeast *p*-nitrophenylphosphatase was not stimulated by either Na^+ or K^+ up to a concentration of 10 mM with or without Mg^{2+}. The same results were obtained whether the di-

Tris or the disodium salt of *p*-nitrophenylphosphate was used. In the presence of 20 mM Mg^{2+}, Na^+ and K^+ were inhibitors at high concentrations. About 60% inhibition was observed with both ions at a concentration of 0.5 M, suggesting that inhibition is due rather to a nonspecific ionic strength effect. This result confirms our previous observation where an increase in the molarity of the Tris–HCl buffer in the assay medium also decreased the enzymatic activity[2].

Transphosphorylation

In contrast to the nonspecific alkaline phosphatases[19,20], the enzyme was previously reported to lack phosphotransferase activity towards Tris[2]. The study of this reaction was extended to other acceptors such as ammediol, ethanolamine, D-glucose

TABLE IV

TRANSPHOSPHORYLATION

Phosphotransferase activity was assayed with the different acceptors as described previously[2]. Glucose and glycerol solutions were buffered with 0.1 M Tris–HCl (pH 8.2).

Acceptor concn (pH 8.2)	*p-Nitrophenol/P_i*				
	Tris	*Ammediol*	*Ethanolamine*	*Glycerol*	*Glucose*
0.1 M	1.01 1.18*	1.03	1.04	1.03	1.02
0.5 M	1.02 1.53*	1.00	1.03	1.02	1.01

* Values obtained with *E. coli* alkaline phosphatase.

and glycerol. These compounds were also found not to be phosphorylated upon hydrolysis of *p*-nitrophenylphosphate. A ratio *p*-nitrophenol/P_i close to 1 was found at concentrations of the acceptors up to 0.5 M. In the same conditions, *Escherichia coli* alkaline phosphatase showed ratios of 1.18 and 1.53, respectively, with 0.1 and 0.5 M Tris (Table IV).

TABLE V

EFFECT OF ANIONS ON *p*-NITROPHENYLPHOSPHATE HYDROLYSIS

The enzyme was assayed in the presence of different anions (sodium salts) at a concentration of 10 mM in the assay medium. A value of 100 was taken for the activity in the absence of added anions.

Anion (10 mM)	*Activity (%)*
None	100
Cl^-	100
SO_4^{2-}	100
NO_3^-	100
Acetate	100
CN^-	100
F^-	21
Arsenate	6
HPO_4^{2-}	75

Inhibitors

Among several anions tested, AsO_4^{3-} and F^- were found to be inhibitors. Chloride, sulfate, nitrate, acetate and cyanide ions were without effect up to a concentration of 10 mM (Table V).

The enzyme was inhibited by *p*-chloromercuribenzoate. An inhibition of 60% was observed when 1 mM *p*-chloromercuribenzoate was added to the assay medium.

Ouabain, an inhibitor of several mammalian *p*-nitrophenylphosphatases[8-10] had no effect up to a concentration of 5 mM.

DISCUSSION

The properties of yeast *p*-nitrophenylphosphatase have been further characterized.

The specificity of the enzyme has been confirmed on various compounds. (a) The enzyme did not catalyze the hydrolysis of *p*-nitrophenyl sulfate and bis(*p*-nitrophenylphosphate), indicating that it is indeed a phosphomonoesterase. Moreover, these compounds did not affect the hydrolysis of *p*-nitrophenylphosphate. These results confirm the very narrow specificity of the active center for both the *p*-nitrophenol and the phosphate moieties. (b) The enzyme was inactive on acetylphosphate, carbamylphosphate and ATP.

Phosphate compounds showed variable effects towards the hydrolysis of *p*-nitrophenylphosphate. P_i and ATP were competitive inhibitors, whereas PP_i, AMP, carbamylphosphate and acetylphosphate were without effect. The inhibition by ATP is surprising if one considers the lack of ATPase activity of the enzyme. A similar behavior of ATP has been reported by Fujita *et al.*[9] towards a *p*-nitrophenylphosphatase activity occurring in rabbit brain microsomes.

The enzyme was inactive in the absence of Mg^{2+} which was found to be the best activator among several divalent metal ions. Metal ions such as Ca^{2+}, Zn^{2+} and Mn^{2+} strongly antagonized the activation by Mg^{2+}. The inhibition was only partly abolished by increasing the concentration of Mg^{2+}. Na^+ and K^+ showed no specific effects such as those observed with mammalian *p*-nitrophenylphosphatases. In particular K^+ did not stimulate the activity, whether Mg^{2+} was present or not, as well with the sodium salt as with the Tris salt of *p*-nitrophenylphosphate.

By these properties, yeast *p*-nitrophenylphosphatase has only few similarities with the *p*-nitrophenylphosphatase activities associated with the Na^+-, K^+- and Mg^{2+}-dependent ATPase found in mammalian cells[7].

Concerning baker's yeast, Nurminen *et al.*[21] have reported that plasma membranes contain a Mg^{2+}-dependent ATPase but no *p*-nitrophenylphosphatase activity was present in their yeast preparation. In fact, as we have discussed previously[2], the *p*-nitrophenylphosphatase does not seem to be present in all yeasts. The only other examples of specific *p*-nitrophenylphosphatases in yeasts are those described by Stadtman[22] and by Gorman and Hu[3]. Very recently a specific *p*-nitrophenylphosphatase has also been purified from *Streptococcus mutans*[24].

Preliminary results obtained on our yeast preparation indicate that the enzyme is not located outside the surface of the cell wall. Intact cells do not show activity towards *p*-nitrophenylphosphate at alkaline or neutral pH. After freeze-thawing with liquid nitrogen, 80–90% of the activity is found in heavy sediments (15

min, 6000 × *g*). The enzyme can be solubilized by autolysis of freeze-thawed cells[2] or by toluene-induced autolysis. A similar behavior has been observed by Tonino and Steyn-Parvé[4] for the nonspecific alkaline phosphatase of *Saccharomyces carlsbergensis*. This enzyme was more or less soluble according to the age of the cultures and was clearly demonstrated to be intracellular.

The mammalian *p*-nitrophenylphosphatase–ATPase systems are believed to regulate the active transport of Na^+ and K^+ across membranes. Such systems may also occur in yeasts; however, the lack of specific effects of these ions on the hydrolysis of *p*-nitrophenylphosphate does not support this view. The observed antagonistic effect of Ca^{2+} or Zn^{2+} towards the activation by Mg^{2+} and the inhibition by ATP may be related to a physiological role of the enzyme. Further studies on the cellular localization of the enzyme and on its properties in its bound form may provide further information in order to understand its physiological role.

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