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MULTIPLE MOLECULAR FORMS OF PHOSPHOPROTEIN PHOSPHATASE

III. PHOSPHORYLASE PHOSPHATASE AND PHOSPHOHISTONE PHOSPHATASE OF RABBIT LIVER

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Summary

1. Phosphoprotein phosphatase (phosphoprotein phosphohydrolase EC 3.1.3.16) in the soluble fraction of rabbit liver which catalyzes the dephosphorylation of muscle phosphorylase *a* and phosphohistone (*P*-histone) was resolved into three active fractions by NaCl gradient elution from a DEAE-cellulose column (Fraction I, II and III in order of elution). They have different relative reaction rates for the two substrates and different degrees of stimulation by Mn^{2+}. Apparent K_m values of Fraction I, II and III were 15, 20 and 16 μM for phosphorylase *a*, and 6.9, 5.3 and 4.4 μM for *P*-histone, respectively (with Mn^{2+} in the assay mixture).

2. On sucrose density gradient centrifugation Fraction I and II were revealed to contain a major peak (7.0 S and 7.8 S, respectively) and a minor peak (4.0 S) of activity, while Fraction III contained only one peak (5.8 S). Freezing and thawing in the presence of 0.2 M mercaptoethanol dissociated all three fractions into subunits of similar molecular size (3.4 S), with concomitant enhancement of phosphorylase phosphatase activity. The K_m values all became essentially the same (20 μM for phosphorylase *a* and 16 μM for *P*-histone).

3. The phosphorylase phosphatase and *P*-histone phosphatase activities could not be separated with any of the procedures described. Competition between the two phosphoprotein substrates was observed with some of the fractions.

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Introduction

Recent reports [1—4] suggest that phosphoprotein phosphatase from rabbit skeletal muscle or bovine heart muscle may have a broad substrate specificity catalyzing the dephosphorylation of glycogen synthetase D, phosphohistone (*P*-histone), phosphorylase *a* and the phospho-form of phosphorylase *b* kinase. Separation of *P*-histone phosphatase and/or *P*-protamine phosphatase into more than one component by DEAE-cellulose chromatography has, however, been reported in rat liver [5] and brain [6].

In our preliminary studies (Kobayashi, M. and Bishop, J.S., unpublished)* into the relationship between the multiple forms and the substrate specificity of the phosphatase, phosphorylase phosphatase and *P*-histone phosphatase activities in the soluble fraction of rat liver were resolved by DEAE-cellulose chromatography into three fractions with different degrees of stimulation by Mn^{2+}. Both phosphatase activities were co-eluted on gel filtration of each fraction, showing the existence of components with different molecular size, but the same activity.

The present paper extends these observations of the three forms of liver phosphoprotein phosphatase, which may possibly have a common catalytic subunit. Multiple forms of rabbit skeletal muscle phosphatase have been reported [3,7].

Methods

Preparation of substrates

Rabbit muscle phosphorylase *a* labeled with ^{32}P was prepared by the method described previously [3].

P-histone, unlabeled and labeled with $^{32}P_i$ was prepared by the method of Meisler and Langan [5] modified by Kato and Bishop [1].

Assay of phosphatase activities

P-histone phosphatase activity was measured by the method of Kato and Bishop [1] except that 0.1 M NaCl was included in the reaction mixture as an activating factor.

Phosphorylase phosphatase activity was measured by the method described previously [3] except that 2.5 mM caffeine was used instead of 2.5 mM theophylline as an activating factor. When indicated, 5 mM $MnCl_2$ was included in the reaction mixture. The concentration of the substrates was calculated on the basis of alkali-labile [^{32}P] phosphate content.

One unit of activity of phosphorylase phosphatase and *P*-histone phosphatase was defined as the amount catalyzing the hydrolysis of 1 nmole of [^{32}P] orthophosphate from the respective substrates per min at 30°C.

The reaction rate was proportional to enzyme concentration in the standard 4-min incubation except in the case of crude extracts before $(NH_4)_2SO_4$

* Part of these preliminary results were reported as an poster presentation at the 9th Int. Congr. Biochem. (1973), Stockholm.

fractionation, in which poor proportionality was observed for phosphorylase phosphatase. Under other conditions, the time course was linear up to at least 12 min.

Preparation of the phosphatase

Phosphoprotein phosphatase of the soluble fraction of rabbit liver was prepared by essentially the same procedure as that of Meisler and Langan [5] except that EDTA was included at concentrations of 5 mM in the homogenizing solution and 1 mM in the buffer employed after $(NH_4)_2SO_4$ fractionation. 10 mM mercaptoethanol was used instead of 1 mM dithiothreitol in the buffer except in that used for the dialysis of the concentrated phosphatase fractions to be stored, and the step of calcium phosphate gel treatment was omitted because of a great loss in yield at that step.

After resolution of the three phosphatase fractions by DEAE-cellulose column chromatography the most active fractions of each peak were pooled (Fig. 1), and the phosphatase fractions were collected by $(NH_4)_2SO_4$ precipitation (55% satn), dissolved in small volume of 20 mM Tris · HCl buffer (pH 7.5) containing 1 mM EDTA and 1 mM dithiothreitol, dialyzed against the same buffer, and stored at −20° C.

The results of a representative purification are summarized in Table I.

Other methods

Sucrose density gradient centrifugation [8] was carried out as described by Kato and Sato [7]. NaCl concentration was determined by measuring the sodium amount flamephotometrically. Protein concentration was determined by Folin-Lowry method [9].

Results

Resolution of three active fractions of the phosphatase

Chromatography of $(NH_4)_2SO_4$ fraction (30—50% satn) on a DEAE-cellulose column resolved phosphorylase phosphatase and *P*-histone phosphatase into three active fractions, Fraction I, II, and III eluted at NaCl concentration of 0.10—0.13 M, 0.15—0.22 M and 0.23—0.30 M, respectively; the peaks of both phosphatase activities were detected in the same fractions (Fig. 1).

When the activities assayed in the presence and absence of 5 mM $MnCl_2$ were compared, both phosphatase activities of Fraction III were regularly stimulated by the presence of Mn^{2+}. The degree of stimulation by Mn^{2+} of Fraction II varied from preparation to preparation; Fraction I was least affected, sometimes slightly inhibited, by this ion. The relative reaction rate of phosphorylase phosphatase compared with *P*-histone phosphatase was greatest in Fraction III and least in Fraction II. On rechromatography of each of the three fractions on smaller columns, after concentration by $(NH_4)_2SO_4$ precipitation and dialysis, the activity peak of each fraction was detected at the same range of NaCl concentration as the origin.

When a crude extract (78 000 × *g* supernatant fraction) was chromatographed in a similar manner, essentially the same results were obtained as

TABLE I

PURIFICATION OF PHOSPHORYLASE PHOSPHATASE AND *P*-HISTONE PHOSPHATASE FROM RABBIT LIVER

The purification procedure is described under Methods. The starting material was 76 g rabbit liver. The data for the $(NH_4)_2SO_4$ fraction and the DEAE-cellulose fractions were normalized on the basis of the starting scale, though a portion of 78 000 × *g* supernatant fraction corresponding to 65 g liver was used for $(NH_4)_2SO_4$ fractionation and processed further. DEAE-cellulose fractions imply the preparations concentrated from the pools of peak fractions indicated in Fig. 1 and dialyzed. Only the activities measured in the presence of 5 mM $MnCl_2$ are shown.

Fraction	Total protein (mg)	Phosphorylase phosphatase			*P*-histone phosphatase		
		Total activity (units)	Spec. act. (units/mg protein)	Recovery (%)	Total activity (units)	Spec. act. (units/mg protein)	Recovery (%)
13 000 × g supernatant	5384	524	0.097	100	1300	0.242	100
78 000 × g supernatant	4484	662	0.148	126	1099	0.245	85
$(NH_4)_2SO_4$	1517	2963	1.95	565	932	0.614	72
DEAE-cellulose							
Fraction I	31.1	71	2.27	13.5	41	1.33	3.2
Fraction II	76.4	260	3.40	49.6	212	2.77	16.3
Fraction III	38.7	582	15.0	111	60	1.56	4.6

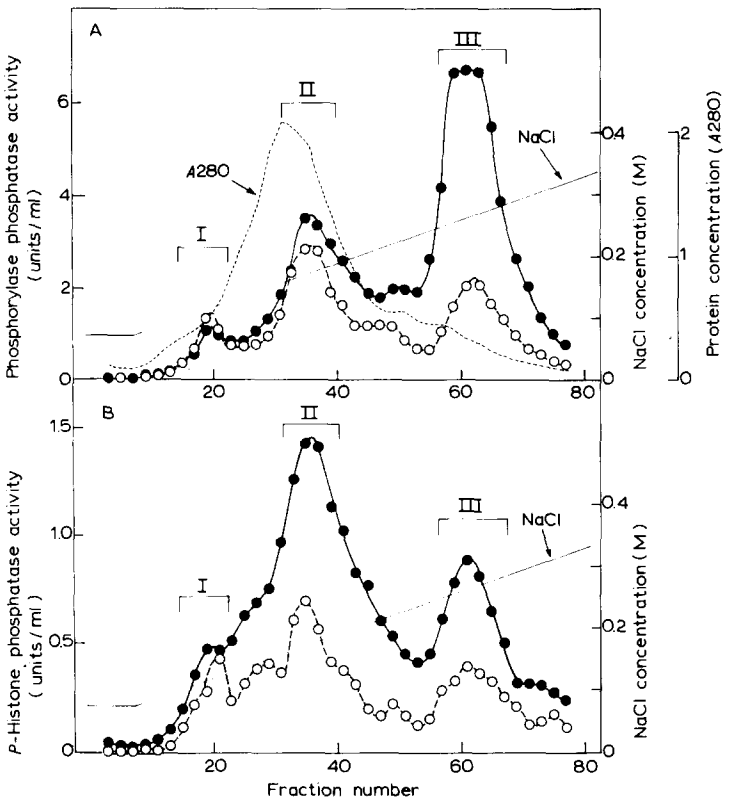

Fig. 1. DEAE-cellulose chromatography of phosphoprotein phosphatase ($(NH_4)_2SO_4$ fraction). $(NH_4)_2SO_4$ fraction (30—50% satn) from 78 000 × *g* supernatant fraction corresponding to 65 g liver, after dialysis, was put on a DEAE-cellulose (Selectacel, Type 20) column (2.3 cm × 14 cm) equilibrated with 20 mM Tris · HCl buffer (pH 7.5) containing 1 mM EDTA and 10 mM mercaptoethanol. After washing the column successively with 150 ml of the same buffer and the buffer containing 0.07 M NaCl, elution was performed with 800 ml of a linear gradient of NaCl (0.07—0.35 M) in the above buffer. Fractions of 10 ml were collected and the activity of phosphorylase phosphatase (A) and *P*-histone phosphatase (B) was measured in the presence and absence of 5 mM $MnCl_2$. The basic reaction mixture for phosphorylase phosphatase contained: 50 mM imidazole · HCl buffer (pH 7.4), 1.7 mM dithiothreitol, 0.07% bovine serum albumin, 2.5 mM caffeine and 31 μM ($4.8 \cdot 10^5$ cpm/nmole) of [^{32}P]phosphorylase *a*; that for *P*-histone phosphatase contained: 50 mM imidazole · HCl buffer (pH 7.4) 1.7 mM dithiothreitol, 0.07% bovine serum albumin, 0.1 M NaCl and 21 μM ($1.3 \cdot 10^5$ cpm/nmole) of [^{32}P]*P*-histone. Incubation was carried out in a final volume of 60 μl for 4 min at 30°C. 10- and 30-μl aliquots of the fractions were used for the assay of phosphorylase phosphatase and *P*-histone phosphatase, respectively. Peak fractions as indicated were pooled and the enzyme fractions were collected and stored as described under Methods. ●———●, plus Mn^{2+} activity; ○- - - - -○, minus Mn^{2+} activity.

described above (Fig. 2), suggesting that these three forms are not artifacts formed during the purification procedure, but may exist in vivo.

Kinetic results

Apparent K_m values for phosphorylase *a* and *P*-histone of the three fractions are summarized in Table IIa. The K_m values for phosphorylase *a* were larger than those observed with muscle phosphatase preparations [3]; that may be due to the heterologousness of tissues from which the enzyme and substrate were derived. Inclusion of manganese in the reaction mixture resulted in some

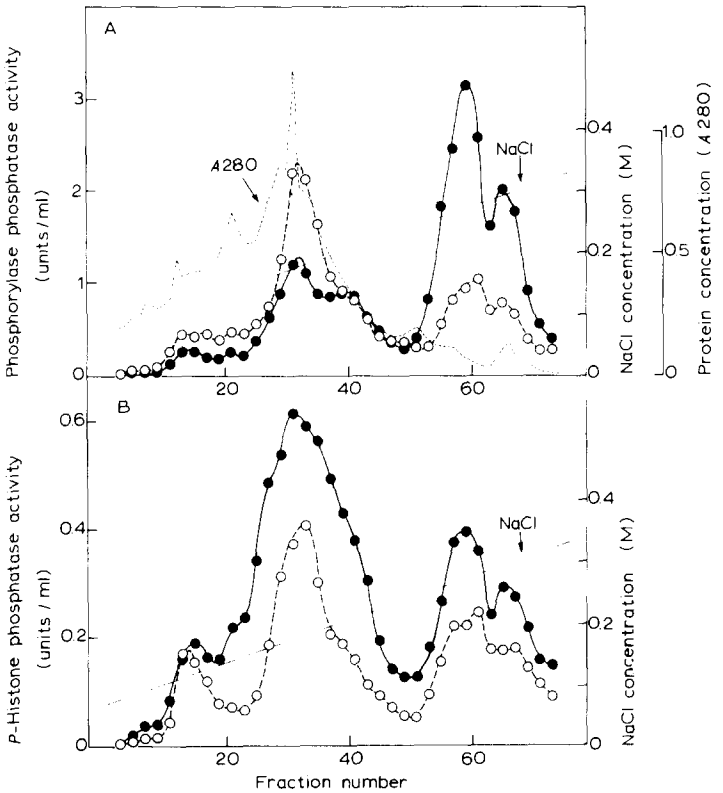

Fig. 2. DEAE-cellulose chromatography of phosphoprotein phosphatase (crude extract). 78 000 × *g* supernatant of liver homogenate corresponding to 7.5 g liver was chromatographed on a DEAE-cellulose column (1.6 cm × 9 cm) in a similar manner as in Fig. 1. Elution was performed with 400 ml linear gradient of NaCl (0.07—0.35 M). 5-ml fractions were collected and activity of phosphorylase phosphatase (A) and *P*-histone phosphatase (B) were measured as described under Fig. 1. ●———●, plus Mn^{2+} activity; ○- - - - -○, minus Mn^{2+} activity.

decrease of K_m values for phosphorylase *a* (Fraction I and III) and increase for *P*-histone (all fractions). Inhibition of *P*-histone phosphatase reaction at higher concentrations of the substrate was observed in some preparations of Fraction III, similar to the results with the "larger form" of muscle phosphatase [7], as shown in the foot note to Table II.

Optimal reaction rates of both phosphatase activities of all the three fractions were observed at pH values near neutral (pH 6.6—7.8); the presence of Mn^{2+} resulted in some broadening of the optimal pH values of phosphorylase phosphatase to the acidic side (data not shown).

Sucrose density gradient centrifugation studies

Fig. 3 shows the typical sedimentation profiles of phosphorylase phosphatase and *P*-histone phosphatase activities of the three fractions in 5—25% linear gradients of sucrose. Fraction I was revealed to contain a major peak of 7.0 S and a minor peak of 4.0 S; similarly Fraction II contained a 7.8-S major peak and 4.0-S minor peak of both phosphatase activities. Fraction III showed only one activity peak of 5.8 S. In every case the two phosphatase activities

TABLE II

COMPARISON OF K_m VALUES FOR PHOSPHORYLASE *a* AND *P*-HISTONE

Fraction I, II and III were treated with mercaptoethanol as described under Fig. 4. Phosphorylase phosphatase activity and *P*-histone phosphatase activity of untreated fractions (a) and mercaptoethanol-treated fractions (b) were measured after appropriate dilution in the presence and absence of 5 mM $MnCl_2$ in the reaction mixtures described under Fig. 1. Eight different concentrations of phosphorylase *a* (3.2—37 μM) and *P*-histone (2.1—25 μM) were used, and K_m values were calculated from the double reciprocal plots.

Fraction	K_m for phosphorylase *a* (μM)		K_m for *P*-histone (μM)	
	Plus Mn^{2+}	Minus Mn^{2+}	Plus Mn^{2+}	Minus Mn^{2+}
(a) Untreated fractions				
Fraction I	15	25	6.9	3.6
Fraction II	20	20	5.3	0.93
Fraction III	16	33	4.4*	3.6*
(b) Mercaptoetahanol-treated fractions				
Fraction I	20	20	16	8.0
Fraction II	20	20	16	8.0
Fraction III	20	20	16	10

* At concentrations over 10 μM reaction rate decreased to 80% (plus Mn^{2+}) and 70% (minus Mn^{2+}) of the maximum.

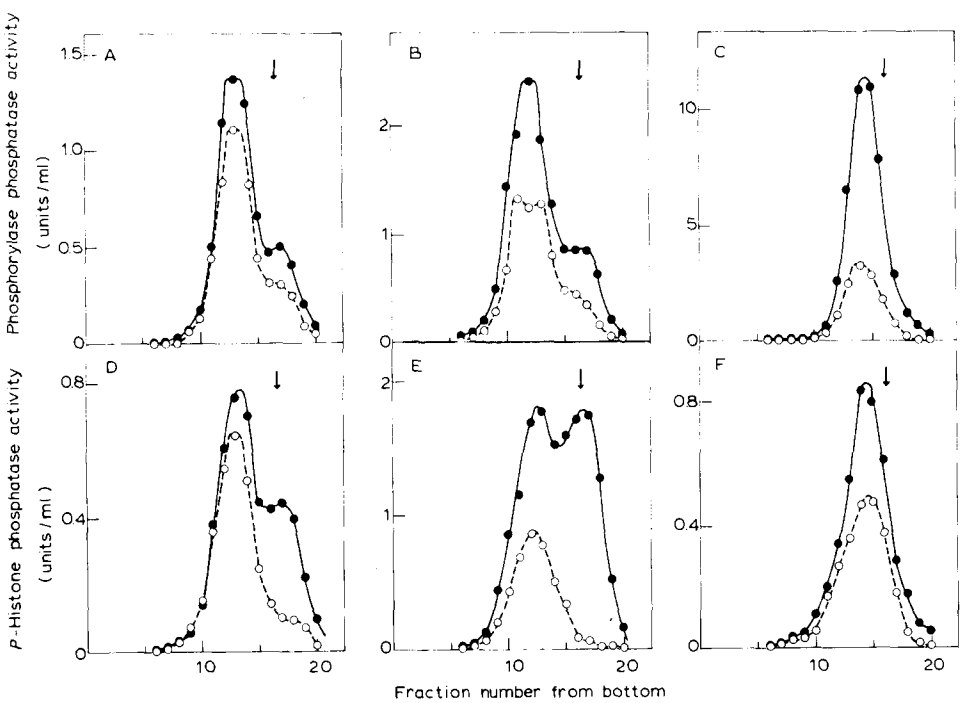

Fig. 3. Sedimentation profiles of phosphorylase phosphatase activity and *P*-histone phosphatase activity of phosphoprotein phosphatase fractions in a sucrose density gradient. 150-μl aliquots of Fraction I, II and III (protein content: 0.97, 1.3 and 0.73 mg, respectively) were layered over a 4.2-ml linear gradient of sucrose (5—25%) and centrifuged. Phosphorylase phosphatase activity (A, B and C showing that of Fraction I, II and III, respectively) and *P*-histone phosphatase activity (D, E and F showing that of Fraction I, II and III, respectively) were measured as described under Fig. 1 using 10-μl aliquots of the 0.2 ml fractions. ●———●, plus Mn^{2+} activity; ○- - - - -○, minus Mn^{2+} activity. Arrows indicate the position of bovine serum albumin used as a reference protein.

co-migrated, although the relative reaction rates of them and the sensitivity to Mn^{2+} differed between subfractions.

Dissociation of the phosphatase fractions

On the analogy of the observations with muscle phosphatase [3,7] Fraction I, II and III were frozen and thawed in the presence of 0.2 M mercaptoethanol. This resulted in dissociation of all the three fractions into subunits of the same molecular size of 3.4 S, and, at the same time, a marked increase (up to several-fold) of Mn^{2+}-independent activity of phosphorylase phosphatase occurred (Fig. 4) (Compare the scales at the ordinates with those in Fig. 3.). Both phosphatase activities, which were originally stimulated by Mn^{2+} to various extents, were either independent or slightly inhibited by it after dissociation. The effect of mercaptoethanol treatment on total phosphatase activities before separation by sucrose density gradient centrifugation are summarized in Table III. Increase in *P*-histone phosphatase activity was less marked, compared to phosphorylase phosphatase; the apparent increased activity of mercaptoethanol-treated Fraction III may be due to the loss of substrate inhibition described above. The degree of activation differed from preparation to preparation. After the treatment much of the proteins became insoluble and could be removed by centrifugation as reported with muscle phosphatase [7].

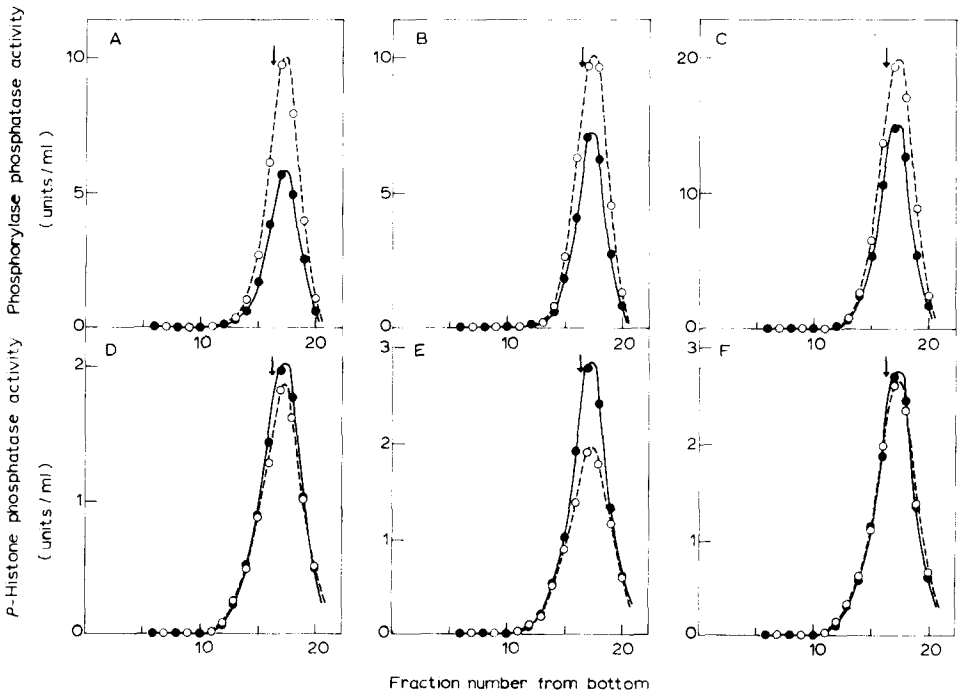

Fig. 4. Sedimentation profiles of phosphorylase phosphatase activity and *P*-histone phosphatase activity of the mercaptoethanol-treated phosphoprotein phosphatase fractions. Fraction I, II and III were frozen for 15 min at −20° C in the presence of 0.2 M mercaptoethanol. After removal of insoluble proteins formed by centrifugation, sucrose density gradient centrifugation was carried out with the clear supernatant fractions (protein contents of Fraction I, II and III: 0.11, 0.41 and 0.12 mg, respectively) and phosphatase activities were measured as described under Fig. 3. The state of dilution of each fraction is equivalent to that in Fig. 3. Panel A—F and symbols correspond to those in Fig. 3.

TABLE III

EFFECT OF MERCAPTOETHANOL TREATMENT ON THE ACTIVITY OF PHOSPHORYLASE PHOSPHATASE AND *P*-HISTONE PHOSPHATASE

Fraction I, II and III were treated with mercaptoethanol as described under Fig. 4. Phosphorylase phosphatase activity and *P*-histone phosphatase activity of the untreated fractions (a) and the mercaptoethanol-treated fractions (b) were measured in the presence and absence of 5 mM $MnCl_2$ after appropriate dilution. The state of dilution of each fraction is equivalent between (a) and (b). Phosphatase activities are expressed in terms of units/ml of the diluted fractions, while the values of protein concentration indicate those of the original solution before dilution.

Fraction	Protein (mg/ml)	Phosphorylase phosphatase activity (units/ml)		*P*-histone phosphatase activity (units/ml)	
		Plus Mn^{2+}	Minus Mn^{2+}	Plus Mn^{2+}	Minus Mn^{2+}
(a) Untreated fractions					
Fraction I	8.64	1.74	1.21	1.09	0.56
Fraction II	11.9	3.58	1.21	3.10	0.94
Fraction III	6.45	9.42	2.33	1.08	0.70
(b) Mercaptoethanol-treated fractions					
Fraction I	0.97	3.22	4.76	1.02	1.03
Fraction II	3.26	4.28	6.86	1.68	1.35
Fraction III	1.02	8.23	13.05	1.44	1.71

K_m values for the two substrates, which originally showed some difference between the fractions (Table IIa), became essentially the same for all the three fractions after mercaptoethanol treatment (Table IIb). The property of substrate inhibition observed in *P*-histone phosphatase activity of Fraction III was lost and normal hyperbolic substrate-saturation curves were obtained as in other fractions after this treatment.

When DEAE-cellulose chromatography was carried out with the $(NH_4)_2SO_4$ fraction, after freezing and thawing in the presence of 0.2 M mercaptoethanol, only one major peak at an NaCl concentration of 0.13—0.21 M of the two phosphatase activities was observed (Fig. 5).

It could be concluded here that from Fraction I, II and III catalytically active subunits with very similar properties are derived.

Competitive nature of phosphorylase a and P-histone

In order to test the possibility that phosphorylase phosphatase activity and *P*-histone phosphatase activity are attributed to the same enzyme protein the effect of unlabeled *P*-histone on phosphorylase phosphatase activity of Fraction I, II and III was investigated. These experiments encountered a difficulty that precipitate formation occurred on mixing of the two substrates in the same assay tubes. This was effectively prevented, in the absence of Mn^{2+}, by inclusion of 0.1 M NaCl in the assay mixture for phosphorylase phosphatase, disregarding its inhibitory effect*. Fig. 6 shows the competitive nature of

* Phosphorylase phosphatase activity of Fraction I, II and III in the presence of 0.1 M NaCl were 74, 96 and 53% of the control, respectively; the corresponding values for the mercaptoethanol-treated fractions were 63, 67 and 59%, respectively.

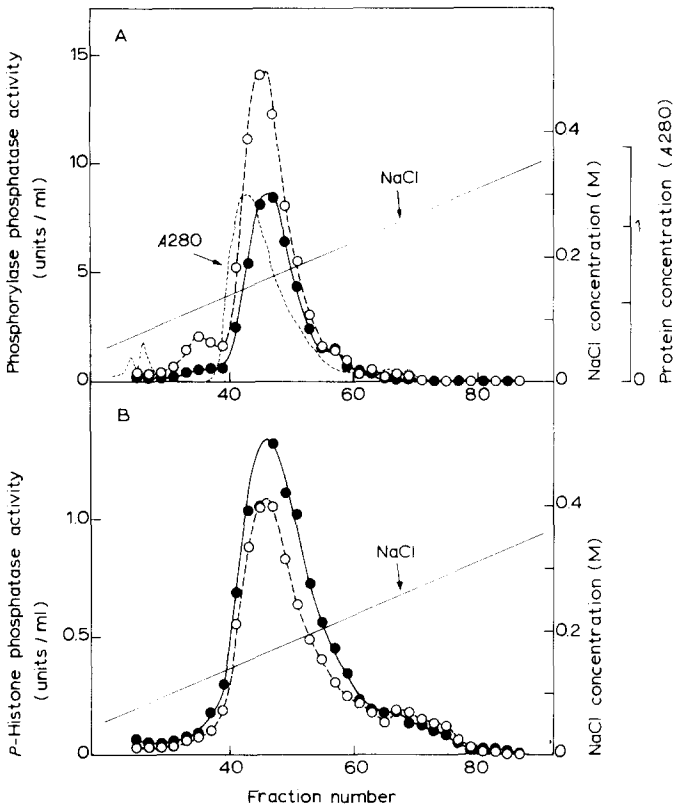

Fig. 5. DEAE-cellulose chromatography of mercaptoethanol-treated phosphoprotein phosphatase. A preparation of $(NH_4)_2SO_4$ fraction (30—50% satn) corresponding to 8.6 g liver was treated with mercaptoethanol as described under Fig. 4. Chromatography on a DEAE-cellulose column (1.6 cm × 6 cm) was carried out in a similar manner as described for Fig. 1 using 500 ml of a 0—0.4 M linear gradient of NaCl. 5-ml fractions were collected and phosphorylase phosphatase activity (A) and *P*-histone phosphatase activity (B) were measured as described under Fig. 1. The total phosphatase activities put on the column were 1344 units (plus Mn^{2+}) and 1846 units (minus Mn^{2+}) in respect of phosphorylase phosphatase and 201 units (plus Mn^{2+}) and 222 units (minus Mn^{2+}) in respect of *P*-histone phosphatase; the corresponding values of a equivalent amount of the $(NH_4)_2SO_4$ fraction before the treatment with mercaptoethanol were 325, 270, 173 and 114 units, respectively. ●———●, plus Mn^{2+} activity; ○- - - - -○, minus Mn^{2+} activity.

unlabeled *P*-histone on phosphorylase phosphatase activity of Fraction I and II. Unusual results were obtained with Fraction III and mercaptoethanol-treated fractions (not shown), the presence of *P*-histone showing some stimulatory effect. The mechanism for this latter observation is not clear, but may be a direct effect of *P*-histone on the enzyme protein. Basic proteins such as histones might interact with other proteins, as suggested by the precipitate formation with phosphorylase *a*, in addition to the simple competition for the active site of the phosphatase. Despite this unclear effect the positive results observed here, together with other results, may suggest the identity of phosphorylase phosphatase and *P*-histone phosphatase.

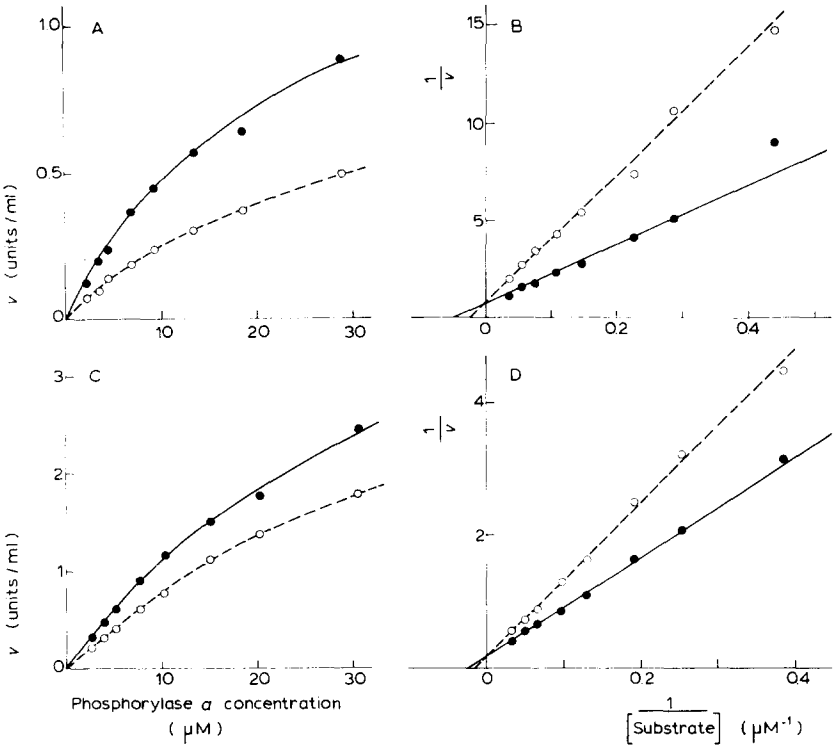

Fig. 6. Effect of *P*-histone on phosphorylase phosphatase activity of Fraction I and II. The reaction mixture (60 μl) contained 50 mM imidazole · HCl buffer (pH 7.4), 1.7 mM dithiothreitol, 0.07% bovine serum albumin, 2.5 mM caffeine, 0.1 M NaCl and 10 μl of appropriately diluted Fraction I (8.0 μg protein) (A) and Fraction II (9.2 μg protein) (C); phosphorylase *a* concentration was varied as indicated. B and D show the double-reciprocal plots of A and C, respectively. ●———●, control; ○- - - - -○, plus unlabeled *P*-histone (100 and 50 μg, corresponding to final concentrations of 62 and 31 μM, for the assay of Fraction I and II, respectively).

Discussion

Present results indicate the existence of at least three forms of phosphoprotein phosphatase with different molecular size in the soluble fraction of rabbit liver. The nature of the minor components with lower molecular size (4.0 S) in Fraction I and II is not as yet clear; they may represent a fourth form or dissociated forms, comparable to those caused by mercaptoethanol treatment, formed during the purification procedure.

The three forms are resolved by DEAE-cellulose chromatography, while Meisler and Langan [5] obtained in rat liver only two *P*-histone phosphatase peaks by essentially the same procedure. The reason for the difference between these two observations is not clear, even if the difference of the substrates used is taken into account; their two peaks seem to correspond to our Fraction II and III in respect of NaCl concentrations at which they were eluted but to Fraction I and II in respect of the molecular size*.

* Preliminary studies (Kobayashi, M. and Bishop, J.S., unpublished) with rat liver phosphatase gave essentially the same results as reported here, in which the major components of Fraction I, II and III had molecular weight of 180 000, 180 000 and 70 000, respectively, on gel filtration, while that of their Fraction A and B [5] was 185 000 and 196 000, respectively. Gross calculation of molecular weight from the sedimentation coefficient [8] of the 7.0-, 7.8-, 5.8-, 4.0- and 3.4-S component reported here gives approximate molecular weight of 139 000, 164 000, 105 000, 60 000 and 47 000, respectively.

The three forms were also observed in a crude extract of liver, suggesting their existence in vivo.

The mercaptoethanol treament released from Fraction I—III catalytically active subunits with very similar properties. At present, the nature of the subunits cleaved off the active subunits is not clear; it might also be the dissociation of identical subunits. Moreover, neither the direct evidence for the possible identity of the active subunits dissociated from the original forms nor the reconstruction of them from the dissociated subunits has yet been accomplished. It is tempting, however, to speculate a possible model, in which a common catalytic subunit is bound with different regulatory proteins that give some differential properties between the multiple forms of the phosphatase.

Divalent metal ions were reported to be competitive inhibitors of muscle phosphorylase phosphatase with respect to phosphorylase *a* [10]. In our recent observations with muscle phosphatase (unpublished) the Mn^{2+}-insensitive form was easily rendered Mn^{2+}- (or Mg^{2+}-)sensitive by preincubation with ATP, apparently through chelation of the endogenous metal(s) from the enzyme protein. With the metal-depleted enzyme Mn^{2+} increased the maximal velocity of phosphorylase phosphatase activity but decreased the affinity for phosphorylase *a*. Phosphorylase phosphatase activity of the enzyme fully activated by preincubation with Mg^{2+} and ATP [13,14] and dialyzed, which was supposed to be saturated with metal(s), however, was inhibited competitively by Mn^{2+} in accordance with the observation by Martensen et al. [10]. No change in molecular size was observed during the inactivation or activation procedures (unpublished results). Assuming the similar situation in the case of liver phosphatase, the observed differential sensitivity to Mn^{2+} does not seem to be a crucial difference in properties between the phosphatase forms; it may only reflect the proportion of apo-enzyme to holo-enzyme protein. The change in the sensitivity to Mn^{2+} by the mercaptoethanol treatment may be explained by introduction of some metal(s), probably derived from other proteins denatured during this procedure, into the apo-enzyme protein(s). Mn^{2+} may also interact with the substrate proteins, as suggested by much easier crystal formation of phosphorylase *a* in its presence than in its absence. This may cause some differential Mn^{2+}-sensitivity between phosphorylase phosphatase and *P*-histone phosphatase observed in elution or sedimentation profiles shown.

The relationship between the forms reported in this paper and those reported by Merlevede and co-workers [11,12] in dog liver has not been extensively investigated, but the inactivation and reactivation of phosphorylase phosphatase forms by preincubation with ATP and ATP plus Mg^{2+}, respectively, reported by them may also be caused by chelating off and re-introduction of metal(s) as discussed above.

The exact relationship between the multiple forms of liver phosphatase reported here and those of muscle phosphatase [3,7] has not been elucidated, either. However, some structural similarities are suggested by the similar effect of mercaptoethanol treatment.

Despite some unusual observations not clearly explained the positive results regarding the competitive nature of phosphorylase *a* and *P*-histone in phosphatase reaction reported here, together with other observations, which include co-elution from DEAE-cellulose columns, co-sedimentation in sucrose

density gradients and the same change in molecular weight by mercaptoethanol treatment, suggest the identity of phosphorylase phosphatase and *P*-histone phosphatase, offering an additional support to a current concept that a single phosphoprotein phosphatase catalyzes the dephosphorylation of several phosphoproteins [1—4]. The apparent difference of the ratio of phosphorylase phosphatase over *P*-histone phosphatase between the early purification steps with increase in yield only of the former (Table I) may be explained by the existence of some kind of inhibitor(s) acting on the substrate, phosphorylase *a*, in the crude preparations, as suggested by poor proportionality of the reaction rate to protein concentration described under Methods. A similar increase in yield of phosphorylase phosphatase during purification was also reported by others [13].

Acknowledgements

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