Phosphorylase Phosphatase Activity in *Saccharomyces cerevisiae* 257

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A phosphatase, active towards phosphorylase *a* and phosphorylated proteins casein and histone II-A, was isolated from *Saccharomyces cerevisiae* 257. The enzyme dephosphorylated glycogen phosphorylase from commercial yeast rendering it inactive. The protein phosphatase activity was not influenced by any metal ions. Phosphorylase phosphatase activity was slightly stimulated by *p*-nitrophenyl phosphate and inhibited by heparin.

Introduction

The reversible phosphorylation of proteins is recognized to be the major mechanism for control of biological functions (Krebs, 1985). Protein phosphatases, the counterpart of protein kinases, play a key role in regulating the phosphorylation levels of cellular proteins (Cohen, 1989). Four major serine/threonine phosphatases (types 1, 2A, 2B and 2C) have been described in mammalian cells according to criteria based on substrate specificity and sensitivity to endogenous inhibitors (Ingebritsen and Cohen, 1983; Shenolikar, 1994). Based on these criteria Cohen *et al.* (1989) have suggested that yeast extracts contain phosphatases similar to mammalian phosphatases 1, 2A and 2C. However, little information is available concerning the properties of any of the yeast enzymes described thus far, and it is not clear if they correspond to the forms found in mammalian cells.

The enzymes involved in glycogen synthesis and degradation, glycogen synthase, and glycogen phosphorylase, are regulated by reversible phosphorylation (Ingebritsen *et al.* 1983). Phosphorylase, as other enzymes in biodegradative pathways, is activated by phosphorylation, while glycogen synthase is, on the contrary, activated by dephosphorylation.

A protein phosphatase, partially purified from yeast extracts, dephosphorylating yeast phosphorylase, was described by Wingender-Drissen and Becker (1983). Activity of the enzyme was found to be dependent on Mg^{2+} and Mn^{2+} showing that the enzyme is a type 2C according to the classification of Ingebritsen and Cohen (1983).

In this work a presence of a phosphatase in *Saccharomyces cerevisiae* active on yeast phoshorylase is demonstrated. In contrast to other phosphorylase phosphatases studied, enzyme activity was independent of any metal ions.

Materials and Methods

Strain and growth conditions

S. cerevisiae strain was kindly provided by Prof. P. Venkov from the Institute of Molecular Biology (IMB), Bulgarian Academy of Sciences (IMB culture collection 257) and originally obtained from Prof. A. Hinnen collection (AH :220, *MATa* leu2–3 leu2–112 his3 trp1 *pho3 pho5)*. The strain was grown in 500 ml Erlenmeyer flasks with 100 ml medium under slow rotary agitation at 28 °C on YEPD medium (4% Bacto-yeast extract, 2% Bacto-peptone and 2% glucose).

Preparation of phosphoprotein substrates

Unlabeled phosphohistone II-A from calf thymus (Sigma) was prepared as described by Meisler and Langan (1969). Phosphocasein from bovine milk (Sigma), phoshorylase *a* from rabbit muscle (Sigma) and yeast phosphorylase *a* were prepared as described by Hemmings (1981) and Manhart and Holzer (1988), respectevily, using unlabeled ATP and 0.05 mg cAMP-dependent protein kinase

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from bovine heart (Sigma). Preparations were assayed for their content of alkali-labile phosphate according to Meisler and Langan (1969).

Enzyme assays

Phosphatase assay. Phosphatase activity was assayed with *o*-phospho-DL-serine (Ser(P)) and *p*-nitrophenyl phosphate (*p*NPP) as substrates, respectively. The reaction mixture contained 100 µl enzyme solution, 100 µl of 20 mM Tris (hydroxymethyl) amino methane-HCl, pH 7.4, and 100 µl 2 mM Ser(P) or *p*NPP. When unlabeled phosphoproteins were used as substrates the assay mixture contained 100µl of the respective phosphoprotein instead of Ser(P) or *p*NPP. After incubation for 15 min at 37 °C the reaction was terminated by adding 900 µl of reagent solution (an aqueous mixture of ammonium molybdate and zinc acetate at pH 5.0) according to the method of Bencini *et al.* (1983) for one-step spectrophotometric orthophosphate assay. The molar absorption coefficient for the phosphomolybdate complex at 350 nm was 7200 $M^{-1}cm^{-1}$. One unit of enzyme activity was defined as 1 nmol of inorganic phosphate (Pi) liberated from the substrate per min at 37 °C.

Phosphorylase assay. Phosphorylase activity was assayed as described by Fosset *et al.* (1971). The reaction mixture contained 100 µl enzyme solution, 100 µl substrate solution (0.15 M glucose-1-phosphate and 2% glycogen in 50 mM sodium succinate buffer, pH 5.8) and 100 µl 50 mM sodium succinate buffer, pH 5.8. After incubation for 10 min at 30 °C 900 µl of the reagent solution of Bencini *et al.* (1983) was added for the determination of the liberated inorganic phosphate. One unit of enzyme activity is the amount of enzyme releasing 1 µmol of inorganic phosphate (Pi) from glucose-1-phosphate.min^{-1} at 30 °C.

Inactivation of yeast phosphorylase. Phosphorylase phosphatase activity was measured as well by following the inactivation of yeast phosphorylase. Aliquots of phosphorylase and phosphatase, preliminary buffered in Tris buffer, pH 7.4, were mixed (final volume of 1 ml) and incubated at 30 °C. At appropriate intervals 100 µl samples were taken, mixed with glycogen phosphorylase assay mixture and estimated for phosphorylase activity. A blank with distilled water instead of phosphatase was assayed under the same conditions.

Activation of yeast phosphorylase by cAMP-dependent protein kinase. After 30 min inactivation of yeast phosphorylase, 3.6 mM ATP, 3.6 mM $MgCl_2$, 1.8 mM cAMP and 20 µg of protein kinase were added to the inactivated phosphorylase (final volume of 1 ml) and 100 µl sampels were withdrawn at intervals for determination of phosphorylase activity.

Protein assay

Protein concentration was estimated as described by Bradford with BSA as standard.

Enzyme preparation

Preparation of cell free extract. Yeast cells of an exponentially grown culture (20 h) were harvested by centrifugation (at 6 000×*g* for 10 min at 4 °C), washed twice and then ressuspeneded in a minimal volume of 20 mM Tris-HCl pH 7.4, containing 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM dithiothreitol (buffer A). The cell suspensions obtained were disrupted in a homogenizer. The resulting homogenates were centrifuged at 12000×*g* (20 min at 4 °C) and the supernatant fluids (crude extract) were used as a starting material for enzyme purificaton.

DEAE-cellulose chromatography. The crude extract after dialysis against buffer A was loaded onto a DEAE-cellulose column (1.8×10 cm) equilibrated with the same buffer. Elution of the bound phosphatases was achieved with a linear 0–0.6 M NaCl gradient in 60 ml of the initial buffer. Fractions of 1.2 ml collected at a flow rate of 25 ml h^{-1} were tested for P-Ser and pNPP phosphatase activity. Active fractions were pooled and concentrated.

Ser(P)-agarose column chromatography. Ser(P) phosphatase fractions after the DEAE-cellulose chromatography were applied to a Ser(P)-agarose column (1×6 cm) previously equilibrated with buffer A. The enzyme activity was eluted with 1 M NaCl in the same buffer after a linear 0–0.6 M NaCl gradient. Fractions of 1.2 ml were collected and used for further investigations.

Preparation of glycogen phosphorylase. Yeast phosphorylase was obtained from Baker's yeast as described by Fosset *et al.* (1971).

Results and Discussion

Chromatographic separation of phosphatase activities

DEAE-cellulose chromatography of the crude extract revealed several phosphatase activity peaks (Fig. 1). Ser(P) activity was eluted in peaks *P-1* and *P-3* whereas *p*NPP activity was eluted in peaks *P-2* and *P-3.* The two major alkaline phosphatase activities (*P-2* and *P-3)* represented specific *(pho13)* and nonspecific (*pho*8) alkaline phosphatases, respectively, and were described in separate publications. Fractions containing only Ser(P) phosphatase activity from the *P-1* peak were pooled and applied on a Ser(P)-agarose column chromatography. Trace amounts of *p*NPP-hydrolysing activity still present after the DEAE-cellulose chromatography were completely removed with the Ser(P)- agarose step. The full Ser(P) phosphatase activity was retained on the column, whereas most of the concomitant proteins were eluted in the unabsorbed fractions and in the NaCl (0–0.6 M) gradient fractions as well. (Fig. 2).

Activity of P-1 phosphatase with various phosphoproteins

The data in Table I illustrate that the *P-1* phosphatase can dephosphorylate a wide spectrum of phosphorylated by the c-AMP-dependent protein kinase proteins, yeast phosphorylase *a,* phosphorylase *a* from rabbit muscle, histone II-A and casein, showing considerably high activity towards yeast phosphorylase. This observation, coupled with the fact that the phosphorylase dephosphorylating activity co-eluted with the peak of casein phosphatase activity on Ser(P)-agarose (Fig. 2), indicates that the same enzyme catalyzes each of these reactions. The partially purified enzyme did

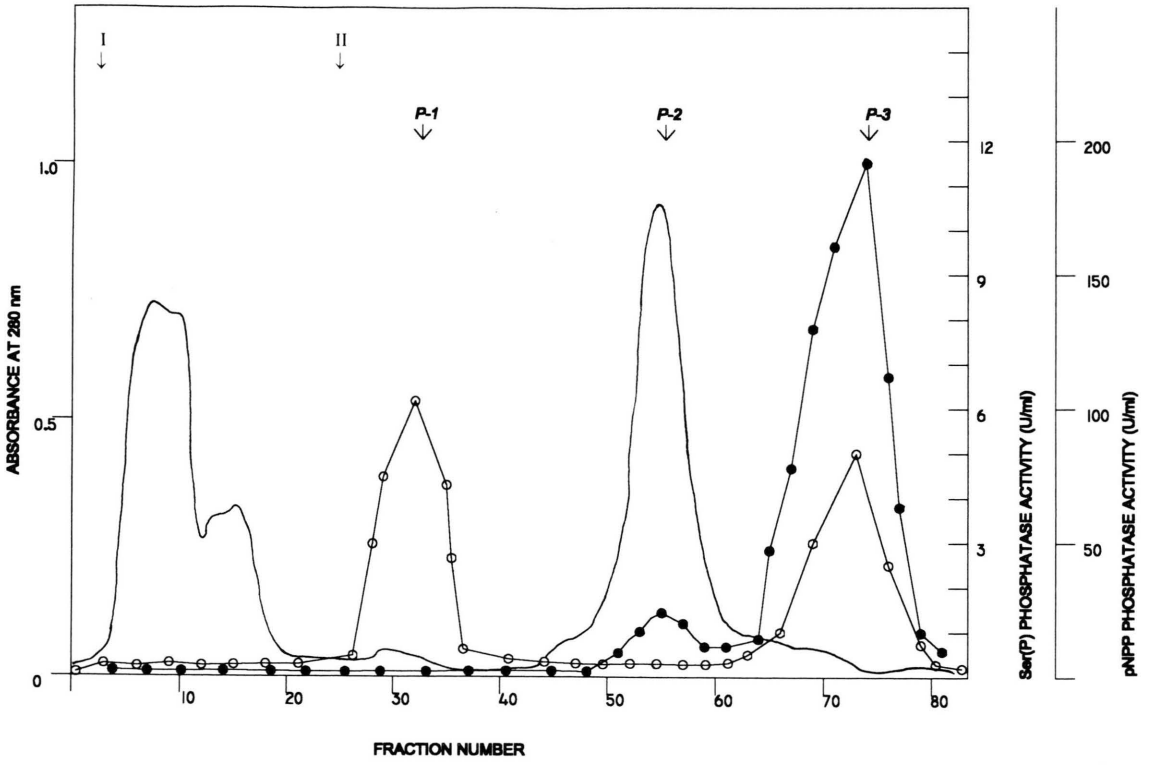

Fig. 1. DEAE-cellulose chromatography. *S. cerevisiae* cell extracts were applied to a DEAE-cellulose column (1.8×10 cm). The elution and the assay of Ser(P) phosphatase (○) and *p*NPP phosphatase (●) activities was carried out as described in Materials and Methods. I, start of the elution with buffer A (20 mM Tris-HCl , 1 mM EDTA, 1 mM dithiothreitol, 0.5 mM PMSF, pH 7.4); II, start of the salt gradient (0–0.6 M NaCl, 60 ml in buffer A, pH 7,4). Protein concentration was monitored by transmission at 280 nm (—).

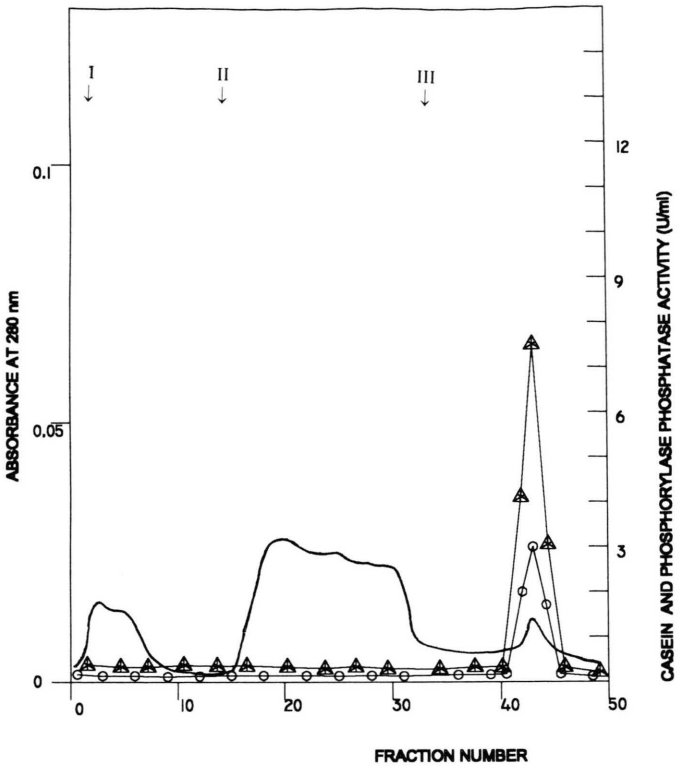

Fig. 2. Ser(P)-agarose column chromatography. Ser(P) phosphatase fractions after the DEAE-cellulose chromatography were applied to a Ser(P)-agarose column (1 × 6 cm). Casein phosphatase (○) and yeast phosphorylase phosphatase (△) activities were eluted and assayed as described in Materials and Methods. I, start of the elution with buffer A; II, start of the salt gradient (0–0.6 M NaCl, 60 ml in buffer A); III, start of the elution with 1 M NaCl in buffer A. Protein concentration was monitored by transmission at 280 nm (—).

Table I. Phosphoprotein activity of the partially purified yeast protein phosphatase. Assays were performed with the Ser(P)-agarose fraction as described in Material and Methods. Data were pooled to give a mean ± SEM of within 10–15%.

Substrate	Specific activity ($nmol.min^{-1}.mg^{-1}$ protein)
Yeast phosphorylase *a*	18.5
Phosphorylase *a* from rabbit muscle	10.7
Casein	10.9
Histone II-A	9.8

not act with non-protein phosphate esters such as *p*-NPP (2 mM) (data not shown).

Reversibility of inactivation of yeast phosphorylase by P-1 phosphatase

In the example presented (Fig. 3), a decrease from 540 mU/mg to 42.8 mU/mg of phosphorylase activity was observed after 30 min incubation with *P-1* phosphatase . Reversibility of the reaction was proved by adding cAMP-dependent protein kinase thus demonstrating that inactivation i.e. dephosphorylation of yeast phosporylase was due to phosphatase action and not to proteolysis (Fig. 3).

Effect of some activators and inhibitors on protein phosphatase activity

S. cerevisiae protein phosphatase acitvity was measured in the presence of some compounds that have been observed to affect the activity of protein phosphatases *in vitro* (Cohen, 1989).

The effect of Mg^{2+}, Mn^{2+} and Ca^{2+} was determined using Ser(P), phosphocasein and yeast phosphorylase *a* as substrates in order to asses the cation dependence of the enzyme (Table II). Ser(P) and protein phosphatase acitvities were independent of divalent metal ions and the chelating agent EDTA did not change the initial enzyme activities. The broad spectrum protein phosphatase inhibitor NaF decreased Ser(P), casein and phosphorylase phosphatase activities up to 84%, 60% and 51%, respectevily.

Activation / inhibition by *p*NPP and heparin are listed as useful methods for distinguishing protein phosphatases when phosphorylase *a* is used as a

Table II. Effect of some activators and inhibitors on *S. cerevisiae* phosphatase activity. All results are represented as percent of initial activity with no additions and the effectors listed below are presented at the indicated final concentration. Values of 6.0 U/mg; 11 U/mg and 18 U/mg correspond to 100% of Ser(P) phosphatase, casein phosphatase and yeast phosphorylase phosphatase activities, respectively. Data were pooled to give a mean ±SEM of within 10–15%. Mean values are given.

Effectors	**(mM)**	**Activity (%)**		
		P-Ser-phosphatase	Casein phosphatase	Yeast phosphorylase phosphatase
None		100	100	100
Ca^{2+}	10	95	83	85
Mg^{2+}	10	103	70	82
Mn^{2+}	10	52	117	77
EDTA	10	92	116	107
NaF	10	84	60	51
pNPP	1			128
Heparin	10μg/ml		–	39

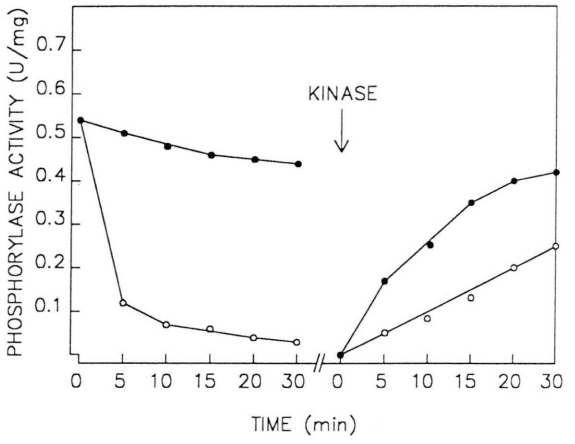

Fig. 3. Inactivation of yeast phosphorylase by Ser(P) phosphatase. Yeast phosphorylase was incubated at 30 °C without (●) and with (○) Ser(P) phosphatase. Aliquots were taken at the indicated times from the reaction mixture and assayed for phosphorylase activity (see Methods). After 30 min, protein kinase from bovine heart (Sigma) was added and the reversibility of the reaction was tested. Data (three determinations) were pooled to give a mean ± SEM of within 10–15%.

substrate (Cohen, 1989). *S. cerevisiae* 257 phosphorylase phosphatase activity was slightly activated (128%) by *p*NPP (Table II). The *p*NPP stimulation of phosphorylase phosphatase activity was reported for the first time by Goris and Merlevede (1988) as a feature of ATP-Mg^{2+}- dependent phosphatases. They found that phosphorylase phosphatase activity was stimulated severalfold by *p*NPP at an optimal concentration of 1 mM and this effect was synergistic with the action of a deinhibitor protein. *p*NPP was used as an effector for the characteristic of the archaebacteruim *Sulfolobus solfataricus* protein phosphatase as well (Kennelly *et al.*, 1993). Since the enzyme looked like protein phosphatases type 2C *p*NPP did not change its activity.

On the other hand, *S. cerevisiae* phosphorylase phosphatase activity was inhibited up to 39% by the sulfated polysaccharide heparin (Table II). This finding is in agreement with observations made by other authors. Gergely *et al.* (1984) showed that the inhibition of phosphatase type 1 by heparin is complete (5 to 10 μg/ml heparin caused 50% inhibition) and not mimicked by closely related polysaccharides. As it was shown that heparin inhibits protein phosphatase type 1 and activates or has no effect on protein phosphatase type 2, Pelech and Cohen (1985) suggested that basic proteins and heparin can be employed like inhibitors -1 and -2 to distinguish protein phosphatases types 1 and 2A. In contrast, heparin did not affect the activity of the catalytic subunit of a novel serine/threonine protein phosphatase from bovine brain designated as type 3 (Honkanen *et al.*, 1990).

In summary, a phosphorylase phosphatase activity not dependent on Me^{2+} ions was found in *S. cerevisiae 257.* In some respect, independence of divalent metal ions, activation by *p*NPP and inhibition by heparin, the enzyme bears some resemblance to the eukaryotic protein phosphatases type 1 (Cohen, 1989). This functional resemblance is, however, limited. Moreover, novel forms of

protein phosphatases have been described, that do not fit into a definitive classification at both biochemical and molecular levels (Honkanen *et al.*, 1990).

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