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MULTIPLE MOLECULAR FORMS OF PHOSPHOPROTEIN PHOSPHATASE FROM RABBIT SKELETAL MUSCLE

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SUMMARY

1. Phosphohistone phosphatase activity of rabbit skeletal muscle was separated into two fractions (phosphatase I and phosphatase II) by Sephadex G-150 chromatography. The molecular weights estimated using a Sepharose 6B column for phosphatase I and phosphatase II were about 300 000 and 150 000, respectively.

2. Phosphatase I was active in the absence of divalent metal ions and was not inhibited by EDTA or EGTA. However, the activity of phosphatase II was almost totally dependent on Mn^{2+} or Co^{2+} with K_m of 0.8 and 0.5 mM, respectively. Freezing of phosphatase I in the presence of 200 mM mercaptoethanol resulted in a change of the molecular size to that of phosphatase II with the disappearance of substrate (*P*-histone)-inhibition property, which was observed in phosphatase I at a concentration higher than 10 μM of phosphohistone (based on the alkali-labile ^{32}P content).

3. Glycogen synthetase-D phosphatase activity, which converts the D form of glycogen synthetase (UDPglucose:glycogen 4-α-glucosyltransferase, EC 2.4.1.11) to the I form, was also found in the two fractions. Synthetase-D phosphatase activity of phosphatase I was less dependent on Mn^{2+} than that of phosphatase II, which was essentially inactive without the metal ion.

4. Existence of the two types of phosphohistone phosphatase was shown in crude extract of rat liver, kidney, brain, heart muscle and skeletal muscle.

INTRODUCTION

The phosphoprotein phosphatase (phosphoprotein phosphohydrolase, EC 3.1.3.16) of skeletal muscle which catalyzes the conversion of the phospho-(D) form to the dephospho-(I) form of glycogen synthetase is reported to be identical to the phosphatase for phosphohistone [1] and for the phospho-(active) form of phosphorylase kinase [2]. The similar phosphatase of heart muscle is also reported to have a broader substrate specificity [3].

On the other hand, chromatography on DEAE-cellulose resolved the phosphoprotein (*P*-histone and *P*-protamine) phosphatase activity of rat cerebral cortex into three fractions [4], and recently the phosphatase of rat liver was separated into three

peaks by DEAE-cellulose chromatography, which exhibited a different metal ion requirement [5].

The present paper reports two types of the phosphoprotein phosphatase of rabbit skeletal muscle which can be separated by gel filtration or sucrose density gradient centrifugation.

MATERIALS AND METHODS

UDP-[U-^{14}C]glucose was obtained from International Chemical and Nuclear Corp. Carrier-free [^{32}P]phosphoric acid was from Japan Radioactive Isotope Assoc. Glucose-6-*P*, dithiothreitol, ATP and crystalline bovine serum albumin were obtained from Seikagaku Kogyo, Tokyo. Glyceraldehyde-3-*P* dehydrogenase, 3-*P*-glycerate kinase, cyclic AMP and 3-*P*-glyceric acid were from Boehringer. UDP-glucose and rabbit liver glycogen (Type III) were from Sigma.

[γ-^{32}P]ATP was prepared as described [1].

Phosphohistone, labelled with [^{32}P]ATP, was prepared by the previous method [1] which is essentially that of Meisler and Langan [6], using calf thymus histone obtained from Sigma (Type IIA). Glycogen synthetase-D of rabbit skeletal muscle was purified by the method of Brown and Larner [7].

Phosphatase activities for *P*-histone and glycogen synthetase-D were measured by the methods previously described [1], except that 0.1 M NaCl was included in the reaction mixture for histone phosphatase assay.

Phosphoprotein phosphatase from rabbit skeletal muscle was prepared by the previous method [1] with modifications. Manganese and EDTA were not included in the buffers used. The enzyme activity was eluted from the DEAE-cellulose column with 50 mM Tris–HCl buffer (pH 7.5), containing 50 mM mercaptoethanol and 400 mM NaCl. The activity from the column was precipitated by the addition of the equal volume of saturated solution (pH 7.0) of $(NH_4)_2SO_4$ at 4 °C. The precipitate was dissolved in 1/10–1/20 volume of 50 mM Tris–HCl buffer (pH 7.5) containing 10 mM mercaptoethanol, and dialyzed overnight against the same buffer. Because of poor recovery of the activity when Mn^{2+} is absent in the buffer during the chromatography [1], the present preparations were less pure than those obtained by the previous method. Histone phosphatase activity in these preparations (40–50 mg protein/ml), assayed with and without 5 mM Mn^{2+}, was 1.5–2 and 0.5–0.7 units (nmoles of $^{32}P_i$/min), respectively, per mg of protein. The enzyme solution was stored at −20 °C or directly applied to a Sephadex G-150 column. Most studies were done with the enzyme purified by the column.

Sucrose density gradient centrifugation [8] was performed at 4 °C at 36 000 rev./min for 16 h with a swinging bucket rotor (RPS 40) in a Hitachi 55PA ultracentrifuge. The samples (0.1–0.2 ml) were layered on 5–25% (w/v) sucrose linear gradient (4.2 ml) in 50 mM Tris–HCl buffer (pH 7.5), containing 10 mM mercaptoethanol. Bovine serum albumin was used for the calculation of sedimentation coefficients.

Protein concentration was measured by biuret [9] or by the Folin-Lowry method [10] following precipitation in 5% trichloroacetic acid with bovine serum albumin as a standard.

RESULTS

When the $(NH_4)_2SO_4$ fraction of phosphatase which was about 3-fold stimulated by Mn^{2+} was eluted from a Sephadex G-150 column, the activity was separated into approximately one part active enzyme and two parts enzyme dependent on Mn^{2+}

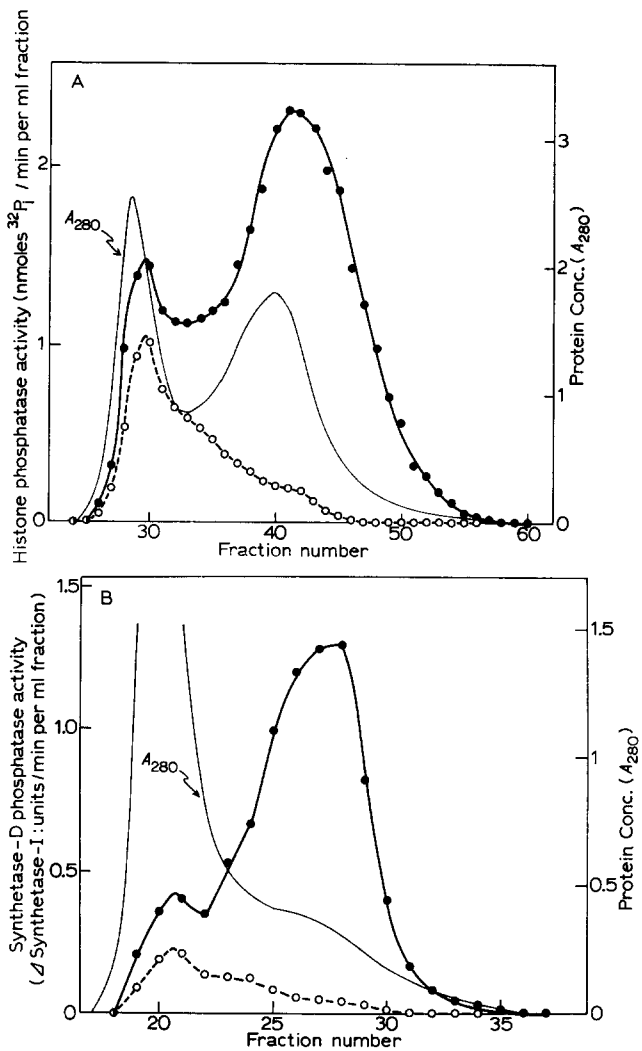

Fig. 1. Elution of two types of phosphoprotein phosphatase from Sephadex G-150 columns. (A) The $(NH_4)_2SO_4$ fraction of skeletal muscle phosphatase after DEAE-cellulose chromatography (5 ml, 200 mg protein) was applied to a Sephadex G-150 column (2.5 cm × 88 cm) and eluted with 50 mM Tris-HCl buffer (pH 7.5), containing 10 mM mercaptoethanol. Histone phosphatase activity (release of $^{32}P_i$) was assayed in 20 μM *P*-histone without (broken line) or with (unbroken line) 5 mM $MnCl_2$ using 10-μl aliquots of a 6-ml fraction collected at a flow rate of 20 ml/h. (B) The similar $(NH_4)_2SO_4$ fraction (1.5 ml, 65 mg protein) was eluted from a Sephadex G-150 column (1.9 cm × 88 cm). Glycogen synthetase-D phosphatase activity (conversion to the I form) was assayed without (broken line) or with (unbroken line) 5 mM $MnCl_2$ using 10-μl aliquots of a 5-ml fraction and is shown as the net increase of the I activity.

(Fig. 1A). The larger enzyme (phosphatase I) was active without Mn^{2+} and the smaller enzyme (phosphatase II) was almost totally dependent on the metal ion for its activity. Glycogen synthetase-D phosphatase activity was also found in the two fractions having a similar activity to histone phosphatase (Fig. 1B), and the activity in phosphatase I and phosphatase II showed similar responses with Mn^{2+} as observed in the activity for *P*-histone of these two fractions. The molecular weight obtained by a calibrated Sepharose 6B column was about 300 000 and 150 000 for phosphatase I and phosphatase II, respectively (not shown).

Density gradient centrifugation of phosphatase I and phosphatase II on 5–25% sucrose linear gradient was performed following collection of each type of phosphatase by $(NH_4)_2SO_4$ (50% satn) precipitations and dialysis. As shown in Fig. 2, phosphatase I sedimented with a $s_{20,w}$ value of 6.7 and phosphatase II, with 3.8*. In the preparation of phosphatase I (Fig. 2A) the presence of a shoulder of enzyme activity ($+Mn^{2+}$) and sedimenting in the same relation to bovine serum albumin as phosphatase II (Fig. 2B) suggests a trace contamination of phosphatase I with phosphatase II. The sedimentation profiles and the less sensitive property to Mn^{2+} of phosphatase I (Fig. 2A) were not affected by pre-incubation with 10 mM EDTA or EGTA at 20 °C for 30 min before being layered on the sucrose gradient solution which contained the same concentration of EDTA or EGTA. Pre-incubation (for 30 min at 20 °C) and centrifugation with Mn^{2+}, Co^{2+}, Ca^{2+}, Mg^{2+} (3 mM) or Zn^{2+} (1 mM) had no effect on the sedimentation coefficient of phosphatase II.

Fig. 3 shows the effect of divalent metal ions on each type of phosphatase. Phosphatase I was slightly stimulated (less then 2-fold) by Mn^{2+}, Mg^{2+} or Ca^{2+} at the concentrations indicated in Fig. 3A. On the other hand, phosphatase II, which showed very low activity without metal ions, was markedly activated by Mn^{2+} or Co^{2+} (Fig. 3B). The apparent K_m for Mn^{2+} and Co^{2+} of phosphatase II was 0.8 and 0.5 mM, respectively. Phosphatase II was slightly stimulated by Zn^{2+} but inhibited at a concentration higher than 1 mM. No effect of Mg^{2+} and Ca^{2+} was observed on phosphatase II (not shown). Phosphatase I was unaffected by Co^{2+} and was strongly inhibited by Zn^{2+} even at a concentration lower than 1 mM (Fig. 3A). Inclusion of 10 mM EDTA or EGTA in the reaction mixture did not inhibit the activity (without metal) of phosphatase I and the independence of the activity on the metal ions did not change with dialysis against the buffer containing 5 mM EDTA. When phosphatase II, which showed a small residual activity without metal ions, was exposed to Mn^{2+} or Co^{2+} (3 mM) overnight at 4 °C, more than 40% of the activity assayed with 5 mM of the metal ion was detected in its absence and in the presence of 10 mM EDTA.

The effect of substrate (*P*-histone) concentration on the activity of phosphatase I or phosphatase II is shown in Fig. 4. Histone phosphatase activity of phosphatase I was inhibited at a concentration of *P*-histone higher than 10 μM (based on the alkalilabile ^{32}P content of the labeled histone) and the inhibition was observed both in the presence and absence of 5 mM Mn^{2+} (Fig. 4A). The substrate saturation curve of phosphatase II was of the usual type with an apparent K_m of 13 μM (Fig. 4B). Mixtures of both types of phosphatase showed the additive enzyme activity.

* The reasons for the discrepancy between the values of molecular weight obtained by gel filtration and those obtained by sucrose density gradient centrifugation are unknown.

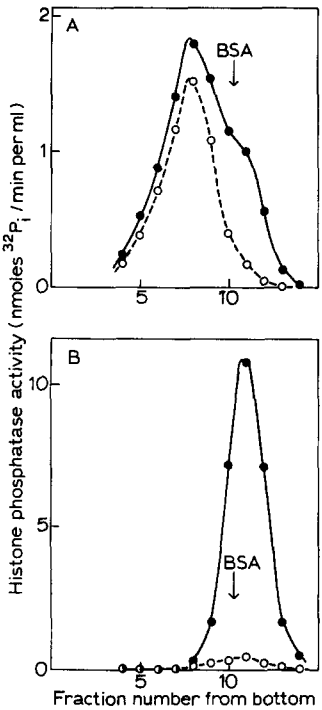

Fig. 2. Sedimentation profiles of phosphatase I and phosphatase II in a sucrose density gradient. Aliquots (0.15 ml) of phosphatase I (1.8 mg protein) (A) or phosphatase II (1.5 mg protein) (B) which were concentrated by the $(NH_4)_2SO_4$ precipitation, resuspended and dialyzed (50 mM Tris–HCl buffer, 10 mM mercaptoethanol), were centrifuged for 16 h at 36 000 rev./min at 4 °C on 5–25% sucrose linear gradient (4.2 ml) in 50 mM Tris–HCl buffer (pH 7.5) containing 10 mM mercaptoethanol. Bovine serum albumin (BSA), which was added as a reference protein, sedimented at the indicated point. Histone phosphatase activity was assayed in 10 (A) or 20 μM (B) of *P*-histone without (broken line) or with (unbroken line) 5 mM $MnCl_2$ using 10-μl aliquots of a 0.29-ml fraction.

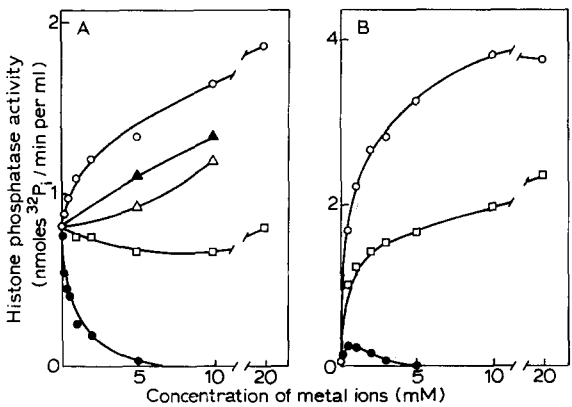

Fig. 3. Effect of divalent metal ions on phosphatase I and phosphatase II. Phosphatase I (18 μg protein) (A) or phosphatase II (5.6 μg protein) (B) of the pooled fractions of the Sephadex G-150 eluate was incubated in a total volume of 60 μl with 50 mM imidazole–HCl buffer (pH 7.4), 1 mM dithiothreitol, 0.07% bovine serum albumin and 100 mM NaCl for 4 min at 30 °C with 10 (A) or 20 μM (B) of *P*-histone in the indicated concentrations of: $MnCl_2$ (○—○), $ZnCl_2$ (●—●), $MgCl_2$ ▲—▲), $CaCl_2$ (△—△) and $CoCl_2$ (□—□).

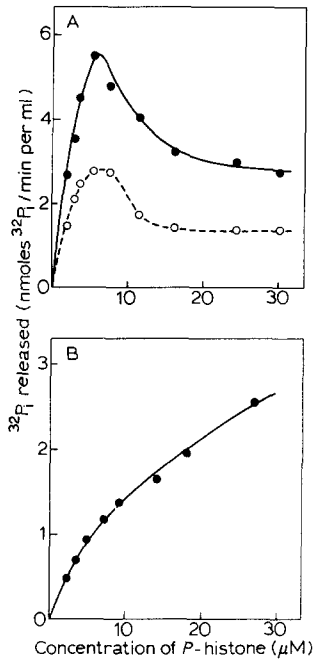

Fig. 4. Inhibition of phosphatase I by a high concentration of substrate (phosphohistone). Phosphatase I (22 μg protein) (A) or phosphatase II (5.6 μg protein) (B) of the pooled fractions of the Sephadex G-150 eluate was incubated with the indicated concentrations of *P*-histone (based on the alkali-labile ^{32}P content of the labeled histone) without (broken line) or with (unbroken line) 5 mM $MnCl_2$.

Maximal histone phosphatase activities of both types of enzyme in the presence and absence of 5 mM Mn^{2+} were observed in the range of pH 7.0–7.4.

Phosphatase I was stable against heating for 8 min at 50 °C. However, the same treatment of phosphatase II resulted in 40% loss of activity. This inactivation was completely protected by the presence of 3 mM Mn^{2+} during the heating.

Both types of phosphatase could be stored at −20 °C in 50 mM Tris–HCl buffer (pH 7.5) containing 10 mM mercaptoethanol for at least 2 months with little loss of activity. However, preparations of phosphatase I which had been stored longer than 1 month were found to have broader sedimentation profiles compared to those of original fresh preparations. The repetition of freezing and thawing 2 or 3 times did not result in the change of the sedimentation profiles. On the other hand, freezing (for 30 min at −20 °C) of the preparation with a high concentration (200 mM) of mercaptoethanol in the buffer resulted in the denaturation of more than 50% protein but about a 2-fold increase of histone phosphatase activity assayed with 20 μM *P*-histone. The increase in activity was explained by the fact that the property of substrate inhibition as observed in the original preparation (Fig. 4A) had disappeared by the freezing with mercaptoethanol (Fig. 5B). A sedimentation profile of thus treated phosphatase I is shown in Fig. 5A. The enzyme activity sedimented with the same sedimentation coefficient ($s_{20,w}$ = 3.8) as phosphatase II. However, the poor stimulability of the activity by the metal ion observed in the original larger enzyme did not

change even after dialysis against the buffer containing EDTA. The freezing of phosphatase II in the same condition showed no effect on the enzyme activity and the sedimentation coefficient. The pre-incubation (for 30 min at 20 °C) of phosphatase I with 200 mM mercaptoethanol did not show the changes as observed above. A highly increased concentration of mercaptoethanol with a decreased volume of water during freezing may result in the cleavage of an enzyme protein (or enzyme–enzyme) complex with the precipitation of the greater part of protein in the preparation.*

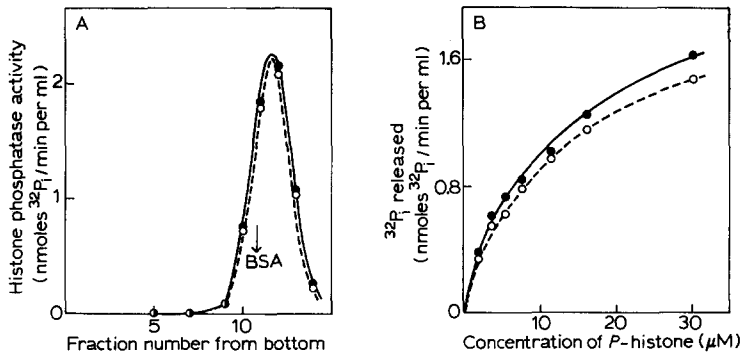

Fig. 5. Influence of freezing in the presence of mercaptoethanol on phosphatase I. Phosphatase I (18.2 mg protein/ml) which had been collected by the $(NH_4)_2SO_4$ precipitation and dialyzed against 50 mM Tris–HCl buffer (pH 7.5) containing 10 mM mercaptoethanol was frozen for 30 min at −20 °C in the presence of additional mercaptoethanol (200 mM). Insoluble protein which precipitated as a result of freezing was removed by centrifugation (5000 × *g*, 10 min). (A) Aliquots (0.15 mg protein in 0.15 ml) of the frozen phosphatase I were centrifuged on the linear gradient of sucrose as described in Fig. 2. The activity of each fraction (0.29 ml) was assayed in 10 μM *P*-histone without (broken line) or with (unbroken line) 5 mM $MnCl_2$. Bovine serum albumin (BSA) sedimented at the indicated point. (B) Aliquots (2.2 μg protein) of the frozen phosphatase I (1.5 mg protein/ml) were incubated with the indicated concentration of *P*-histone without (broken line) or with (unbroken line) 5 mM $MnCl_2$.

To see the general existence of the two types of phosphoprotein phosphatase, sucrose density gradient centrifugation of the crude extract from several rat tissues was carried out. The supernatant (20 000 × *g* for 1 h) of each tissue homogenate in 3 vol. of 0.25 M sucrose containing 10 mM mercaptoethanol was fractionated by $(NH_4)_2SO_4$ (0–50%). The precipitate collected by centrifugation was dissolved in 1/3–1/5 vol. of 50 mM Tris–HCl buffer (pH 7.5), containing 10 mM mercaptoethanol, and dialyzed against the same buffer. Aliquots of the extract were layered on the sucrose solution as described in Fig. 2. As shown in Fig. 6, sedimentation profiles of histone phosphatase activity from liver, kidney, brain and heart muscle indicated the existence of two types of phosphoprotein phosphatase in these tissues as in the skeletal muscle.

* The ethanol precipitation and succesive freezing (without dialysis) used in the previous method [1]for the purification of the phosphatase might have a similar effect on the freezing of phosphatase I preparations in a high concentration of mercaptoethanol as in the present study, because the activity eluted from a Sephadex G-200 column showed a single peak in the previous method, which imaginably contained the original smaller enzyme and the dissociated form of the larger enzyme.

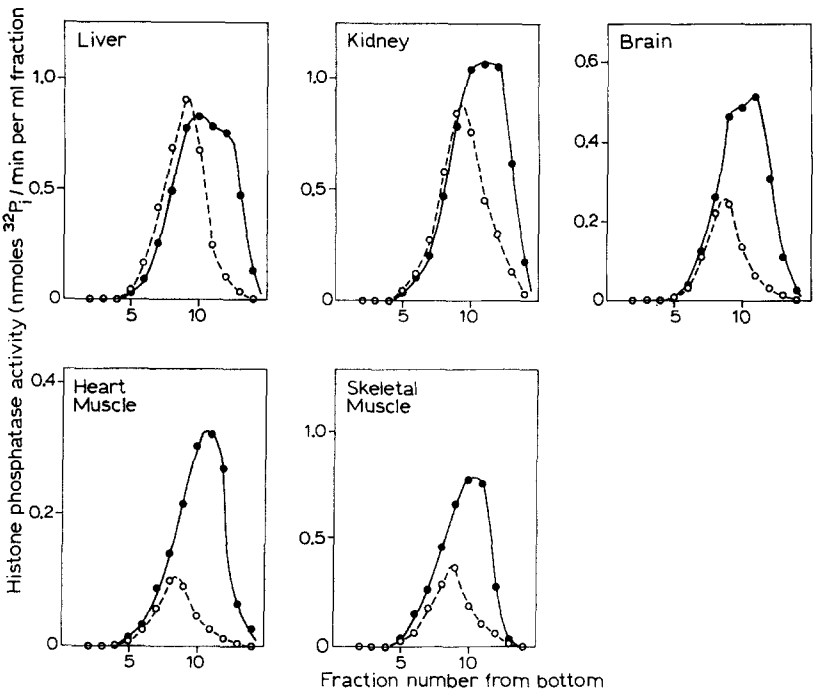

Fig. 6. Sedimentation profiles of histone phosphatase prepared from rat liver, kidney, brain, heart muscle and skeletal muscle in a sucrose density gradient. Aliquots (100 μl, 1.5–2 mg protein) of the $(NH_4)_2SO_4$ fraction of crude extract (see text) from each tissue were centrifuged on the linear gradient solution of sucrose as described in Fig. 2. The enzyme activity was assayed in 15 μM *P*-histone without (broken line) or with (unbroken line) 5 mM $MnCl_2$ using 20-μl aliquots of a 0.29-ml fraction.

DISCUSSION

Present results show the two types of phosphoprotein phosphatase in the skeletal muscle which are separated by the difference between their molecular sizes. The larger enzyme (phosphatase I, apparent mol. wt about 300 000) exhibits less requirement of the metal ions for its activity compared to the smaller enzyme (phosphatase II, mol. wt about 150 000) which is essentially inactive in the absence of Mn^{2+} or Co^{2+}.

Two types of the phosphatase are also observed in rat liver, kidney, brain and heart muscle as well as in the skeletal muscle in crude extract.

Perhaps the inhibition of phosphatase I at a high concentration of the substrate, *P*-histone, is due to the non-specific protein–protein interactions which take place with the basic protein (*P*-histone) and some component of phosphatase I.

The larger enzyme can be dissociated to a small molecular size corresponding to that of the smaller phosphatase (phosphatase II) by freezing with mercaptoethanol. The dissociated form of phosphatase I does not show the inhibition by *P*-histone up to 30 μM, indicating the removal of the component (protein) which may interact with *P*-histone.

At present, it is not clear whether both types of phosphatase contain a common catalytic subunit or not, and if they do, whether the larger enzyme is an oligomer of the smaller enzyme or an associated form of the smaller enzyme with some protein

different from the enzyme, like the regulatory subunit of cyclic AMP-dependent protein kinase [11].

Because of the less sensitive method of glycogen synthetase-D phosphatase assay [1], most of the results were obtained with phosphohistone (corresponding to the phosphorylated lysine-rich histone) as the substrate. However, the separations of glycogen synthetase-D phosphatase and phosphorylase phosphatase* activities into two fractions similar to those of histone phosphatase indicate that the two types of phosphatase are very similar, possessing the broad substrate specificity.

Although we have no direct evidence indicating the interconversion of each type of phosphatase, the present results may suggest the possible existence of two interconvertible forms of phosphoprotein phosphatase and the concentration of each form may be controlled in vivo by metabolic and hormonal stimuli as suggested by Bishop [12].

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* Dissociation of phosphatase I by freezing resulted in a several-fold increase of activities for glycogen synthetase-D phosphatase and phosphorylase phosphatase (Kato, K., Kobayashi, M. and Sato, S., unpublished results).