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DISSOCIATION OF PHOSPHOHISTONE PHOSPHATASES FROM CANINE HEART

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Summary

Phosphohistone phosphatase (phosphoprotein phosphohydrolase, EC 3.1.3.16) of canine heart extract has been separated by DEAE-cellulose chromatography into 4 molecular forms, namely phosphatases A (M_r = 156 000), B (M_r = 161 000), C (M_r = 95 600) and U (M_r = 61 000). ATP inhibited phosphatase A, stimulated phosphatase B and did not significantly affect phosphatase C activity. Phosphatase U requires Mn^{2+} for activity, under which condition ATP is inhibitory. Phosphatases A, B and C, but not phosphatase U, were dissociated by ethanol into catalytic subunits that were inhibited by ATP, insensitive to Mn^{2+}, and had a common molecular weight of 34 800 (phosphatase S). The dissociation was accompanied by an increase of enzymic activity. Chromatography of the ethanol-treated 55% $(NH_4)_2SO_4$ fraction of canine heart extract on DEAE-cellulose demonstrated that the multiple forms of phosphohistone phosphatase could be reduced to two forms: phosphatase U and phosphatase S, which may represent two basic constituents of the multiple forms of phosphohistone phosphatase in canine heart.

Introduction

The enzymic interconversions of active and inactive forms of regulatory enzymes through cycles of phosphorylation and dephosphorylation represent an important mechanism of metabolic control. Enzymes and regulatory proteins subject to such control include phosphorylase kinase, phosphorylase, glycogen

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The abbreviations used are: P-histone, phosphohistone; P-casein, phosphocasein; cyclic AMP, adenosine 3′:5′-monophosphate.

synthetase and pyruvate dehydrogenase [1—4]. The effects of a number of hormones on these enzymic interconversions have been shown to be mediated by clyclic AMP and cyclic AMP-dependent protein kinases, which catalyze the phosphorylation of such substrates as phosphorylase kinase, glycogen synthetase, casein or histone [2,3].

Recently, a potential regulatory role for the corresponding dephosphorylation reactions, which are catalyzed by phosphoprotein phosphatases (phosphoprotein phosphohydrolase, EC 3.1.3.16), has been of increasing interest. Evidence from several laboratories indicates that phosphoprotein phosphatases can exist in multiple forms [5—15] and can exhibit a broad substrate specificity [5—9,14—20]. Multiple forms of phosphoprotein phosphatases can be dissociated by treatment with ethanol [12,13] or β-mercaptoethanol [8,9] to yield a catalytic subunit, with concomitant activation of the enzyme.

We have previously reported that phosphohistone (P-histone) phosphatase activity in the 0—55% ammonium sulfate fraction of canine heart extracts can be resolved by DEAE-cellulose chromatography into four major fractions which were arbitrarily designated as phosphatases A, B, C and U [11]. The enzymic activity of each fraction showed different responses to nucleoside triphosphates and metal ions. In the present study, we examine further the subunit composition of these four different fractions. The results indicate that phosphatases A, B and C, but not phosphatase U, can be dissociated by the ethanol treatment described by Brandt et al. [12] into a common catalytic subunit which has molecular weight of approximately 34 800.

Experimental Procedures

Preparation of ^{32}P-labeled proteins

[γ-^{32}P]ATP was purchased from New England Nuclear. Calf thymus histone mixture (Type II), rabbit skeletal muscle phosphorylase *b* (twice crystallised) and phosphorylase kinase were obtained from Sigma. Vitamin-free casein, purchased from Nutritional Biochemicals, was dephosphorylated, as described by Reimann et al. [21], prior to use. Histone and casein were phosphorylated with [γ-^{32}P]ATP and the partially purified bovine heart cyclic AMP-dependent protein kinase [35]. Phosphorylase *b* was converted to ^{32}P-labeled phosphorylase *a* by a modified method of Torres and Chelala [23]. The alkali-labile [^{32}P]-phosphate content of typical preparations of phosphorylated substrates was 30—40 nmol per mg of histone, 2.5—5 nmol per mg of casein and 8—10 nmol per mg of phosphorylase *a*. The specific radioactivity of the [^{32}P]protiens used in the assay varied from 50 to 500 cpm per pmol of [^{32}P]phosphate.

Enzyme assay

Phosphoprotein phosphatase activity was measured at 30° C in an incubation volume of 50 μl or 100 μl. The standard assay mixture contained 50 mM Tris · HCl, pH 7.4, 1 mM dithiothreitol, 0.1 M KCl and 10 μM [^{32}P]protein (calculated by the [^{32}P]phosphate concentrations; any phosphate present before phosphorylation was not considered). Reactions were initiated by addition of the enzyme in an amount that would release less than 15% of the [^{32}P]protein phosphate in 10 min. At the end of incubation, 20 μl of 8 g/100 ml trichloro-

acetic acid was added to the 50 μl mixture to stop the enzymic reaction. A 50 μl aliquot was then withdrawn from the incubation mixture and [^{32}P]-phosphate released was separated from [^{32}P]protein by the paper chromatographic method described previously [24,35]. One unit of phosphohistone phosphatase activity was defined as the amount of enzyme which catalyzed the formation of 1 nmol of [^{32}P]phosphate per min under the conditions specified above.

Preparation of P-histone phosphatases

1. Crude extract. The supernatant fraction of canine heart was prepared essentially according to the method of Harigaya and Schwartz [25]. Hearts were quickly removed from dogs anesthetized with pentobarbital and washed with ice-cold distilled water. After removal of fatty and connective tissues, approximately 70—140 g of ventricular muscle was cut into small pieces. The heart muscle was mixed with 5 vols. of 10 mM $NaHCO_3$ and homogenized at high speed for 30 s in a Waring blendor. The homogenate was centrifuged at 8700 × *g*, for 20 min, and the supernatant was recentrifuged under the same condition to yield a second supernatant fraction. This second supernatant fraction was then centrifuged at 37 000 × *g* for 30 min. The supernatant obtained was the crude extract (Fraction I) used as an enzyme source for further purification.

2. Ammonium sulfate fractionation. Solid ammonium sulfate was added slowly with stirring to the crude extract to obtain 55% saturation (0.35 g added to each ml). The precipitate was collected by centrifugation, resuspended in 1/10 vol. of Buffer A (20 mM Tris · HCl, pH 7.4, 10 mM β-mercaptoethanol, 2 mM $MgCl_2$, 1 mM EDTA and 50 mM KCl), and dialyzed overnight against the same buffer (Fraction II).

3. DEAE chromatography. 10 ml of Fraction II were applied to a DEAE-cellulose (Whatman DE-52) column (1.5 × 60 cm) previously equilibrated with Buffer A. After sample application, the column was rinsed with Buffer A. Proteins that adsorbed to DEAE-cellulose were eluted by an increasing salt gradient as described by Li [11]. The DEAE-cellulose column resolved P-histone phosphatase into three peaks of activity, namely, peaks A, B and C (Fig. 1). When the fractions were assayed in the presence of 5 mM $MnCl_2$, an additional peak of activity (peak U) appeared between peaks A and B (data not shown). The active fractions of peaks A (fractions 53—66), U (fractions 68—77), B (fractions 84—100) and C (fractions 120—147) were pooled and concentrated by membrane ultrafiltration with a Diaflo membrane (PM 10, Amicon Corporation) to approximately 5 ml. The concentrated fractions were dialyzed overnight against 1 l of Buffer G (20 mM Tris · HCl, pH 7.4, 0.1 M KCl, 10 mM β-mercaptoethanol and 10% glycerol) and stored at 4° C.

Ethanol treatment

Ethanol treatment of various fractions of P-histone phosphatase was essentially that of Brandt et al. [12], with minor modification. One volume of the enzyme was mixed with 5 vols. of 95% ethanol at room temperature, and the mixture was centrifuged immediately at 15 000 × *g* for 10 min. P-histone phosphatase activity in the precipitate was extracted twice with 0.5 volume of Buff-

er G. The extracts were combined and dialyzed for 1—2 h against 200 vols. of Buffer G.

All operations were carried out at 4°C, except where otherwise indicated.

Other methods

$s_{20,w}$ (sedimentation coefficient) values were determined by sucrose density gradient centrifugation as described by Martin and Ames [26]. Centrifugation was carried out with an SW 50.1 swinging bucket rotor run at 39 000 rev./min for 16 h at 4°C. 5 ml of linear sucrose density gradient from 5 to 20% sucrose in 20 mM Tris · HCl, pH 7.4, was employed. Approximately 50 μg of the phosphatase, together with marker proteins, bovine serum albumin (2 mg), pig heart malic dehydrogenase (20 μg) and horse heart cytochrome *c* (0.4 mg) were applied to each gradient in a total volume of 100 μl. After the run, each gradient tube was fractionated and assayed for phosphatase activity and the marker proteins.

Proteins concentration was determined by the method of Lowry et al. [27], following trichloroacetic acid precipitation.

Results

Separation of P-histone phosphatases by DEAE-cellulose column chromatography

DEAE-cellulose column chromatography resolved P-histone phosphatase present in the 55% ammonium sulfate fraction of canine heart extract into three major peaks (A, B and C. Fig. 1A). When less enzyme from each fraction was assayed in the presence and absence of 2 mM ATP, the activity profile shown in Fig. 1B was obtained. At this concentration, ATP markedly stimulated the activity of peak B, inhibited the activity of peak A and had no significant effect on the activity of peak C. When each fraction from the DEAE column was assayed in the presence of 5 mM $MnCl_2$, the activity of peaks A, B and C were all slightly stimulated. Furthermore, an additional peak of P-histone phosphatase activity (fraction U) appeared between peaks A and B (data not shown). The results of a typical separation are summarized in Table I. The data indicate that either in the presence or absence of effectors, fraction B contained the highest activity recovered from DEAE-cellulose chromatography.

Sephadex G-200 gel filtration

The various fractions of phosphatase obtained from DEAE-cellulose chromatography were pooled, then chromatographed separately on a Sephadex G-200 column previously equilibrated in Buffer G. The activity profiles of phosphatase B and U determined by using P-histone as a substrate in the presence and absence of 5 mM $MnCl_2$ are shown in Figs. 2A and 3A, respectively. The phosphatases A and C have a similar activity profile as phosphatase B (data not shown). The data indicate that, when the fractions were assayed in the absence of Mn^{2+}, each phosphatase emerged from the gel filtration column as a single peak of activity. The activity profiles of phosphatases A, B and C were not influenced by Mn^{2+} whereas, in the case of phosphatase U, an additional peak of Mn^{2+}-activated enzyme emerged from the column after a minor peak

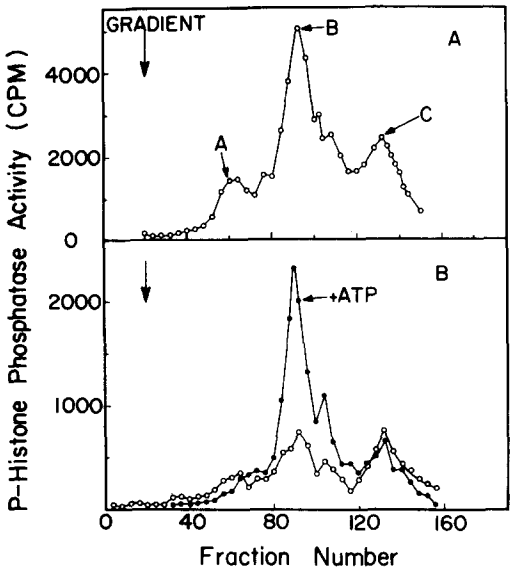

Fig. 1. Column chromatography of the $(NH_4)_2SO_4$ fraction on DEAE-cellulose. 10 ml of the $(NH_4)_2SO_4$ fraction (25 mg/ml) were applied to a DEAE-cellulose column (1.5 X 60 cm). The enzyme was eluted with a KCl gradient as described [11]. Fig. 1A shows the elution profile of P-histone phosphatase activity measured in the absence of ATP with 10-μl aliquots from the indicated fractions. Fig. 1B shows the elution profile of the enzymic activity measured in the presence (●———●) and absence (○———○) of 2 mM ATP with 2-μl aliquots from the indicated fraction. Incubation was carried out at 30°C for 10 min.

of Mn^{2+}-insenstive activity which was the contamination of phosphatase B (Fig. 3A).

The fractions containing the major activity of each phosphatase were pooled after gel filtration chromatography and stored at 4°C for further studies. The

TABLE I

SEPARATION OF VARIOUS FORMS OF PHOSPHOHISTONE PHOSPHATASE FROM CANINE HEART

Various forms of P-histone phosphatase were isolated from 90 g of canine heart and the enzymic activity was measured in the absence and presence of 2 mM ATP or 5 mM $MnCl_2$ as described in the text.

Fraction	Total protein (mg)	Specific activity units/mg			Total activity (unit)		
		Control	+ATP	$+Mn^{2+}$	Control	+ATP	$+Mn^{2+}$
37 000 X *g* supernatant	2985	0.47	0.62	0.6	1403	1851	1791
$(NH_4)_2SO_4$ fraction	875	1.5	3.0	3.1	1312	2623	2711
DEAE-cellulose							
fraction A	37	1.3	0.6	2.1	48	22	77
fraction B	92	5.3	14	12	485	1281	1098
fraction C	29	3.3	4.6	6.4	95	133	185
fraction U	42	1.0	1.5	8.4	42	63	353
Sephadex G-200 fraction B	27	13.7	28	19	370	756	513
Ethanol treatment fraction B	5.2	174	104	182	905	541	946

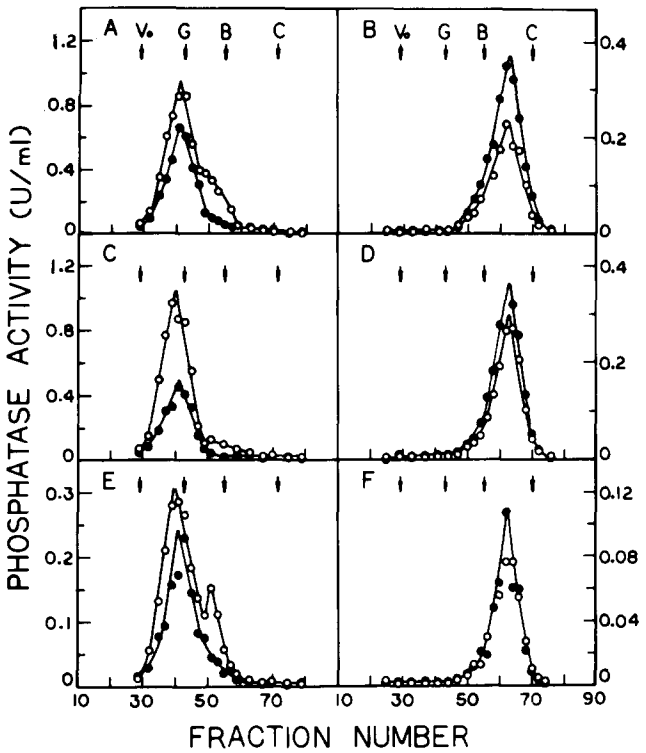

Fig. 2. Sephadex G-200 chromatography of phosphatase B and the ethanol-treated phosphatase B. 5 ml of phosphatase B (15.2 mg protein) obtained from DEAE-cellulose chromatography were applied to a Sephadex G-200 column (2.5 X 61 cm) pre-equilibrated with Buffer G. The column was eluted with the same buffer. Fractions of 3.8 ml were collected. Phosphatase activity toward P-histone (2A), P-casein (2C) and phosphorylase *a* (2E) was measured in the absence (●———●) or in the presence (○———○) of $MnCl_2$ (5 mM for phosphohistone and phosphocasein; 0.5 mM for phosphorylase *a*) as described in the text. The active fractions (31—47) were pooled and concentrated by membrane ultrafiltration to 9.1 ml. 3 ml of the concentrated fraction were treated with ethanol, chromatographed on the same Sephadex G-200 column and fractions collected were assayed for P-histone (2B), P-casein (2D) and phosphorylase *a* (2F) phosphatase activity in the absence (●———●) or in the presence (○———○) of $MnCl_2$ as described above. V_0 refers to void volume. G, B and C indicate the elution volume of bovine γ-globulin, bovine serum albumin and cytochrome *c*, respectively.

activity of phosphatase B was stimulated more than two-fold by 2 mM ATP while this nucleotide concentration inhibited the activity of phosphatase A about 50% and did not significantly affect phosphatase C activity. Only phosphatase U required Mn^{2+} for activity. In the presence of 5 mM Mn^{2+}, 2 mM ATP inhibited phosphatase U activity about 50%. Thus, the responses to ATP of these four enzyme fractions did not change after gel filtration chromatography.

The Stokes radii for phosphatases A, B, C and U, as determined by gel filtration, were 54, 55, 43 and 39 Å, respectively (Fig. 4). The sedimentation coefficient ($s_{20,w}$) for phosphatases A, B, C and U, as determined by sucrose density gradient centrifugation, were 7.0, 7.1, 5.4 and 3.8, respectively.

Dissociation of P-histone phosphatases by ethanol treatment

Phosphatases A, B, C and U, obtained from Sephadex G-200 chromatog-

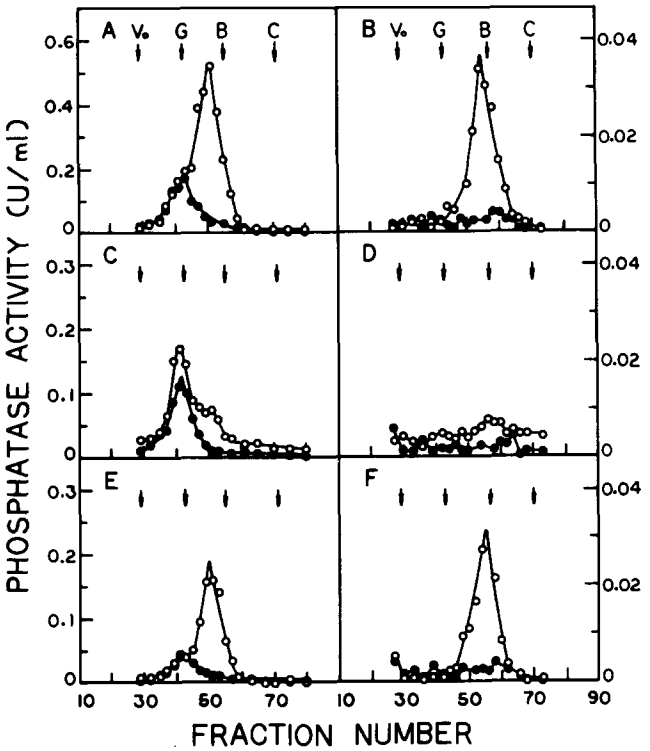

Fig. 3. Sephadex G-200 chromatography of phosphatase U and the ethanol-treated phosphatase U. 5 ml of phosphatase U (15.6 mg protein) obtained from DEAE-cellulose chromatography were chromatographed on a Sephadex G-200 column and fractions collected were assayed for P-histone (3A), P-casein (3C) and phosphorylase *a* (3E) phosphatase activity in the absence (●———●) and presence (○———○) of $MnCl_2$ as described in the legend to Fig. 2. The active fractions (48—59) were pooled and concentrated to 6.1 ml. 3 ml of the concentrated fraction were treated with ethanol, chromatographed on the same Sephadex G-200 column and fractions collected were assayed for P-histone (3B), P-casein (3D) and phosphorylase *a* (3F) phosphatase activity in the absence (●———●) and in the presence (○———○) of $MnCl_2$ as described above. V_0 refers to void volume. G, B and C indicate the elution volume of bovine γ-globulin, bovine serum albumin and cytochrome *c*, respectively.

raphy, were each treated with ethanol, as described by Brandt et al. [12], which increased the activities of phosphatases A, B and C, in the absence of Mn^{2+}, by 110, 250 and 317%, respectively. After ethanol treatment, phosphatase B had a specific activity of 174 units/mg which represented about 400-fold purification from crude extract (Table I). In contrast, the activity of phosphatase U, measured in the presence of 5 mM Mn^{2+}, was decreased 20—30% after the ethanol treatment.

Chromatography of the ethanol-treated phosphatases on a Sephadex G-200 column previously equilibrated in Buffer G resulted in dissociation of phosphatases A, B and C into a smaller molecular size activity. Fig. 2B shows the dissociation of the phosphatase B. The Stokes radii for the ethanol-treated phosphatases A, B and C, as determined by gel filtration, were all 24.5 Å (Fig. 4) and their $s_{20,w}$ values, as determined by sucrose density gradient, were all 3.45. Furthermore, the ethanol-treated phosphatases A, B and C showed similar responses to Mn^{2+} and ATP, being inhibited about 50% by 2 mM ATP and not signifi-

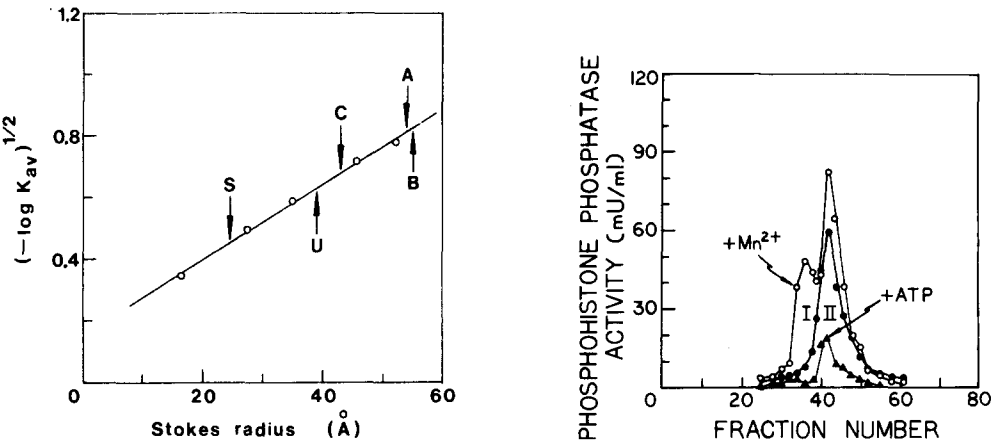

Fig. 4. Estimation of the Stokes radii of phosphatases by gel filtration on Sephadex G-200. The column (1.6 X 84 cm) was eluted with a buffer containing 50 mM Tris · HCl (pH 7.4) and 0.1 M KCl. Fractions of 1.2 ml were collected at a flow rate of 5 ml per hour. The column was calibrated with the following proteins (Stokes radius): bovine γ-globulin (a = 52 Å), yeast alcohol dehydrogenase (a = 46 Å), bovine serum albumin (a = 35 Å), ovalbumin (a = 28 Å) and cytochrome c (a = 17 Å). The data are plotted according to the method of Siegel and Monty [28]. A, phosphatase A; B, phosphatase B; C, phosphatase C; U, phosphatase U; S, phosphatase S.

Fig. 5. Column chromatography of the ethanol-treated $(NH_4)_2SO_4$ fraction on DEAE-cellulose. 2 ml of $(NH_4)_2SO_4$ fraction was treated with ethanol as described in the text, except Buffer A was used instead of Buffer G. One ml of this fraction was applied on a DEAE-cellulose column (0.9 X 27 cm) which was pre-equilibrated with buffer A. The enzyme was eluted by a KCl gradient as described [11] except each chamber contained 60 ml of elution buffer. Fraction volumes of 2.2 ml were collected. Enzymic activity was measured in the absence (●———●) and in the presence of 5 mM $MnCl_2$ (○———○) or 4 mM ATP (▲———▲) with aliquots from the indicated fractions.

cantly affected by 5 mM $MnCl_2$. These data suggest that phosphatases A, B and C consist of a common catalytic subunit.

In contrast, chromatography of the ethanol-treated phosphatase U on Sephadex G-200 column (Fig. 3B) showed a single peak of Mn^{2+}-activated activity contained in an elution volume similar to that of the untreated enzyme. (Compare Figs. 3A and 3B). The data indicate that ethanol treatment did not dissociate phosphatase U into the smaller molecular size, Mn^{2+}-insensitive enzyme obtained after similar treatment of phosphatases A, B and C.

In order to examine further the molecular forms and subunit composition of P-histone phosphatases, the 0—55% $(NH_4)_2SO_4$ fraction was treated with 80% ethanol at room temperature prior to fractionation on a DEAE-cellulose column. Ethanol treatment of the 0—55% $(NH_4)_2SO_4$ fraction resulted in a 2- to 3-fold increase of the enzymic activity. Fig. 5 shows the DEAE-cellulose chromatography of the ethanol-treated 0—55% $(NH_4)_2SO_4$ fraction. Two peaks of P-histone phosphatase were found. Peak I was active only in the presence of Mn^{2+}. Peak II was active without Mn^{2+} and was markedly inhibited by ATP. Chromatography of peak I on a calibrated Sephadex G-200 column yielded a single peak of a Mn^{2+}-activated enzyme with a Stokes radius of 39 Å. Gel filtration chromatography of peak II showed a single peak of a Mn^{2+}-insensitive, ATP-inhibited activity with a Stokes radius of 24.5 Å. Ultracentrifugation of peaks I and II on a 5—20% sucrose gradient resulted in a single peak of a Mn^{2+}-

activated enzyme with an $s_{20,w}$ = 3.8 and a Mn^{2+}-insensitive enzyme with an $s_{20,w}$ = 3.45, respectively. These data indicate that peak I was identical to phosphatase U, and peak II was identical to the smaller molecular size enzyme derived from phosphatase A, B or C. This smaller molecular size, ATP-inhibited, Mn^{2+}-insensitive enzyme, which appears to be a common catalytic subunit of several fractions of P-histone phosphatase, is arbitrarily designated as phosphatase S.

The molecular weight and frictional ratio for various fractions of P-histone phosphatases were calculated according to the procedure of Siegel and Monty [28], using Stokes radius and $s_{20,w}$ values obtained and assuming a partial specific volume of 0.725 for the enzymes. Axial ratio was estimated according to the method of Schachman [29]. The results are summarized in Table II. The frictional and axial ratios suggest that phosphatase S is a globular protein.

Substrate specificity

All fractions obtained by DEAE-cellulose chromatography catalyzed the dephosphorylation of phosphorylase *a* and phosphocasein (P-casein), as well as P-histone, in the presence and absence of 5 mM $MnCl_2$. These activities were co-eluted from the DEAE-column with P-histone phosphatase activity (data not shown).

Ethanol treatment of phosphatases A, B and C also resulted in a 2- to 3-fold increase of the enzyme activity toward P-casein and phosphorylase *a*. On the other hand, ethanol treatment of phosphatase U resulted in a 20—30% decrease of the enzymic activity toward these two phosphoproteins. Chromatography of phosphatases A, B, C and U, either before or after treating with ethanol, on Sephadex G-200 column showed that phosphorylase and P-casein phosphatase were co-eluted with P-histone phosphatase activity (Figs. 2, 3) (data not shown for phosphatases A and C). As shown in Figs. 3C and 3D, the Mn^{2+}-activated phosphatase, however, was much less active toward P-casein than toward P-histone and phosphorylase *a*, indicating that P-casein was a poor substrate for this fraction of the enzyme.

The catalytic properties of the Mn^{2+}-activated P-histone phosphatase (phosphatase U) have been described previously [35]. The mechanism of ATP-stimulation on phosphatase B and the properties change associated with the dissociation of phosphatase B by ethanol to its putative catalytic subunit, phosphatase S, have been studied [30].

TABLE II

PHYSICAL PROPERTIES OF PHOSPHOHISTONE PHOSPHATASES

Fractions	Stokes radius (Å)	$s_{20,w}$	Molecular weight	f/f_0	Axial ratio
Phosphatase A	54	7.0	156 000	1.520	9
Phosphatase B	55	7.1	161 000	1.532	9
Phosphatase C	43	5.4	95 000	1.425	7
Phosphatase U	39	3.8	61 000	1.501	9
Phosphatase S	24.5	3.45	34 800	1.137	4

Discussion

The present findings indicate that P-histone phosphatase activity in a canine heart extract arises from four molecular forms of the enzyme that can be separated by DEAE-cellulose chromatography. These are designated as phosphatase A (M_r = 156 000), B (M_r = 161 000), C (M_r = 95 600) and U (M_r = 61 000). ATP inhibited phosphatase A, stimulated phosphatase B and showed no significant effect on phosphatase C. In contrast to the other three fractions of the enzyme, phosphatase U requires Mn^{2+} for activity. The different responses of these four fractions of the enzyme to ATP and Mn^{2+} indicate that they possess different regulatory properties which could reflect distinct roles for each enzyme in the control of protein dephosphorylation by the cell.

Two laboratories have reported that the multiple forms of phosphoprotein phosphatases isolated from various tissues can be dissociated by either ethanol [12,13] or β-mercaptoethanol [8,9] treatment to yield a single, lower molecular weight form concomitant with activation of enzymic activity. Our studies in canine heart P-histone phosphatase confirm these observations. Phosphatases A, B and C were dissociated by treating with ethanol into a single catalytic subunit (phosphatase S) having a molecular weight of 34 800. This value is in agreement with those reported for ethanol-treated phosphorylase phosphatase isolated from rabbit liver and other sources [12,13]. Phosphatase U, a Mn^{2+}-activated enzyme is neither dissociated nor activated by treating with ethanol. Chromatography of the ethanol-treated 0—55% $(NH_4)_2SO_4$ fraction of canine heart extract on DEAE-cellulose also demonstrated that the multiple forms of the enzyme are reduced to two forms, namely, phosphatases U and S. The data suggest that the multiple forms of the enzyme in canine heart consist of at least two basic catalytic entities.

P-histone phosphatases A, B, C and their catalytic subunit, phosphatase S, can also dephosphorylate P-casein and phosphorylase *a*. Phosphatase S has been purified to apparent homogeneity from the ethanol treated 0—55% $(NH_4)_2SO_4$ fraction in our laboratory [31]. The pure enzyme (M_r = 34 800; specific activity about 2000 unit/mg) is divalent-cation-independent, inhibited by ATP and active towards all three phosphoproteins. Recently Khandelwal et al. [15] have reported the purification of phosphoprotein phosphatase from rabbit liver. Two fractions of the pure enzyme, designated phosphatase I (M_r = 30 500) and phosphatase II (M_r = 34 500) were obtained. Both of these two enzymes can dephosphorylate phosphorylase *a*, P-histone, P-casein and other phosphoproteins. The molecular weight and the broad substrate specificity property of the canine heart phosphatase S are similar to those of phosphatase II obtained from rabbit liver [15]. Whether phosphatases A, B and C consisted of an oligomer of phosphatase S or a combination of phosphatase S with other proteins is not known, but Brandt et al. [12], proposed that the multiple forms of phosphoprotein phosphatase consist of an enzyme-inhibitory protein complex which contains a single catalytic subunit of molecular weight 32 000. Purification of the ethanol-activated phosphorylase phosphatase to homogeneous state [13] and isolation of heat-stable inhibitory protein(s) [32] from rabbit liver where subsequently described. Huang and Glinsmann [33,34] have reported that a partially purified phosphorylase phosphatase from rabbit skeletal muscle

contains heat-stable inhibitory proteins. These observations suggest that phosphatases A, B and C isolated from canine heart may consist of an enzyme-inhibitory protein complex.

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