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COMPARISON OF TWO FORMS OF PIG HEART PHOSPHOPROTEIN PHOSPHATASE

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Summary

A phosphoprotein phosphatase (phosphoprotein phosphohydrolase, EC 3.1.3.16) was partially purified from pig heart using as substrate H2B histone which had been phosphorylated at Ser-32 and Ser-36 by adenosine 3′,5′-monophosphate-dependent protein kinase (EC 2.7.1.37). The enzyme had a molecular weight of approx. 250 000 and was converted to a smaller form with a molecular weight of approx. 30 000 upon treatment with ethanol. Phosphorylase *a* (EC 2.4.1.1) and phosphorylated H1 histone also served as substrates for both forms of the enzyme. The conversion of the large form of the enzyme to the small form decreased the phosphohistone phosphatase activity to 25—50% with a concomitant 7-fold increase in the phosphorylase *a* phosphatase activity. Ser-36 phosphate was removed 6- and 15-fold more rapidly than was Ser-32 phosphate by the large and small forms of the enzyme, respectively. Among Ser-36-containing tryptic phosphopeptides derived from phosphorylated H2B histone, Lys-Glu-Ser(*P*)-Tyr-Ser-Val-Tyr was the shortest phosphopeptide which was dephosphorylated at a significant reaction rate with the phosphoprotein phosphatase. The K_m values for phosphorylated H2B histone and the tryptic phosphopeptide were 23.7 μM and 187.1 μM, respectively, with the large form, and 81.4 μM and 90.0 μM, respectively, with the small form of the enzyme.

Introduction

Evidence has accumulated to support the concept that a single phosphoprotein phosphatase (phosphoprotein phosphohydrolase, EC 3.1.3.16), dephosphorylates glycogen synthase *D*, (EC 2.4.1.11), phosphorylase *a* (EC 2.4.1.1), phosphorylase *b* kinase (EC 2.7.1.38) and other proteins phosphorylated by cyclic AMP-dependent protein kinase (EC 2.7.1.37) including histone, protamine, casein, pyruvate kinase (EC 2.7.1.40) and the inhibitory component of

troponin [1—7]. The phosphoprotein phosphatase exists in multiple forms of different molecular weight in various tissue extracts [8—14]. Brandt et al. [15] have reported that the multiple forms of the phosphoprotein phosphatase in extracts from rabbit liver and rat tissues may be converted upon 80% ethanol treatment to a single smaller form of molecular weight 30 000—35 000. Although the small form of the enzyme from rabbit liver has been purified homogeneously and characterized [1,2], little information is available concerning the purification and properties of the original large form of the enzyme. In this paper, we will describe the partially purification of a high molecular weight form (mol. wt. 250 000) of the phosphoprotein phosphatase from pig heart and its conversion to a smaller form (mol. wt. 30 000) by ethanol treatment. Differences in the substrate specificities of these two forms of the phosphoprotein phosphatase will be described in this paper. These two forms of the phosphoprotein phosphatase will be referred to in the paper as the large form and the small form, respectively.

Materials and Methods

Materials. Calf thymus whole histone, H2B histone and H1 histone were prepared as described previously [16]. Cyclic AMP-dependent protein kinase was purified from an extract of silkworm pupae (*Bombyx mori)* as previously described [17]. [γ-^{32}P]ATP was prepared by the method of Glynn and Chappell [18]. Trypsin and chymotrypsin were obtained from Worthington. Rabbit muscle phosphorylase *a* was purchased from Boehringer Mannheim. Bovine serum albumin, egg yolk phosvitin, jackbean urease and horse heart cytochrome *c* were obtained from Sigma. Human γ-globulin (Fraction II) and ovalbumin (twice crystallized) were products of ICN Pharmaceuticals. Bovine casein (Hammarsten) was purchased from Merck A.G., Darmstadt. Other chemicals were obtained from commercial sources.

Preparation of substrates. ^{32}P-labelled H2B and H1 histones were prepared with [γ-^{32}P]ATP and cyclic AMP-dependent protein kinase as described before [16]. ^{32}P-labelled H2B histone which was used in the present study, contained 53% of total incorporated phosphate at Ser-36 and 43% at Ser-32. The radioactive tryptic and chymotryptic phosphopeptides derived from ^{32}P-labelled H2B histone were prepared and identified by amino acid analysis with a Nihondenshi amino acid analyzer, model 5AH [17].

Enzyme assay. Phosphoprotein phosphatase was incubated in a final volume of 100 μl with ^{32}P-labelled H2B histone, which contained 50 μM alkali-labile phosphate with a specific activity of 2—50 cpm/pmol. The incubation was carried out for 10 min at 30°C in a solution containing 50 mM Tris · HCl at pH 7.2, 50 mM $(CH_3COO)_2Mg$ and 0.5 mM dithiothreitol (standard assay conditions). A blank incubation without enzyme was run simultaneously. The reaction rate was linear with time and directly proportional to the amount of enzyme under conditions in which no more than 30% of the total alkali-labile phosphate was dephosphorylated. All assays were performed in duplicate. The reaction was terminated by the addition of 1 ml of 5 mM silicotungstic acid in 0.0025 M H_2SO_4. The [^{32}P]orthophosphate released was measured by a modification of the procedure of Martin and Doty [19] as described by Titanji [7].

1 unit of phosphoprotein phosphatase was defined as the amount of enzyme which catalyzed the release of 1 nmol of [^{32}P]orthophosphate per min.

Phosphorylase phosphatase was assayed by measuring the inactivation of rabbit muscle phosphorylase *a* as described by Brandt et al. [1]. 1 unit of activity was taken as the amount of enzyme which converted 0.2 mg of phosphorylase *a* per min.

Protein was determined by the method of Lowry et al. [20] with bovine serum albumin as a reference protein. Orthophosphate released from nonradioactive substrates was determined chemically [19].

Enzyme purification. Pig heart was obtained from a local slaughterhouse and kept on ice until use (usually within 1.5 h). All subsequent steps were carried out at 0—4°C unless otherwise stated.

Heart muscle was cut into small pieces and homogenized for 3 min in a Waring Blendor with 3 vols. of Buffer A (50 mM Tris · HCl, pH 7.4, containing 50 mM 2-mercaptoethanol and 1 mM EDTA). To the homogenate, 1 g of Norit A per l was added with gentle stirring. Then, the homogenate was centrifuged for 20 min at 15 000 $\times g$. The supernatant solution was filtered through glass wool to remove fat.

To the filtrate (crude extract), $(NH_4)_2SO_4$ was added to a final concentration of 70% saturation. The pH was maintained at 7.4 during this process by the addition of 2 M Tris base. After 30 min, the precipitate was collected by centrifugation (15 000 $\times g$, 30 min). The pellet was suspended in a minimum volume of Buffer A and dialyzed for 3 h against the same buffer. The dialyzed enzyme solution was diluted with Buffer A to the protein concentration of 20 mg/ml. The pH of the enzyme solution was adjusted to 5.0 with 0.5 M acetic acid and then $(NH_4)_2SO_4$ was added to a final concentration of 20% saturation. After standing for 15 min, the precipitate was collected by centrifugation (30 000 $\times g$, 20 min). From the precipitate, the enzyme was extracted twice with Buffer A by homogenization with a Dounce homogenizer followed by centrifugation (30 000 $\times g$, 20 min).

The enzyme solution was loaded onto a DEAE-Sephadex A-50 column (5 × 5 cm) equilibrated with 0.18 M NaCl in Buffer A. After washing the column with the same buffer until the absorbance at 280 nm returned close to baseline, the enzyme was eluted with 0.35 M NaCl in Buffer A (36 ml/h). The active fractions were pooled and diluted with the same volume of Buffer A and loaded onto a DEAE-Sephadex A-50 column (2.6 × 5 cm) equilibrated with 0.18 M NaCl in Buffer A. After washing with 1 column vol. of the same buffer, elution was performed with a linear concentration gradient of NaCl (0.18—0.5 M) in Buffer A (7 ml fractions, 17 ml/h). The enzyme was eluted at about 0.26 M NaCl. The active fractions were pooled and concentrated in an Amicon ultrafiltration cell equipped with a PM-10 filter membrane, and applied to a Sephadex G-200 column (2.5 × 80 cm) equilibrated with Buffer A. Elution was performed upward with Buffer A (3.8 ml fractions, 16 ml/h). Fractions of the single active peak were pooled and concentrated by ultrafiltration. The phosphoprotein phosphatase was purified about 50-fold (specific activity, 73 units/mg) from the crude extract, with an overall yield of approx. 10%. The enzyme preparation at this stage could be stored for a month with a 20% loss of activity, in the presence of 15% glycerol, at —20°C. Purification and recovery

TABLE I

PURIFICATION OF PIG HEART PHOSPHOPROTEIN PHOSPHATASE

Fraction	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Crude extract	9783	13 990	1.4	100
70% $(NH_4)_2SO_4$, pH 7.4	5786	9 053	1.6	65
20% $(NH_4)_2SO_4$, pH 5.0	1176	2 976	2.5	21
DEAE-Sephadex I	55	2 010	37	14
DEAE-Sephadex II	33	1 691	51	12
Sephadex G-200 * (large form)	16	1 167	73	8
Ethanol	7.6	710	93	5
Sephadex G-150 * (small form)	0.14 **	622	4443	4

* These fractions were used as large and small forms of the enzyme, respectively, in this paper.
** Protein was determined from the absorbance at 280 nm.

of the enzyme through these purification steps are summarized in Table I.

Other procedures. Autoradiograms of radioactive tryptic phosphopeptides from ^{32}P-labelled H2B histone were made as described earlier [16]. The radioactive spots were identified in comparison with authentic samples of the phosphopeptides: Ser(*P*)-Arg, Ser(*P*)-Arg-Lys, Lys-Arg-Ser(*P*)-Arg and Lys-Glu-Ser(*P*)-Tyr-Ser-Val-Tyr-Val-Tyr-Lys. The radioactivity of the radioactive spots was determined by direct paper strip counting using a Packard Tri-Carb liquid scintillation spectrometer, model 3320.

Results

Conversion of the large form of phosphoprotein phosphatase to the small form

The purified phosphoprotein phosphatase appeared as a single peak from a Sephadex G-150 column as shown in Fig. 1A. The molecular weight of the enzyme, as estimated by gel-filtration on a calibrated Sephadex G-200 column, was approx. 250 000. Confirming the results of previous workers [15] with other enzyme sources, the eluted phosphoprotein phosphatase was converted to a smaller form of the enzyme by ethanol treatment. To 1 vol. of the enzyme solution (specific activity, 73 units/mg; protein, 16 mg) 5 vols. of ice-cold 95% ethanol were added. The precipitate was immediately collected by centrifugation at 30 000 $\times g$ for 5 min and dissolved in 50 mM Tris · HCl (pH 7.4)/1 mM dithiothreitol, using a Dounce homogenizer. The enzyme solution was then dialyzed for 20 min against the same buffer and was applied again to a Sephadex G-150 column. The enzyme activity was eluted as a single peak of lower molecular weight as shown in Fig. 1B. The molecular weight of the small form of the enzyme was estimated to be approx. 30 000 (Fig. 2). Since most of the material absorbing at 280 nm remained in the higher molecular weight fractions, the phosphoprotein phosphatase was further purified about 60-fold by ethanol treatment followed by Sephadex G-150 gel-filtration (Table I). The enzyme preparation was free of cyclic AMP-dependent protein kinase and could be stored for a month with a 30% loss of activity in the presence of 15% glycerol at −20°C. On SDS polyacrylamide gel electrophoresis, the enzyme preparation

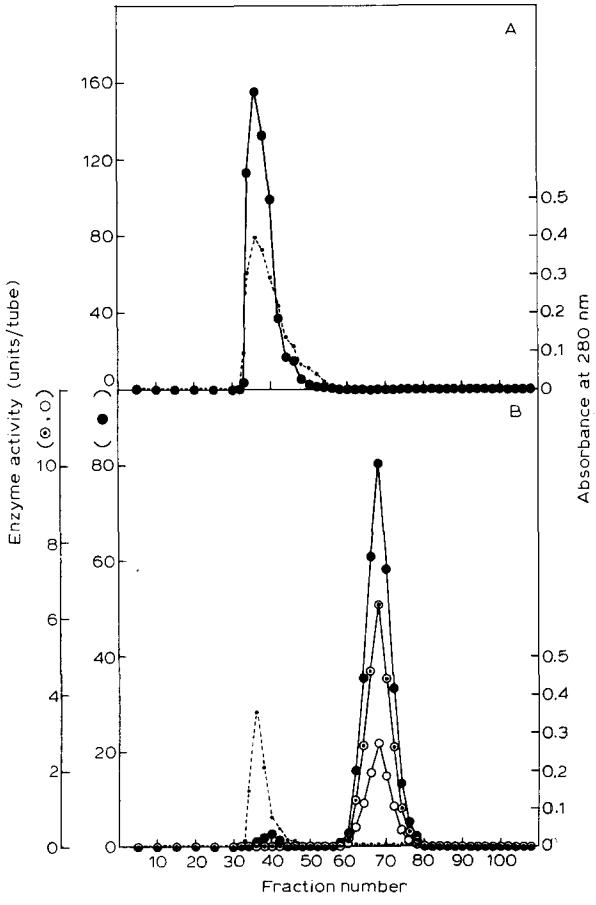

Fig. 1. Gel filtration of phosphoprotein phosphatase on a Sephadex G-150 column. A, before ethanol treatment; B, after ethanol treatment. The purified enzyme (approx. 20 mg protein) before or after ethanol treatment (see text) was applied to a Sephadex G-150 column (2.5 × 79 cm) equilibrated with 50 mM Tris · HCl, pH 7.4 containing 1 mM dithiothreitol. Elution was performed upward with the same buffer and fractions of 3.8 ml each were collected at a flow rate of 16 ml/h. Enzyme activity was determined as described under Materials and Methods. Solid lines with ●, ○ and ⊙ indicate enzyme activity assayed with ^{32}P-labelled H2B histone, ^{32}P-labelled H1 histone and phosphorylase *a*, respectively. The dotted line indicates absorbance at 280 nm.

appeared to be nearly homogeneous; there were one major (mol. wt. approx. 34 000) and severeal minor protein bands.

The effect of the concentration of Mg^{2+} or Mn^{2+} on the enzymatic activities of the two forms of the enzyme is given in Fig. 3. Mg^{2+} or Mn^{2+} permitted maximal enzyme activities of both forms of the enzyme when present in concentrations of 50 mM and 20—30 mM, respectively. Zn^{2+} (at 5 mM) completely inhibited the reactions of both the large and small forms of the enzyme in the presence or absence of 50 mM Mg^{2+}. The effect of pH on the rate of enzymatic dephosphorylation of ^{32}P-labelled H2B histone is shown in Fig. 4. Both forms of the enzyme had a pH optimum in the region of pH 7.0. Tris/maleate buffer was more inhibitory with the small form of the enzyme than with the large form.

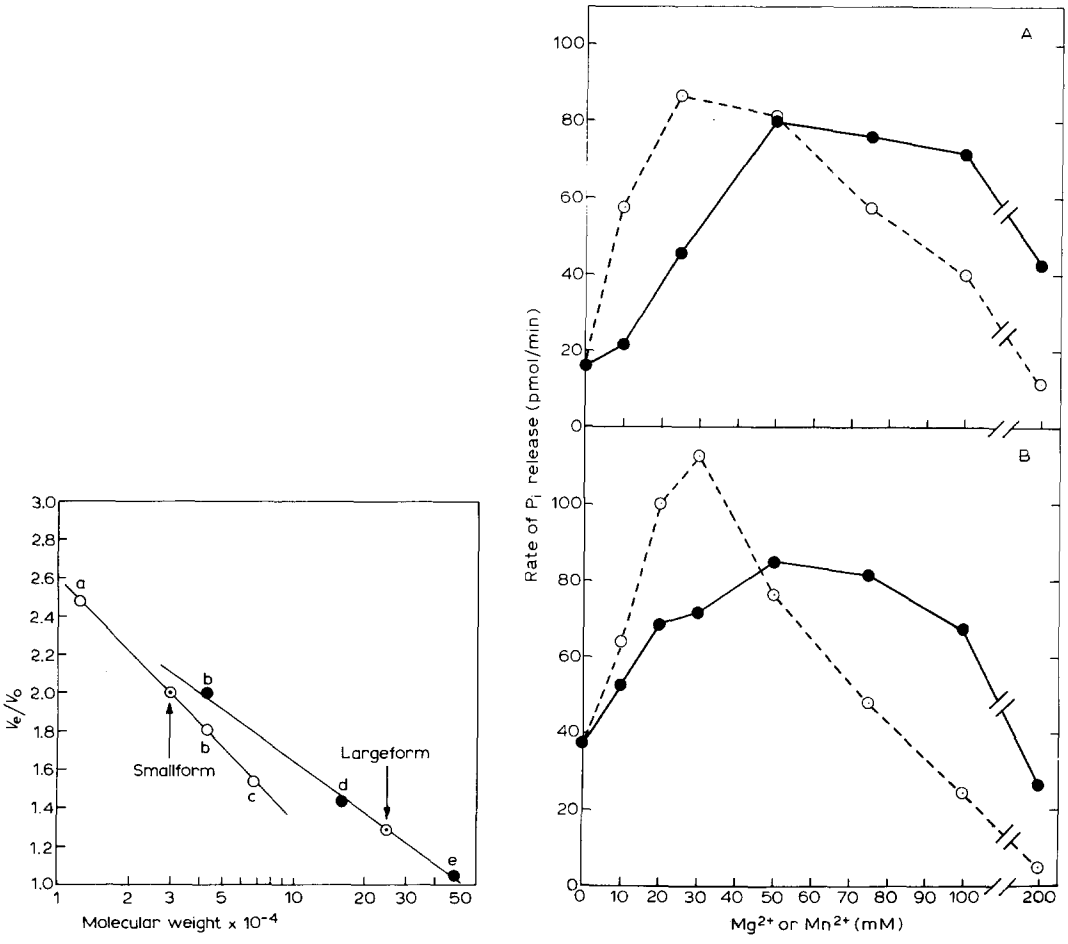

Fig. 2. Molecular weight estimation of two forms of phosphoprotein phosphatase by gel filtration. This was done on columns of Sephadex G-200 (—●—) and of Sephadex G-150 (—○—) calibrated with (a) horse heart cytochrome *c* (12 400), (b) ovalbumin (43 000), (c) bovine serum albumin (68 000), (d) human γ-globulin (160 000), (e) jackbean urease (483 000) as standards. The void volume was determined with Blue Dextran.

Fig. 3. Effect of Mg^{2+} and Mn^{2+} on phosphoprotein phosphatase activity. Initial velocities of dephosphorylation of ^{32}P-labelled H2B histone by the large form (A) or the small form (B) of the enzyme were measured under standard assay conditions except $(CH_3COO)_2Mg$ (—●—) or $MnCl_2$ (- - ○ - -) at an indicated concentration was included.

Substrate specificity.

The phosphoprotein phosphatase was also active with phosphorylase *a* and ^{32}P-labelled H1 histone as shown in Table II. Upon Sephadex G-150 gel filtration after ethanol treatment, elution profiles of the phosphatase measured with phosphorylase *a* and ^{32}P-labelled H1 histone as substrates coincided with that of ^{32}P-labelled H2B histone phosphatase (Fig. 1B). When the phosphatase was measured with phosphorylase *a* as substrate, a 7-fold activation occurred after the ethanol treatment and gel-filtration (Table II) as described by Brandt et al. [15] for rat liver phosphorylase phosphatase. On the other hand, the total

activity for dephosphorylation of ^{32}P-labelled histones was reduced to 50% with ^{32}P-labelled H2B histone and 25% with ^{32}P-labelled H1 histone by the ethanol treatment (Table II). No significant amounts of alkali-labile phosphate (1 mM) from casein or phosvitin were hydrolyzed by either the large or small forms of the enzyme (1 unit/ml) under standard assay conditions. Other low molecular weight phosphate esters (1 mM) such as L-phosphoserine, L-phosphothreonine, *p*-nitrophenylphosphate, glucose 6-phosphate and inorganic pyrophosphate did not serve as substrates for the two forms of the enzyme under the same conditions. Alkaline or acid phosphatase activity was not detected in these enzyme preparations when assayed by the method of Nose et al. [21].

Fig. 5 indicates the time course of [^{32}P]orthophosphate release from ^{32}P-labelled H2B histone by either the large or small forms of the enzyme. Although the large form removed most of the [^{32}P]orthophosphate rather quickly, the small form removed only 50—60% of [^{32}P]orthophosphate at a fast rate and hydrolyzed additional phosphate very slowly. These results suggested that the two forms of the enzyme reacted differently with the two phosphorylated sites (Ser-32 and Ser-36) on H2B histone. This was proven by investigation of the relative rates of dephosphorylation of each of the two sites on ^{32}P-labelled H2B histone (Table III). The large form of the enzyme removed [^{32}P]orthophosphate from Ser-36 six times faster than from Ser-32. With the small form of the enzyme, however, the rate of [^{32}P]orthophosphate release from Ser-36 was 15 times faster than that from Ser-32.

The dephosphorylation of Ser-36 phosphate by the two forms of the phosphoprotein phosphatase was also observed when purified tryptic phosphopeptides containing Ser-36 derived from ^{32}P-labelled H2B histone were used as substrates. As shown in Table IV, two ^{32}P-labelled peptides containing Ser-36, Lys-Glu-Ser(*P*)-Tyr-Ser-Val-Tyr-Val-Tyr-Lys and Lys-Glu-Ser(*P*)-Tyr-Ser-Val-Tyr, served as substrates for both the large and small forms of the enzyme. Removal of the COOH-terminal tripeptide, Ser-Val-Tyr, from Lys-Glu-Ser(*P*)-Tyr-Ser-Val-Tyr resulted in significant reduction of the rate of dephosphorylation by either the large or small forms of the enzyme. On the other hand, a tryptic phosphopeptide containing Ser-32, Lys-Arg-Ser(*P*)-Arg, was dephosphorylated at a rate only a few percent of the rate obtained with ^{32}P-labelled H2B

TABLE II

SUBSTRATE SPECIFICITY OF PHOSPHOPROTEIN PHOSPHATASE BEFORE AND AFTER ETHANOL TREATMENT FOLLOWED BY SEPHADEX G-150 GEL-FILTRATION

Ethanol treatment followed by gel-filtration was performed as described in the text and the legend to Fig. 1. Phosphoprotein phosphatase activities were determined as described under Materials and Methods. One unit of ^{32}P-labelled H1 histone phosphatase activity was defined as the amount of enzyme which catalyzed the release of 1 nmol of P_i from ^{32}P-labelled H1 histone containing 50 μM alkali-labile phosphate under standard assay conditions.

Substrate	Before treatment (large form) (total activity, units)	After treatment (small form) (total activity, units)	Recovery (%)
^{32}P-labelled H2B histone	1167	622	53
^{32}P-labelled H1 histone	83.9	20.1	24
Phosphorylase *a*	7.1	47.1	663

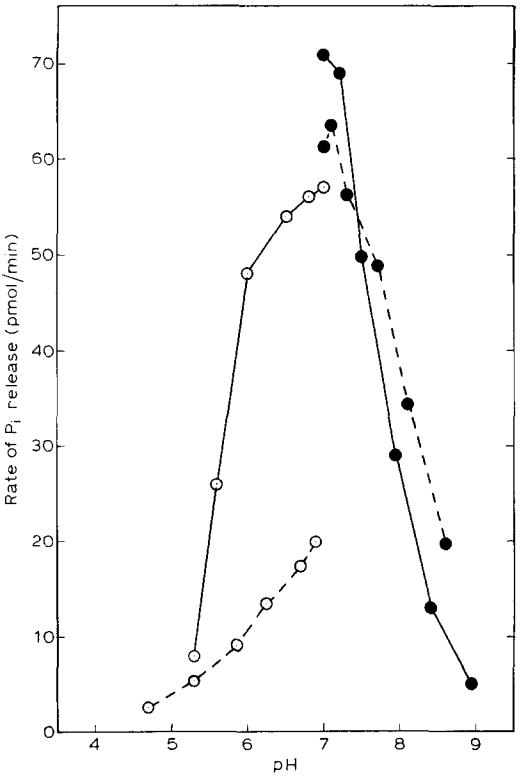

Fig. 4. Effect of pH on phosphoprotein phosphatase activity. Initial velocities of dephosphorylation of ^{32}P-labelled H2B histone by the large form (———) or the small form (- - - - - -) of the enzyme were measured under standard assay conditions except that 50 mM Tris/maleate buffer (○) or Tris · HCl buffer (●) at an indicated pH value was used.

TABLE III

RATES OF [^{32}P]ORTHOPHOSPHATE RELEASE FROM Ser-32 AND Ser-36 OF ^{32}P-LABELLED H2B HISTONE BY TWO FORMS OF PHOSPHOPROTEIN PHOSPHATASE

Incubations were performed under the same conditions as described in the legend to Fig. 5. The radioactive histone was isolated from the reaction mixture and digested with trypsin as described previously [16]. The amount of [^{32}P]orthophosphate at Ser-32 and Ser-36 was analyzed by autoradiograms as described under Materials and Methods. The mean ± S.D. of data of experiments with three separate analyses are given.

Enzyme form	Incubation time (min)	$^{32}P_i$ remaining (nmol)		Rate * of $^{32}P_i$ release (nmol/min)	
		at Ser-32	at Ser-36	from Ser-32	from Ser-36
Large	0	5.25 ± 0.07	6.00 ± 0.07	—	—
Large	1	4.57 ± 0.10	1.84 ± 0.10	0.68	4.16
Large	4	2.93 ± 0.00	0.56 ± 0.00	—	—
Small	0	5.25 ± 0.07	6.00 ± 0.07	—	—
Small	3	5.03 ± 0.08	2.51 ± 0.08	0.07	1.16
Small	10	4.36 ± 0.00	0.54 ± 0.00	0.09	—

* Rate was calculated from the amount of [^{32}P]orthophosphate release during the indicated incubation time.

histone by the two forms of the enzyme. No dephosphorylation of Ser(*P*)-Arg was detected.

Kinetic studies

The effects of increasing concentration of ^{32}P-labelled H2B histone and a radioactive tryptic phosphopeptide, Lys-Glu-Ser(*P*)-Tyr-Ser-Val-Tyr, on the reaction velocity of the two forms of the phosphoprotein phosphatase were studied. The apparent K_m and V values determined under standard assay conditions are summarized in Table V. The apparent K_m values for ^{32}P-labelled H2B histone of the large and small forms of the enzyme were 23.7 μM and 81.4 μM, respectively. The apparent K_m values for the tryptic phosphopeptide were 187.1 μM with the large form and 90.0 μM with the small form of the enzyme. Since the [^{32}P]orthophosphate released from ^{32}P-labelled H2B histone would be coming from two phosphorylated sites (Ser-32 and Ser-36) at different rate, kinetic constants of the dephosphorylation of Ser-36 phosphate in ^{32}P-labelled H2B histone was calculated (values in parentheses in Table V) for comparison with those for the tryptic phosphopeptide containing Ser-36 phosphate. The estimated K_m values for Ser-36 phosphate in ^{32}P-labelled H2B histone of the large and small forms of the enzyme were 13.3 μM and 45.7 μM, respectively.

The V/K_m ratio was calculated for ^{32}P-labelled H2B histone and the radioactive tryptic phosphopeptide to provide an arbitrary yardstick for quantitating the "specificity" of the two forms of the protein phosphatase towards the substrates in a manner analogous to that used in measuring the specificity of proteases towards synthetic substrates [23]. As shown in Table V, the V/K_m

TABLE IV

RELATIVE RATES OF PHOSPHOPROTEIN PHOSPHATASE ACTIVITY ON PHOSPHOPEPTIDES DERIVED FROM ^{32}P-LABELLED H2B HISTONE

Incubations were performed under standard assay conditions as described under Materials and Methods with a protein phosphatase concentration of 1.2 units/ml. 50 μM of each phosphopeptide were used. Released [^{32}P]orthophosphate was measured as described under Materials and Methods. The rate of dephosphorylation of each phosphorylated site (Ser-32 and Ser-36) in ^{32}P-labelled H2B histone was estimated from the data presented in Table III. The rate with ^{32}P-labelled H2B histone (50 μM) was taken as 100.

Substrate	Rate	
	Large form	Small form
^{32}P-labelled H2B histone ([^{32}P]Ser-36 and [^{32}P]Ser-32)	100	100
^{32}P-labelled H2B histone ([^{32}P]Ser-36)	86	94
^{32}P-labelled H2B histone ([^{32}P]Ser-32)	14	6
Lys-Glu-[^{32}P]Ser-Tyr-Ser-Val-Tyr-Val-Tyr-Lys (36)	81	21
Lys-Glu-[^{32}P]Ser-Tyr-Ser-Val-Tyr (36)	70	33
Lys-Glu-[^{32}P]Ser-Tyr (32)	4	2
Lys-Arg-[^{32}P]Ser-Arg (32)	3	2
[^{32}P]Ser-Arg	0	0

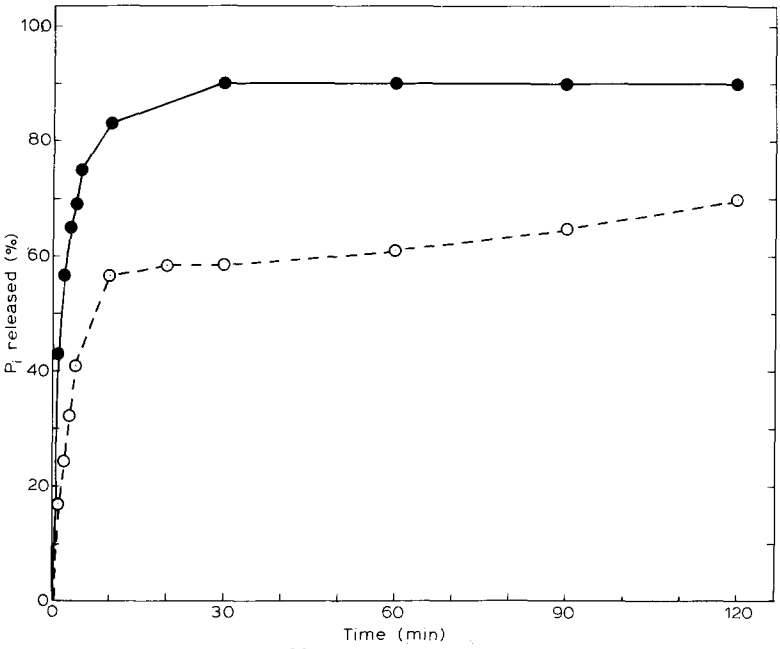

Fig. 5. Dephosphorylation of ^{32}P-labelled H2B histone by two forms of phosphoprotein phosphatase. The reactions were carried out under standard assay conditions except that ^{32}P-labelled H2B histone containing 11.25 nmol alkali-labile phosphate was incubated with either 3.4 units of the large form of the enzyme (—●—) in a reaction volume of 240 μl or 2.8 units of the small form (- - ○ - -) in a reaction volume of 480 μl. Released [^{32}P]orthophosphate was measured as described under Materials and Methods.

TABLE V

KINETIC CONSTANTS FOR ^{32}P-LABELLED H2B HISTONE AND A TRYPTIC ^{32}P-LABELLED PEPTIDE

Phosphopeptide dephosphorylation was measured under standard assay conditions as described under Materials and Methods with 1.4 units/ml of either the large or small forms of phosphoprotein phosphatase. Kinetic constants were estimated by fitting the data to the Michaelis-Menten equation using the method of least squares [22]. Values are averages (with the range) of values for two separate experiments. Values in parentheses are kinetic constants for Ser-36 phosphate in ^{32}P-labelled H2B histone. The rate of dephosphorylation of Ser-36 phosphate in ^{32}P-labelled H2B histone was estimated from the data presented in Table III.

Substrate	Enzyme form	K_m (μM)	V (pmol/min per unit)	Ratio V/K_m
^{32}P-labelled H2B histone	Large	23.7 ± 2.1 *	1410 ± 82	59
		(13.3 ± 1.4 **)	(1263 ± 41)	
^{32}P-labelled peptide ***	Large	187.1 ± 5.9	1636 ± 49	9
^{32}P-labelled H2B histone	Small	81.4 ± 0.8 *	2604 ± 63	32
		(45.7 ± 0.5 **)	(2476 ± 52)	
^{32}P-labelled peptide ***	Small	90.0 ± 0.5	952 ± 70	11

* Values are expressed as concentrations of total [^{32}P]orthophosphate in ^{32}P-labelled H2B histone.

** Values are expressed as concentrations of [^{32}P]orthophosphate at Ser-36 in ^{32}P-labelled H2B histone.

*** Lys-Glu-Ser(^{32}P)-Tyr-Ser-Val-Tyr

ratios for the tryptic phosphopeptide were 15% and 34% of that of ^{32}P-labelled H2B histone with the large and small forms of the enzyme, respectively.

Discussion

The molecular weights, 250 000 and 30 000, of the two forms of pig heart phosphoprotein phosphatase described here do not coincide with the molecular weights, 150 000 and 70 000, of the bovine heart enzyme partially purified by Nakai and Thomas [5] and Thomas et al. [14]. The difference in molecular size of the enzyme may be attributed to partial dissociation of the large form of the enzyme to smaller forms of intermediate molecular weight by several purification procedures such as 30% ethanol precipitation. Lee et al. [24] recently reported evidence that multiple forms of the phosphoprotein phosphatase in rat liver extracts are artifacts, generated by partial proteolysis of a single high molecular weight (approx. 220 000) form during liver homogenization.

Using phosphorylase *a* as substrate, the conversion of the large form (mol. wt. 250 000) of the pig heart phosphoprotein phosphatase to a smaller form of mol. wt. 30 000 greatly activated phosphorylase phosphatase activity as previously described [15] with enzymes from other sources (Table II). However, when ^{32}P-labelled H2B and H1 histones were used as substrates, the conversion resulted in the inactivation of the enzyme. It is not known at this time whether the large form of the phosphoprotein phosphatase is really one enzyme, but there is no indication that phosphorylase *a* and the phosphohistones are dephosphorylated by different enzyme species. Tan and Nuttal [25] and Kikuchi et al. [26] reported that ethanol treatment of rat liver extract produced a large activation of phosphorylase phosphatase activity with no corresponding increase in synthetase activity.

The two forms of pig heart phosphoprotein phosphatase described in this paper removed the phosphate preferentially from Ser-36 in H2B histone (Table III). Antoniw et al. [9] resolved the rabbit skeletal muscle phosphoprotein phosphatase into four forms with molecular weights of 300 000, 170 000, 75 000 and 46 000 by gel filtration on Sephadex G-200 after several purification steps including 30% ethanol precipitation. They have stated that these four forms of the phosphoprotein phosphatase dephosphorylated the two phosphorylation sites on H2B histone (Ser-32 and Ser-36) at the same rate. They however did not present experimental evidence in support of the statement and the reasons for the discrepancies with our findings are unclear.

The apparent K_m value for ^{32}P-labelled H2B histone of the small form of the enzyme was more than 3 times that of the large form (Table V) indicating that "uncommon parts" of the large form, which were removed (or changed) by ethanol treatment, increased the affinity of the enzyme for the substrate. With the large form, the tryptic phosphopeptide containing Ser-36, Lys-Glu-Ser(*P*)-Tyr-Ser-Val-Tyr exhibited a K_m value 14 times that for Ser-36 phosphate in ^{32}P-labelled H2B histone. With the small form, however, K_m for the tryptic phosphopeptide was only twice that for Ser-36 phosphate in ^{32}P-labelled H2B histone. These results indicate that a peptide sequence in ^{32}P-labelled H2B histone other than Lys-Glu-Ser(*P*)-Tyr-Ser-Val-Tyr interacted with the "uncommon parts" of the large form to increase the affinity of the enzyme for the substrate.

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