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CHARACTERIZATION OF MULTIPLE FORMS OF HISTONE PHOSPHATASE IN RAT LIVER

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Summary

By using chromatography on DEAE-cellulose, aminohexyl-Sepharose 4B and Sephadex G-200, rat liver extract was shown to contain at least three fractions, IA, IB and II, of histone phosphatase. Fractions IA and II are probably the same enzymes as the previously described glycogen synthase phosphatase and phosphorylase phosphatase, respectively, but IB exhibits noticeable activities only with phosphohistone as substrate. Approximate molecular weights of 69 000, 300 000 and 160 000 were determined by gel filtration on Sephadex G-200 for IA, IB and II, respectively.

Introduction

A previous study in this laboratory demonstrated that a fraction of rat liver extract capable of dephosphorylating glycogen synthase D and phosphorylase *a* gave two fractions of phosphoprotein phosphatase when it was chromatographed on DEAE-cellulose [1]. Since the fast-eluting fraction contained the bulk of synthase phosphatase activity recovered from the column and the slow-eluting fraction contained the bulk of phosphorylase phosphatase activity, they were designated "glycogen synthase phosphatase" and "phosphorylase phosphatase", respectively [1]. These observations, however, were apparently in contrast to those of Killilea et al. [2], who reported that in rabbit liver, a single phosphoprotein phosphatase with a molecular weight of 34 000 was responsible for the dephosphorylation of both synthase D and phosphorylase *a*. Apparently, the same phosphatase has also been shown to catalyze the dephosphorylation of phosphohistone [3].

In order to obtain further insights into the nature of rat liver phosphoprotein phosphatase, we compared the chromatographic patterns of histone

phosphatase activity from rat liver extract with those of synthase phosphatase and phosphorylase phosphatase activities.

Materials and Methods

DEAE-cellulose chromatography of rat liver phosphoprotein phosphatase. The phosphoprotein phosphatase fraction was prepared from rat liver extracts by acid and $(NH_4)_2SO_4$ fractionation as described previously [1]. The fraction, 100—200 mg in protein, was applied to a DEAE-cellulose column (DE-52, 1.5 × 12 cm) previously equilibrated with 10 mM glycylglycine buffer (pH 7.4) containing 5 mM mercaptoethanol and 2% (v/v) glycerol. The column was first washed with 50 ml of the same buffer, and proteins that adsorbed to the column were eluted with a linear gradient of 0—0.5 M NaCl in the buffer. The eluate was passed through a continuous-flow counter current dialyzing apparatus (Biomed Instruments, Model D-1) for dialysis against the starting buffer; 10-ml fractions were then collected at a flow rate of 30 ml/h. These and other preparative operations were all conducted at 2—4°C.

Partial purification of cyclic AMP-dependent protein kinase. This was done by chromatographing the phosphoprotein phosphatase fraction from rat liver on DEAE-cellulose, Using histone as substrate, two fractions of protein kinase were found (Fig. 1), but only the major one was pooled, because it contained little phosphoprotein phosphatase activities (Fig. 1; also compare Figs.1 and2).

Preparation of ^{32}P-labeled phosphohistone. Calf thymus whole histone (Sigma, type II-A) was phosphorylated with [γ-^{32}P]ATP. The cyclic AMP-dependent protein kinase was prepared as described, and the conditions for incuba-

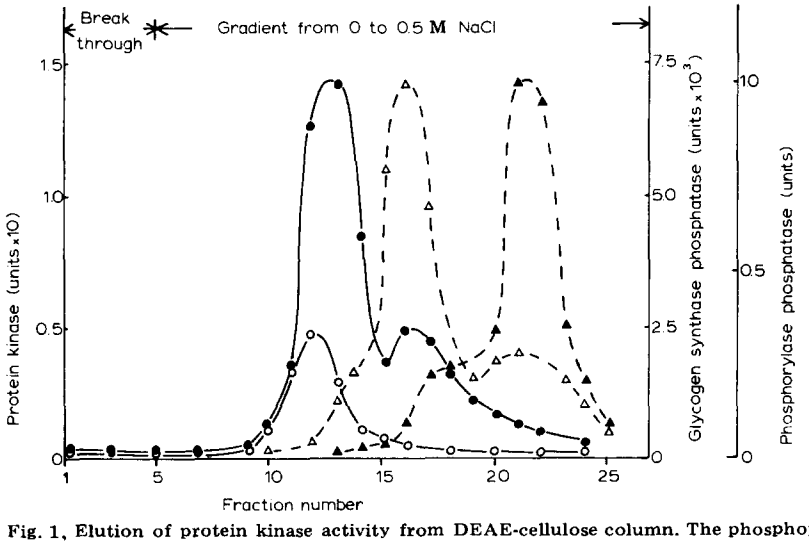

Fig. 1, Elution of protein kinase activity from DEAE-cellulose column. The phosphoprotein phosphatase fraction from rat liver was chromatographed on DEAE-cellulose as described in the text, and the fractions collected were assayed for protein kinase in the presence (●———●) and absence (○———○) of 4 μM cyclic AMP. One unit of the enzyme was defined as the amount which catalyzed the incorporation of 1 nmol of phosphate/min. The fractions were also assayed for glycogen synthase phosphatase (△———△) and phosphorylase phosphatase (▲———▲).

tion were those of Meisler and Langan [4] as modified by Kato and Bishop [5]. After 10 h at 30°C, the reaction was terminated by the addition of 1 vol. 50% (w/v) trichloroacetic acid. The precipitate was collected by centrifugation, and washed as described by Meisler and Langan [4]. The final precipitate was dissolved in 0.2 mM EDTA (pH 7.0), dialyzed against the same solvent, and stored at −20°C until use.

Assay of histone phosphatase. A modification of the method of Meisler and Langan [4] was used for the measurement of histone phosphatase activity. The reaction mixture contained, in a final volume of 0.12 ml, 50 mM Tris · HCl (pH 7.5), 1 mM dithiothreitol, 0.1 M NaCl, 10 mM $MgCl_2$, 60 μM (based on alkali-labile ^{32}P) ^{32}P-labeled phosphohistone and enzyme. After 5 min at 30°C, the reaction was terminated by the addition of 0.1 ml 20 mM silicotungstic acid in 0.01 M H_2SO_4, and the mixture was centrifuged to remove precipitated protein. To 0.1 ml supernatant solution was added 25 μl 5% (w/v) ammonium molybdate in 2 M H_2SO_4, and the phosphomolybdate complex formed was extracted with isobutanol/benzene (1 : 1, v/v). An aliquot of the extract was placed on a Millipore filter, and radioactivity was measured in a Beckman liquid scintillation counter. One unit of the enzyme was defined as the amount which catalyzed the release of 1 nmol of phosphate/min.

Assay of other enzymes. Glycogen synthase phosphatase and phosphorylase phosphatase were assayed by measuring the formation of synthase I from synthase D and the formation of phosphorylase *b* from phosphorylase *a*, respectively. Detailed procedures were described in the previous paper [1], and units of these enzymes were defined also as described previously [1]. Protein kinase activity was determined with calf thymus whole histone as substrate. The procedure was similar to that employed for the preparation of ^{32}P-labeled phosphohistone except that 0.25 ml of the assay mixture was incubated for 10 min. The final precipitate was collected on a Millipore filter and counted for radioactivity in a Beckman liquid scintillation counter. Acid and alkaline phosphatases were assayed with *p*-nitrophenyl phosphate as substrate according to Neil and Horner [6] and Dabich and Neuhaus [7], respectively.

Estimation of molecular weights. Histone phosphatase fractions obtained from DEAE-cellulose chromatography were concentrated and applied to a Sephadex G-200 column (2.5 × 65 cm) previously equilibrated with 10 mM glycylglycine (pH 7.4) containing 5 mM mercaptoethanol and 2% (v/v) glycerol. The same buffer was used for elution, and 4-ml fractions were collected at a flow rate of 10—15 ml/h. The column was calibrated with Jack bean urease, beef liver catalase, rabbit muscle lactate dehydrogenase, horse radish peroxidase and horse heart cytochrome *c*.

Chemicals and commercial enzymes. [γ-^{32}P]ATP was purchased from the Radiochemical Center, Amersham, U.K. Aminohexyl-Sepharose 4B was the product of Pharmacia. The enzymes used for molecular weight estimation were all from Boehringer, Mannheim. The source of other chemicals was described previously [1,8].

Results

DEAE-cellulose chromatography

Preliminary studies in this laboratory demonstrated that the phosphoprotein

phosphatase fraction prepared as described above dephosphorylated phosphohistone in addition to glycogen synthase D and phosphorylase *a*. To explore the possible relationship between the histone phosphatase activity and the other two phosphoprotein phosphatase activities studied previously [1], the above fraction was chromatographed on a DEAE-cellulose column with results reported in Fig. 2. While the majority of synthase phosphatase and phosphorylase phosphatase activities emerged from the column each as a single peak, histone phosphatase activity was resolved into two fractions, one largely overlapping the synthase phosphatase and the other co-eluting with the phosphorylase phosphatase. These two fractions of histone phosphatase activity, designated I and II in order of elution, were not preparative artifacts since the same two fractions of activity were also observed when freshly prepared rat liver extracts were applied to the column.

In a separate experiment, fractions recovered from the DEAE-cellulose column were assayed for acid and alkaline phosphatases. As shown in Fig. 3, their elution profiles were dissimilar to that of histone phosphatase.

Histone phosphatase II

When the fractions containing histone phosphatase II obtained from DEAE-cellulose chromatography was further chromatographed on Sephadex G-200, phosphatase activities toward phosphohistone and phosphorylase *a* eluted from the column as one peak (Fig. 4). In addition, the ratio of the two activities were similar before and after the gel filtration. Histone phosphatase II thus appears to be the same enzyme as the phosphorylase phosphatase already described in the previous paper [1]. By the Sephadex G-200 gel filtration method, the molecular weight of this phosphatase was found to be approx.

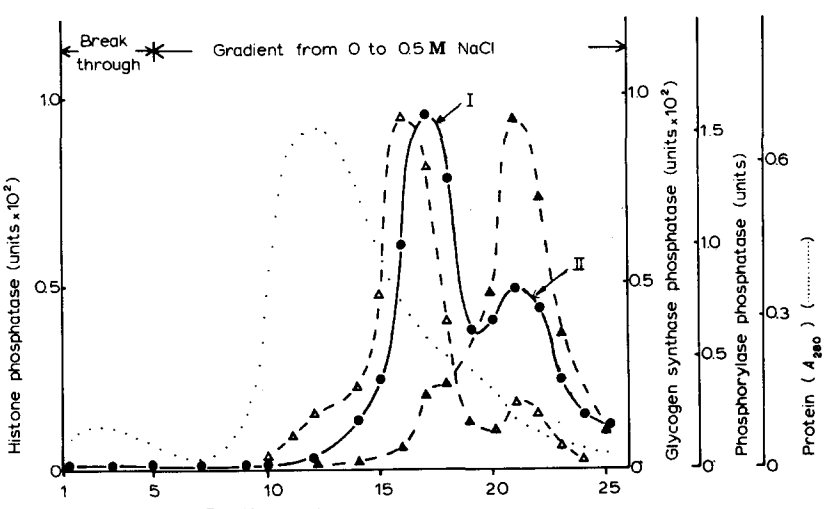

Fig. 2. Elution of histone phosphatase, glycogen synthase phosphatase and phosphorylase phosphatase activities from DEAE-cellulose column. The phosphoprotein phosphatase fraction was chromatographed on DEAE-cellulose as described in the text, and the fractions collected were assayed for histone phosphatase (●———●), synthase phosphatase (△———△) and phosphorylase phosphatase (▲———▲).

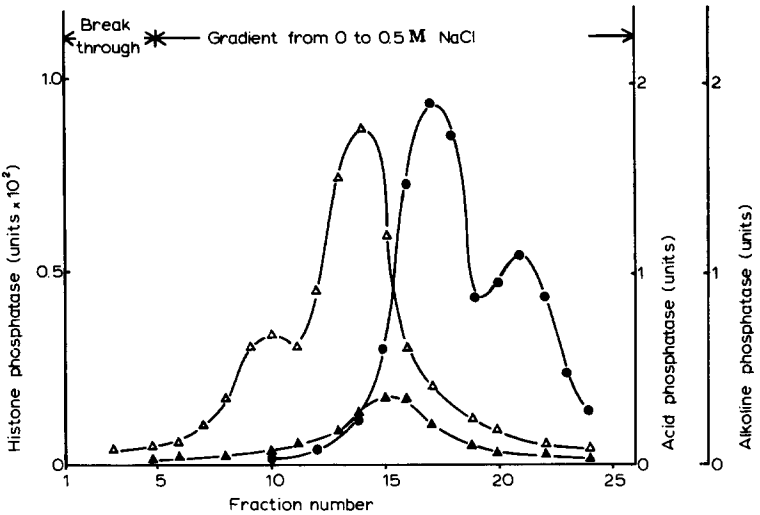

Fig. 3. Elution of acid and alkaline phosphatase activities from DEAE-cellulose column as compared with elution of histone phosphatase activity. The phosphoprotein phosphatase fraction was chromatographed on DEAE-cellulose as described in the text, and the fractions collected were assayed for acid phosphatase (△——△), alkaline phosphatase (▲——▲) and histone phosphatase (●——●). Unit of acid and alkaline phosphatases was defined as the amount which catalyzed the release of 1 nmol of phosphate/min.

160 000. It is also noteworthy that histone phosphatase II (phosphorylase phosphatase) as eluted from the Sephadex G-200 column exhibits little, if any, synthase phosphatase activity.

Histone phosphatase I

When the fraction containing histone phosphatase I obtained from DEAE-

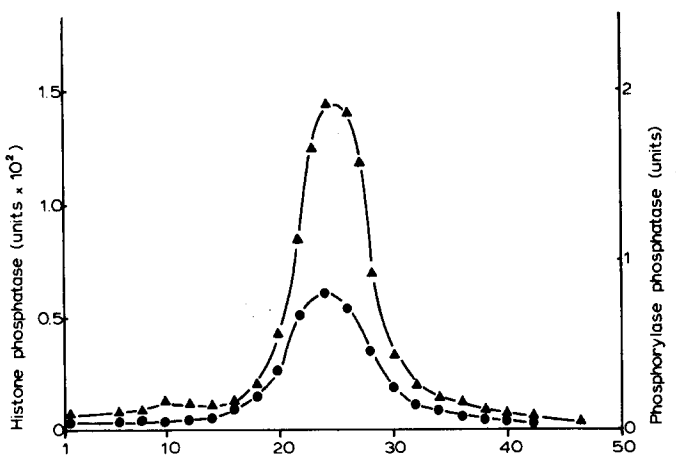

Fig. 4. Gel filtration of histone phosphatase II on Sephadex G-200. Fractions 20—23 obtained from DEAE-cellulose chromatography (Fig. 2) were pooled, concentrated to a few ml using a concentration column (DEAE-cellulose) and applied to a Sephadex G-200 column (2.5 X 90 cm) that had been equilibrated with 10 mM glycylglycine buffer (pH 7.4) containing 5 mM mercaptoethanol and 2% (v/v) glycerol. Elution was conducted with the same buffer at a flow rate of 10—15 ml/h, and the eluate was collected in 10-ml fractions. These fractions were assayed for histone phosphatase (●——●) and phosphorylase phosphatase (▲——▲).

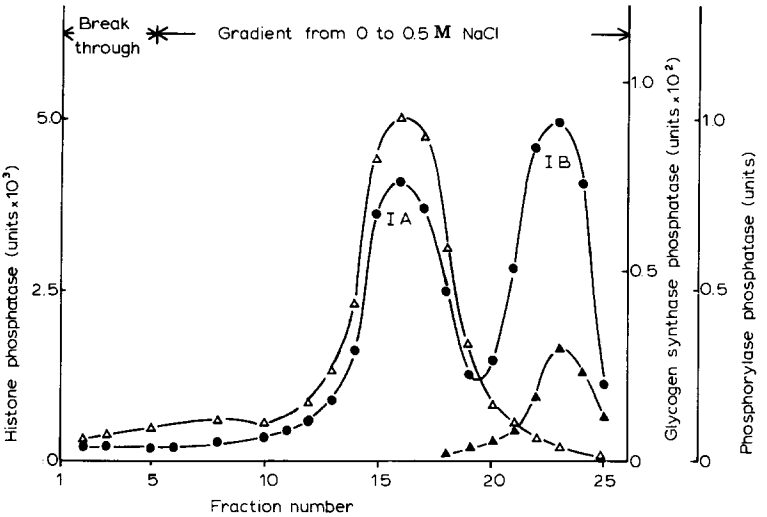

Fig. 5. Histone phosphatase I as chromatographed on aminohexyl-Sepharose 4B. Fractions 15—18 obtained from DEAE-cellulose chromatography (Fig. 2) were pooled and applied to an aminohexyl-Sepharose 4B column (1.5 X 4 cm) previously equilibrated with 10 mM glycylglycine buffer (pH 7.4) containing 5 mM mercaptoethanol and 2% (v/v) glycerol. The column was washed with 50 ml of the same buffer, and proteins were eluted with a linear gradient of 0—0.5 M NaCl in the buffer. Fractions of 10 ml were collected at a flow rate of 40 ml/h, and these fractions were assayed for histone phosphatase (●———●), synthase phosphatase (△———△) and phosphorylase phosphatase (▲———▲).

cellulose chromatography was further fractionated using an aminohexyl-Sepharose 4B column, histone phosphatase activity was resolved into two fractions as shown in Fig. 5. The fast-eluting fraction designated histone phosphatase IA co-eluted with a single-peaked synthase phosphatase, and the two activities again eluted together when peak IA fraction was further chromatographed on Sephadex G-200 (unpublished data). These observations suggest that it is the synthase phosphatase already described in the previous paper [1] that exhibits histone phosphatase IA activity.

In contrast, the slow-eluting fraction of histone phosphatase I, designated IB, was almost free of synthase phosphatase activity. Although it was capable of dephosphorylating phosphorylase *a*, the ratio of phosphatase activities with phosphorylase *a* and phosphohistone was much smaller for this phosphatase than for histone phosphatase II (phosphorylase phosphatase).

Histone phosphatases IA and IB readily separated from each other on a Sephadex G-200 column. The data obtained indicate that the molecular weights of IA (synthase phosphatase) and IB are approx. 69 000 and 300 000, respectively.

Discussion

The previous study in this laboratory has shown that the synthase phosphatase activity of rat liver extract can be separated chromatographically from the phosphorylase phosphatase activity [1]. The present study demonstrates that the chromatographic patterns of histone phosphatase activity are not entirely

identical with those of synthase phosphatase and phosphorylase phosphatase activities, thereby providing additional evidence for the highly complex nature of rat liver phosphoprotein phosphatase.

Confirming the results of Meisler and Langan [4], the histone phosphatase activity of rat liver extracts was recovered by DEAE-cellulose chromatography into two fractions, I and II. Histone phosphatase II is probably the enzyme that has been described in the previous paper [1] as phosphorylase phosphatase, but histone phosphatase I was further resolved on aminohexyl-Sepharose 4B into IA and IB. While histone phosphatase IA is apparently the same enzyme as the previously reported synthase phosphatase [1], IB is unique in that it exhibits noticeable activities with phosphohistone as substrate but not with synthase D or phosphorylase *a*. Rat liver thus contains at least three forms of phosphoprotein phosphatase differing from one another physically and also in relative activities toward different phosphoprotein substrates. The molecular weights of histone phosphatase IA (synthase phosphatase), IB and II (phosphorylase phosphatase) are approx. 69 000, 300 000 and 160 000, respectively. Meisler and Langan [4], however, reported the molecular weight of rat liver histone phosphatase to be 190 000. The reason for the discrepancy between the two observations is presently unknown.

The molecular basis for the physical and catalytic multiplicity of rat liver phosphoprotein phosphatase is as yet unclear. According to Brandt et al. [9], treatment of rat liver extracts with ethanol converts multiple forms of phosphorylase phosphatase into a single form of lower molecular weight, about 32 000. The same low molecular weight enzyme isolated from rabbit liver also dephosphorylated synthase D and phosphohistone in addition to phosphorylase *a* [2,3,10]. Since the ethanol treatment also resulted in a marked increase in phosphorylase phosphatase activity [9], Brandt et al. [9] proposed that phosphoprotein phosphatase is present in tissue extracts in an inactive form consisting of a complex of the low molecular weight form (the catalytic subunit) with an inhibitor protein. Of interest in this regard is our preliminary observation that histone phosphatases IB and II yielded a catalytically active protein of lower molecular weight, 30 000—40 000, when they were each treated with ethanol. Due to the treatment, the phosphorylase phosphatase activities of IB and II were increased 3.6- and 1.2-fold, respectively, but little changes were observed for their histone phosphatase activities. Conversion into lower molecular weight forms upon ethanol treatment has also been reported for multiple forms of phosphoprotein phosphatase from canine heart [11].

It is therefore reasonable to assume that the multiple forms of rat liver phosphoprotein phosphatase either derive from different regulatory proteins attached to a catalytic protein, or represent a series of oligomeric enzymes. In either case, such a multi-subunit structure must be essential to afford substrate specificity for the enzyme, since the different forms of rat liver phosphoprotein phosphatase as revealed by the present work appear to have more rigorous substrate specificities than does the low molecular weight enzyme. Further systematic studies are in progress in this laboratory to purify each of these enzymes and to elucidate their molecular structure.

Acknowledgements

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