Biochimica et Biophysica Acta, 614 (1980) 413—424

BBA 69029

USE OF FLUORIDE TO INACTIVATE PHOSPHORYLASE *a* PHOSPHATASES FROM RAT LIVER CYTOSOL

PRESENCE OF FLUORIDE-INSENSITIVE GLYCOGEN SYNTHASE-SPECIFIC PHOSPHATASE

CHARLES W. MACKENZIE, III *, GREGORY J. BULBULIAN † and JONATHAN S. BISHOP **

Department of Internal Medicine and the General Clinical Research Center, College of Health Sciences, University of Minnesota, Minneapolis, MN 55455 (U.S.A.)

(Received November 7th, 1979)

Key words: Fluoride inactivation; Phosphorylase a phosphatase; Glycogen synthase phosphate Mg^{2+}; Mn^{2+}; (Rat liver)

Summary

The diverse metal requirements for activity of the phosphoprotein phosphatases (EC 3.1.3.16) concerned with glycogen metabolism in rat liver were postulated to reflect the diverse binding intensities of their essential metal(s). After inactivation by fluoride, three of these phosphatases had similar metal requirements in contrast to a fourth phosphatase. Further similarities led to a grouping of these enzymes into two general types.

Phosphatases designated type 1 consisted of three enzymes which had the following properties: (1) preference for glycogen phosphorylase *a* as a substrate; (2) molecular weights in excess of 100 000; (3) conversion to an active 30 000 dalton 'subunit' form upon selective denaturation by 80% ethanol; (4) diverse degrees of stimulation by metals (Mg^{2+} and Mn^{2+}); and (5) changes to an absolute dependence upon added Mn^{2+} (but not Mg^{2+}) for activity of both the holoenzyme and the subunit after a demetallating treatment with fluoride in EDTA.

The phosphatase designated type 2 exhibited the following properties; (1) preference for glycogen synthase D as a substrate; (2) molecular weight of

* Present address: Department of Pharmacology, University of Nebraska Medical Center, Omaha, NE 68106, U.S.A.

** To whom correspondence should be addressed at: Box 504 Mayo Building, University of Minnesota Hospitals, Minneapolis, Minneapolis, MN 55455, U.S.A.

† Deceased.

The D and I forms of glycogen synthase are termed *a* and *b*, respectively, in IUB recommendation.

50 000; (3) no conversion to an active, 30 000 dalton subunit form upon selective denaturation by 80% ethanol; (4) complete metal-dependence upon either Mg^{2+} or Mn^{2+}; and (5) no change to an absolute dependence on added Mn^{2+} for activity after a demetallating treatment with fluoride in EDTA.

Introduction

Multiple species of phosphoprotein phosphatases (EC 3.1.3.16) have been isolated from a number of mammalian tissues, including liver [1—3], skeletal muscle [4,5], cerebral cortex [6] and brown fat cells [7]. While these species show differences in substrate specificity and metal requirements, collectively they are thought to have a common active 'catalytic subunit' [1,8].

The proteins which bind the catalytic subunit(s) to form the 'holoenzyme' are thought to confer substrate specificity by inhibiting, or regulating, the catalytic subunit activity [9,10]. These proteins share a number of properties: (1) denaturation by 80% ethanol at room temperature [11—13] or by high concentrations of 2-mercaptoethanol [14]; (2) trypsin sensitivity [11,14]; but may be heat-labile [10]. In addition other heat-stable, phosphatase-inhibitor proteins have been isolated [11,15—21], some of which show an enhancement [20,21] or a reduction [20] of inhibitory activity after phosphorylation by cyclic AMP-dependent protein kinase. One example of such an inhibitor, is specifically inactivated (dephosphorylated) by a manganese-requiring phosphatase [16,19].

Compared to the stimulation by metals of some holoenzyme phosphatases, the lack of metal dependence of the catalytic subunit [22] might suggest that the primary role of metals is to interact with the regulatory subunit proteins which inhibit certain activities of the catalytic subunit. However, evidence has recently been presented suggesting that the catalytic subunit itself may contain essential metal [23].

The objective of the present study was to define further the metal requirements of glycogen-related phosphoprotein phosphatases both in their holoenzyme and their catalytic subunit forms, in order to establish new criteria for their classification. For this study we used the property of fluoride (in EDTA) which inactivates glycogen phosphorylase *a* phosphatases [24] by demetallation [25]. It was found that three of the four phosphatases studied from rat liver cytosol could be classified together because of similar metal requirements after fluoride/EDTA treatment. Also, fluoride inactivation of the more abundant type 1 enzymes unmasked and so helped to identify the presence of the type 2, a glycogen synthase-specific phosphatase among proteins of the crude cytosol.

Methods

Preparation of substrates

Rabbit skeletal muscle phosphorylase *a*, ^{32}P-labeled, was prepared [26] from five-times crystallized phosphorylase *b*, [27], using phosphorylase *b* kinase [28] and [γ-^{32}P]ATP [29]. Glycogen synthase D was purified as the glycogen-

protein complex from rabbit skeletal muscle by the method of Smith et al. [30] and from dog liver by a method to be described in detail elsewhere. The latter method was similar to the one used previously for the I-form [31], but several modifications were made to minimize phosphatase contamination and to obtain native synthase in the D-form without an incubation step with ATP. The liver enzyme had an activity ratio of 0.07 (activity minus glucose-6-*P*/activity plus glucose-6-*P*) and was contaminated with less than 25 munits of Mn^{2+}-dependent synthase phosphatase per unit of synthase.

Enzyme analysis

Phosphorylase *a* phosphatase activity was measured by the rate of release of $^{32}P_i$ from ^{32}P-labeled muscle phosphorylase *a* essentially by the method of Kato et al. [14], except that this substrate was used at 4—8 μM as judged by ^{32}P concentration. 1 unit of activity was defined as 1 nmol of $^{32}P_i$ released per min at 30°C.

Glycogen synthase D phosphatase activity, using liver glycogen synthase D from dog at a concentration of 0.5 units/ml, was measured essentially by the method of Kikuchi et al. [32], with 0.1% bovine serum albumin/0.1% glycogen and the indicated metal or EDTA supplements in the reaction mixture. Muscle synthase D was used at 2 units/ml in imidazole buffer, as previously described [33]. Glycogen synthase was assayed according to Smith et al. [30]. 1 unit of activity here was defined as conversion of 1 unit of synthase D to the I-form per min at 30°C.

Preparation of the phosphatase fractions

The phosphoprotein phosphatases from rat liver cytosol were resolved by ion-exchange chromatography on DEAE-cellulose (Whatman DE-52) followed by gel filtration, essentially as described by Kobayashi et al. [1] using the buffers of Kikuchi et al. [32]. All procedures were carried out at 4°C, except where otherwise stated. Livers from fed Sprague Dawley male rats (300 g) were homogenized by a Dounce procedure at 0°C in 4 vols of buffer (62.5 mM glycyl-glycine, pH 7.4/62.5 mM EDTA/500 mM sucrose). The low speed extract (17 500 × *g*, 10 min) was recentrifuged at 113 000 × *g*, 60 min. The proteins of the supernatant cytosol were fractionated by addition of solid $(NH_4)_2SO_4$. The precipitate obtained between 30 and 60% saturation of this salt was dissolved in buffer 2 (10 mM glycyl-glycine, pH 7.4/5 mM 2-mercaptoethanol/20% glycerol) and applied to a Sephadex G-25 column equilibrated with buffer 2.

The desalted phosphatases, in a portion equivalent to 8 g of liver, were resolved into three fractions by DEAE-cellulose chromatography, using 3 vols DE-52 mixed with 2 vols Sephadex G-25, as described in the legend to Fig. 1. Each fraction was concentrated by ultrafiltration (Amicon) and was individually applied to a calibrated column of Sephacryl S-200, equilibrated with buffer 3 (20 mM Tris-HCl, pH 7.4/1 mM EDTA/2 mM $MgCl_2$/10 mM 2-mercaptoethanol/20% glyceral). Gel filtration, as described in the legend to Fig. 2, resolved the activity of fractions I and II into two phosphatases of different molecular size; those greater than 100 000 daltons (fractions IA and IIA) were

distinct from those of about 50 000 daltons (fractions IB and IIB). However, fraction IB, which was unstable, was not studied further; and fraction IA was studied without the gel filtration of fraction I. Fraction III contained a single phosphatase of about 105 000 daltons (fraction IIIA). The individual phosphatases were concentrated by ultrafiltration (Amicon), dialyzed for 48 h against buffer 2 and stored at −70 °C.

To study the metal requirements of the catalytic subunits generated from fractions IA, IIA, and IIIA, a separate resolution of the phosphatases was made on a larger scale. For this preparation, 5.7 g of protein was applied to DEAE-cellulose (2.2 × 37 cm) and eluted with 800 ml gradient (0—0.5 M NaCl). The separate treatment of each fraction, with 80% ethanol at room temperature was performed essentially as described by Brandt et al. [11]. The crude catalytic subunits derived from fractions IIA and IIIA were less metal-dependent than their respective holoenzymes (data not shown), as described by Kato et al. [14]. Following gel filtration, over 90% of the recovered activity from each fraction was found in a discrete peak of lower molecular weight (29 000—31 000) (data not shown) and could be stimulated 3- to 8-fold by manganese. However, fraction IIB appeared different from the others, since the activity surviving the ethanol treatment (about 20%) remained at 50 000 daltons (data not shown). Therefore fraction IIB, after ethanol treatment, was not studied further.

The usefulness of an acidification step to fractionate cytosol proteins before chromatography, reported by Kikuchi et al. [32] was tested. An aliquot of the supernatant cytosol (113 000 × *g*) was adjusted to pH 5.2 with 1 N acetic acid. After 20 min at 0°, one half of the sample was centrifuged (10 min at 12 000 × *g*). Both the pH 5.2 supernatant fraction and the uncentrifuged sample were neutralized prior to using the same $(NH_4)_2SO_4$ concentration and chromatography steps that were described above for the untreated cytosol.

Fluoride/EDTA pre-treatment of phosphatases

It was found that exposure to fluoride, or EDTA, or both agents together, resulted in varying degrees of a time-temperature dependent inactivation of these phosphatases, which was similar to that reported for inorganic pyrophosphate and EDTA by others [23]. Based on these studies (data not shown), a standard procedure was adopted for inactivation of the fractionated and the unfractionated phosphatases by overnight dialysis at 4 °C against buffer 2 to which 50 mM KF and 1 mM EDTA had been added. The fluoride/EDTA was then removed by exhaustive dialysis against buffer 2. The dialyzed enzymes were diluted at least 20-fold and assayed immediately without freezing. The catalytic subunits were supplemented with bovine serum albumin (1 mg/ml) for stability during this procedure.

Materials

Uridine diphospho-[U-^{14}C]glucose (250 mCi/nmol) was obtained from ICN and inorganic [^{32}P]phosphate (70 Ci/mg) was obtained from Amersham. Sephacryl S-200, ultrafine, and Sephadex G-25, medium, were obtained from Pharmacia, DEAE-cellulose (DE-52) from Whatman and ultrafiltration membranes (PM-10) from Amicon. All other supplies were obtained from commercial sources in the highest purity available.

Results

The metal requirements of some phosphorylase *a* phosphatases partially resolved from rat liver cytosol changed markedly following further steps in partial purification. Following initial resolution by DEAE-cellulose chromatography (Fig. 1a), as was first shown by Kobayashi et al. with enzymes from rabbit liver, the activities of these phosphatases were characteristically modulated by manganese [1]. The extent of the manganese modulation was expressed as the ratio of $+Mn^{2+}$ activity to +EDTA activity. The ratios of the initial fractions (I, II, and III) were about 0.25, 1.2, and 2.0 (Fig. 1a).

Gel filtration resolved fraction I (Fig. 2a) into two subfractions: (IA), a high molecular weight, manganese-independent species (ratio, 1.0); and (IB), a more labile, low molecular weight species which, like the unresolved fraction I (Table Ia, fraction I-before), was more active with EDTA than with manganese (ratio, 0.25).

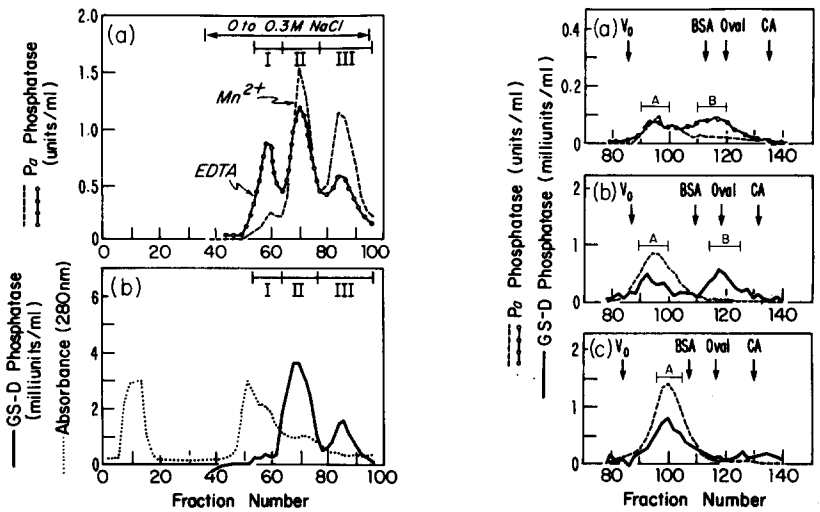

Fig. 1. Metal stimulation of liver phosphoprotein phosphatases. Cytosol protein (229 mg), equivalent to 8 g of rat liver, was applied to a DEAE-cellulose column (2.0 × 25 cm) equilibrated with buffer 2. Elution was with 400 ml of buffer 2, containing 0—400 mM NaCl as the indicated linear gradient. Fractions of 4.4 ml were collected. Protein concentration was monitored by absorbance at 280 nM (· · · · · ·). (a) phosphorylase *a* phosphatase with 5 mM $MnCl_2$ (- - - - - -) or 5 mM EDTA (o———o). (b) liver glycogen synthase D phosphatase with 5 mM $MgCl_2$ (———). Three enzyme fractions (I, II, and III), with peak activities eluting at 0.10, 0.16, and 0.24 M NaCl, respectively, were collected as indicated. They were concentrated by Amicon ultrafiltration and dialyzed against buffer 2 before gel filtration (Fig. 2).

Fig. 2. Gel filtration chromatography of phosphoprotein phosphatase fractions. Fractions of phosphoprotein phosphatase from rat liver cytosol resolved by DEAE-cellulose chromatography (Fig. 1) were applied to a column (2.6 × 90 cm) of Sephacryl S-200, superfine, which was equilibrated and eluted with buffer 3, in 2.2 ml fractions. (a) Fraction I (28 mg protein); (b) fraction II (14 mg protein); (c) fraction III (9 mg protein). Assays as in Fig. 1. Synthase phosphatase activity of fraction I was undetectable after chromatography. The indicated calibration standards were used: BSA (bovine serum albumin: 68 000 daltons); oval (ovalbumin: 45 000 daltons); and CA (carbonic anhydrase: 30 000 daltons). The indicated fractions, with activity peaks A and B, were collected as pools.

TABLE I

COMPARISON OF METAL REQUIREMENTS FOR ACTIVITY OF PHOSPHOPROTEIN PHOSPHATASE FRACTIONS

Phosphorylase *a* phosphatase activity (P*a*Pase) and glycogen synthase phosphatase activity (GSPase) was measured: a, using each phosphatase as holoenzyme; or b, using the catalytic subunit derived from each holoenzyme phosphatase. Parallel assays were done with muscle phosphorylase *a*, or with liver glycogen synthase D, as substrate in the presence of EDTA or metal ion(s) at the indicated concentration(s), before and after pretreatment with fluoride/EDTA.

Enzyme sample	Fluoride/ EDTA treatment	a						b				
		EDTA (5 mM)	$MgCl_2$ (5 mM)	$MnCl_2$ (1 mM)	$MnCl_2$ (5 mM)	$MnCl_2$ (1 mM) + $MgCl_2$ (5 mM)	Ratio: * GSPase / P*a*Pase	EDTA (5 mM)	$MgCl_2$ (5 mM)	$MnCl_2$ (5 mM)	$MnCl_2$ (1 mM) + $MgCl_2$ (5 mM)	Ratio: * GSPase / P*a*Pase
P*a*Pase		units/mg						units/ml				
I	Before	0.100	0.075	0.080	0.055	0.065	0.6	0.27	0.44	0.83	—	2.1
	After	0.006	0.006	0.055	0.066	0.061	2.3	0.02	0.04	1.11	—	4.1
IIA	Before	0.32	0.46	1.20	1.53	1.30	3.2	0.33	0.45	1.34	—	2.4
	After	0.00	0.06	0.54	1.84	1.61	4.1	0.02	0.04	0.61	—	4.0
IIB	Before	0.049	0.146	0.097	0.146	0.146	28.0	—	—	—	—	—
	After	0.000	0.123	0.061	0.123	0.123	34.0	—	—	—	—	—
IIIA	Before	2.0	4.0	53.0	73.0	65.0	3.5	0.07	0.09	0.58	—	3.1
	After	0.0	0.0	64.0	91.0	78.0	4.1	0.01	0.03	0.64	—	4.2
GSPase		munits/mg						munits/ml				
I	Before	0.015	0.040	0.040	0.005	0.040	—	—	0.19	—	1.75	—
	After	0.000	0.000	0.117	0.039	0.139	—	—	0.14	—	4.54	—
IIA	Before	0.09	0.88	0.69	1.71	4.17	—	—	0.32	—	3.23	—
	After	0.04	0.17	0.62	2.92	6.58	—	—	0.12	—	2.47	—
IIB	Before	0.00	2.23	4.32	2.33	4.08	—	—	—	—	—	—
	After	0.12	2.20	3.34	2.57	4.12	—	—	—	—	—	—
IIIA	Before	1.0	7.0	174.0	82.0	229.0	—	—	0.14	—	1.77	—
	After	0.0	0.0	281.0	91.0	320.0	—	—	0.09	—	2.69	—

* Ratio of phosphatase activities (GSPase/P*a*Pase): Panel a, using $MgCl_2$ (5 mM) + $MnCl_2$ (1 mM); Panel b, using $MnCl_2$ (5 mM) for P*a*Pase and $MgCl_2$ (5 mM) + $MnCl_2$ (1 mM) for GSPase.

Fraction II (fig. 2b) also contained two species (IIA, IIB) both of which were manganese-stimulated (data minus Mn^{2+} not shown). The smaller species (fraction IIB) had selective activity as a glycogen synthase phosphatase. Fraction III (Fig. 2c) had only a high molecular weight, manganese-dependent species (IIIA) (data minus Mn^{2+} not shown). After fractions II and III had been subjected to gel filtration, in the presence of Mg^{2+}-EDTA, the ratios of manganese stimulation (+5 mM Mn^{2+}/+5 mM EDTA) had increased by 4-fold and 16-fold to become 4.8 and 36.0 respectively (Table Ia, fractions IIA, before; and IIIA, before). Based on the recently accepted premise that phosphoprotein phosphatases are metalloenzymes, the working hypothesis was that three phosphatase fractions (IA, IIA, IIIA) differed primarily in the strength with which they bound the same intrinsic essential metal. The next step was to compare the metal requirements for total reactivation with manganese or magnesium of these individual enzymes from their same metal-free state.

Metal requirements after fluoride demetallation of type 1 phosphatases

All three of these phosphorylase *a* fractions (IA, IIA, and IIIa) had the same kind of metal requirements in the metal-free state, and so they were designated type1 enzymes. Thus, after fluoride/EDTA treatment, the three fractions were inactive not only in the presence of EDTA but also in the presence of magnesium (Table Ia). However, they were reactivated with manganese ($K_a < 100$ μM, data not shown). Such reversible inactivation constituted the indirect evidence in this study that these three phosphatases were metalloenzymes. Of the metals tested besides Mn^{2+}, only Co^{2+} (not shown) was also able to reactivate the enzymes; Mg^{2+}, Ca^{2+}, Fe^{2+}, Zn^{2+}, Cd^{2+}, Cu^{2+} and Ni^{2+} were ineffective (data not shown). Next, the three catalytic subunits from each of the three type 1 enzymes were treated with fluoride/EDTA. These subunits had become essentially inactive in assays containing EDTA or magnesium, but were fully reactivated by manganese (Table Ib). This result indicated that the catalytic subunit contained the binding site for the intrinsic essential metal of these phosphatases.

Metal requirements of type 1 synthase phosphatase activity

The synthase phosphatase activity of type 1 enzymes had a complete dependence upon metal, since this activity was not seen in the absence of metal either during chromatography (data not shown) or as isolated, concentrated fractions (Table Ia, except for fraction I). This dependence was satisfied by magnesium except when these enzymes had been completely demetallated, whether by purification (fraction IIIA, before), or by fluoride/EDTA (fractions I and IIA, after). However, not withstanding the above observations, manganese could reactivate both their phosphorylase and their synthase phosphatase activities, thereby substituting for magnesium. These data indicate that binding of metal at a second site, which is substrate-specific and distinct from that of essential metal, is required for the synthase phosphatase activity of the type 1 enzymes. Further evidence for this second site was provided by complex modulations of synthase phosphatase activity at saturating concentrations of metal (Table Ia). For instance, while magnesium stimulated the synthase phosphatase activity only slightly in the presence of 1 mM manganese with fractions

I and IIIA, the two metals acted synergistically for fraction IIA. This synergism was not seen with this enzyme when phosphorylase *a* was substrate. Also, whereas an increase in the manganese concentration from 1—5 mM inhibited the phosphatase activity of fraction IIIA with liver glycogen synthase, such an increase in manganese concentration caused only slight further activation with phosphorylase *a* and also with glycogen syntase from a different organ (muscle) (data not shown). It is impossible to conclude from the present data where the second metal binding site required for synthase phosphatase activity was located, whether at a second site on the phosphatase molecule or at a separate site on the substrate molecule, or both.

Identification of type 2, Mg^{2+}/Mn^{2+}-requiring synthase-specific phosphatase

In contrast to the type 1 phosphatases described above, fraction IIB (Fig. 2b) was of a different type. This 50 000 (±3000, $n = 11$) dalton enzyme was not converted to an active 30 000 dalton subunit upon treatment with 80% ethanol at room temperature (not shown). Although essentially inactive without metal, it was equally activated with either magnesium or manganese (Table Ia). These metal requirements were not changed to a specific manganese-dependence even by intensive efforts to demetallate it, as by prolonged dialysis (7 days) against either fluoride/EDTA or 0.5 mM 1,10-orthophenanthroline. Failure to affect the magnesium reactivation indicated that the enzyme had not contained a very tightly-bound intrinsic metal. It displayed a preference for glycogen synthase as substrate, having a substrate activity ratio (synthase phosphatase/phosphorylase phosphatase) 10-fold greater than that for the other phosphatase fractions. However, this specificity was 5-fold greater for synthase from liver than from muscle (data not shown). The properties, being different from those of type 1, are the basis for classifying fraction IIB as a type 2 phosphatase. It was concluded that this type 2 phosphatase resembled the Mg^{2+}-requiring synthase-specific phosphatase reported by Kikuchi et al. [32].

Uselfulness of the pH 5.2 precipitation step for purification of the synthase-specific phosphatase

It next became of interest to show why these results (Fig. 1) differed from those of Kikuchi et al. [32] who reported less phosphorylase *a* phosphatase in fractions I and II, and less synthase phosphatase in fraction III. Except for the use of G-25 Sephadex mixed with DE-52 (2 : 3, v/v) for improved flow characteristics, and omission of the on-line, counter-current dialysis of eluate from the DE-52 column, the chromatographic conditions reported here closely followed those published [32]. It was reasoned that the differences were due to either (1) use of an extra, acid precipitation step to fractionate the cytosol (at pH 5.2); or (2) failure to detect type 1 phosphorylase *a* phosphatases which had become demetallated during purification, or to both explanations.

To study the value of the pH 5.2 step, we compared DE-52 chromatograms of the cytosolic phosphatases with and without removal of the acid-insoluble cytosolic proteins. Most of the phosphorylase phosphatase activity in fractions I and II was removed by pH 5.2 isoelectric precipitation, while the magnesium-dependent synthase phosphatase activity in fractions II and III appeared unchanged (Fig. 3). However, because fraction III was especially

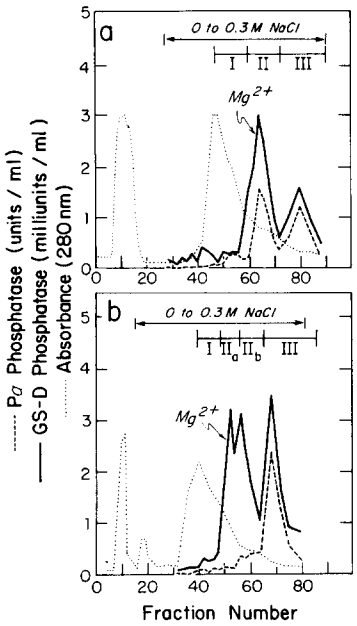

Fig. 3. Effect of pH 5.2 precipitation step on DEAE-cellulose chromatography of liver phosphoprotein phosphatases. Cytosol proteins, equivalent to 8 g rat liver, were chromatographed, after acidification of cytosol (pH 5.2) and after either a: No centrifugation or b: removal of precipitated proteins by centrifugation. The cytosol proteins were then neutralized and prepared for parallel chromatographies on DEAE-cellulose as in Fig. 1. Assays: phosphorylase *a* (P*a*) phosphatase with 5 mM $MnCl_2$ (- - - - -); glycogen synthase D (GS-D) phosphatase with 5 mM $MgCl_2$ (———); protein A_{280} (· · · · · ·).

susceptible to demetallation (inactivation) during resolution (Fig. 1a), only one fourth of its actual synthase phosphatase activity was recognized if assayed in the presence of only Mg^{2+}, without added Mn^{2+} (Fig. 4a, a'). Thus, the differences between this work and that of Kikuchi et al. [32] are explained on both counts. Furthermore, the acidification step did not appear to change the sizes of the phosphatases, since the gel filtration chromatography profiles of the phosphatase fractions of the acidified aliquot which was not centrifuged (data not shown) were the same as those of the untreated extract (Fig. 2).

Quantitation of type 1 and type 2 synthase phosphatases in activity profiles

The stability of these enzyme activities (+Mn^{2+}) throughout fluoride/EDTA inactivation suggested that, if the type 1 phosphatases were demetallated before chromatography, the relative amounts of both type 2 and total synthase phosphatases could be estimated by assaying with magnesium or with magnesium plus manganese, respectively. For this study, an aliquot of the extract equal to that used in Fig. 3a was pretreated with fluoride/EDTA prior to chromatography on a DEAE-cellulose column of identical size to those used before (Figs. 1, 3). Phosphorylase *a* phosphatase was detected in fractions II and III in Mn^{2+}-containing assays (Fig. 4a'), but no activity was detectable

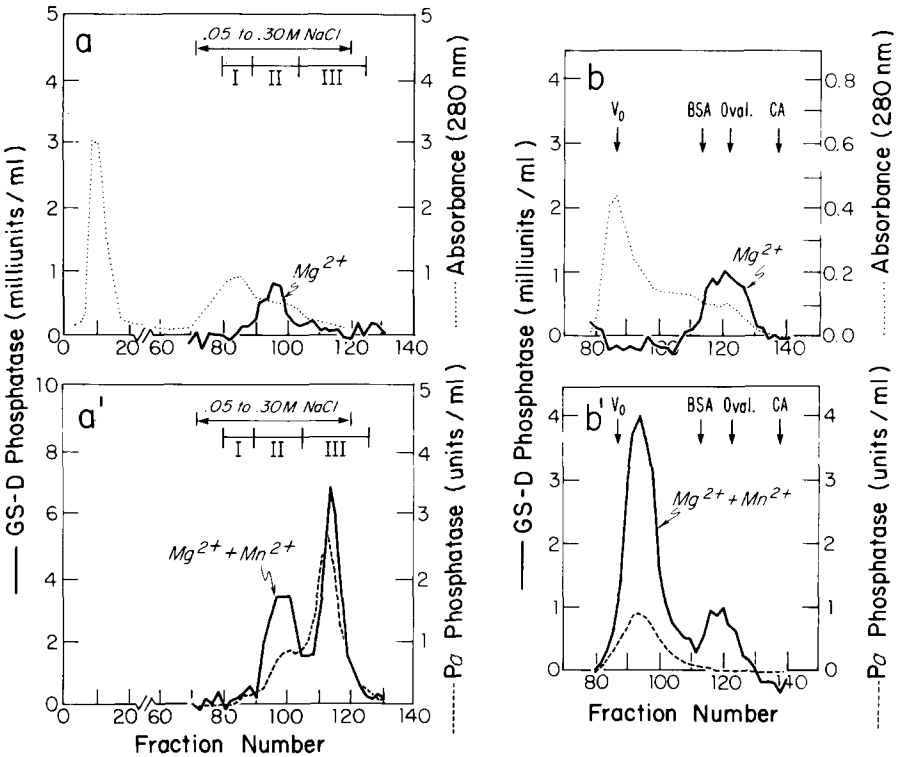

Fig. 4. Metal reactivation of fluoride/EDTA treated phosphoprotein phosphatases of liver cytosol. Acidified, non-centrifuged, cytosol proteins equivalent to 8 g rat liver, as in Fig. 3a, were treated with fluoride/EDTA (see Methods) and chromatographed as in Fig. 1 on DEAE-cellulose (a and a'). Fraction II from this chromatogram was further resolved by gel filtration as in Fig. 2 (b and b'). Assays: a and b: glycogen synthase D (GS-D) phosphatase with 5 mM $MgCl_2$ (———) and protein A_{280} (· · · · · ·). a' and b': glycogen synthase D (GS-D) phosphatase with 5 mM $MgCl_2$ plus 1 mM $MnCl_2$ (———), and phosphorylase *a* (*Pa*) phosphatase with 5 mM $MnCl_2$ (- - - - - -).

without manganese (data not shown). When type 2 synthase phosphatase was assayed using only magnesium (Fig. 4a), only a single peak was observed located in fraction II. When total synthase phosphatase was assayed using magnesium plus manganese the activity in fraction II increased 4-fold (Fig. 4a'). Gel filtration of fraction II confirmed that the Mg^{2+}-requiring synthase phosphatase was entirely of a small size (Fig. 4b). On the other hand, the Mn^{2+}-dependent activity was 3-times more abundant and was present as the large-sized, type 1 phosphatase (Fig. 4b'). Fraction III had about as much synthase phosphatase activity as fraction II (Fig. 4a'), whereas fraction I had negligible activity. By this rough chromatographic analysis it appeared that the type 2 synthase phosphatase accounted for about 12% of the total synthase phosphatase activity in the liver cytosol.

Discussion

These studies used the recently described action of fluoride to reversibly inactivate or demetallate, a metalloenzyme. This new action involved a reason-

able extension of the known property of fluoride to interact strongly with transition metals because of the high intensity of its negative charge. The greater effectiveness of fluoride compared to EDTA may be explained since the smaller F^- can more easily reach a metal located at a poorly accessible binding site. However, this role of fluoride is different from its conventional role as a reversible inhibitor of the metal requirements of a variety of phosphohydrolases [34] and other enzymes such as enolase and phosphoglucomutase [35] through formation of a fluoro-metallo-substrate complex.

The phosphoprotein phosphatases related to glycogen metabolism exist as several species with similar substrate capabilities. This multiplicity has made the search difficult for the most relevant enzymes suited to evaluate possible regulated pathways for hormone action. This report indicates a preliminary basis for grouping as type 1 enzymes the three major species of phosphorylase *a* phosphatase (fraction IA, IIA, IIIA) found in rat liver cytosol. Despite their original diversity, these enzymes after demetallation had similar metal requirements, both as the holoenzyme form and as the individually derived, 30 000 dalton, active catalytic subunit form. The restoration of their activity by metal ion (manganese) provided the necessary indirect evidence that each form was a metalloenzyme, which substantiated our working hypothesis. This work complements recent similar results from other laboratories, exemplified by a synthase phosphatase from muscle [25] and by the catalytic subunit phosphatase derived from the crude, unresolved, phosphorylase *a* phosphatases of skeletal muscle [23] or heart muscle [36]. These enzymes were thought to be metalloenzymes on the basis of similar indirect evidence after being inactivated by incubation with various agents such as fluoride [25], inorganic pyrophosphate [23,26], or nucleotide polyphosphates [23,25,36]. Although manganese (and also cobalt, unpublished data) reactivated each of these enzymes, the essential metal of the common catalytic subunit is not identifiable without direct evidence.

Besides the binding site for the essential metal, a second site for metal interaction was indicated because of a modulation of synthase phosphatase (rather than phosphorylase) phosphatase activity of the type 1 enzymes by saturating concentrations of either magnesium or manganese. These data could indicate metal interaction at a second site on the phosphatase molecule, as is known for magnesium in Zn^{2+} -Mg^{2+}-alkaline phosphatase from *E. coli* [37]. Alternatively, the site for this substrate-specific effect could have been the synthase molecule, since the reactivity of synthase itself, as a glucosyltransferase, is known to be modulated by these two metals [38].

In contrast to the type 1 phosphatases which were not reactivated by magnesium, fraction IIB was classified type 2 because of its different properties including a complete requirement for either magnesium or manganese and a selective activity as a synthase phosphatase. In these regards, fraction IIB seemed to resemble other recently reported enzymes: both the Mg^{2+}-requiring synthase-specific phosphatase of liver, Kikuchi et al. [32] and the Mg^{2+}/Mn^{2+}-dependent casein (or synthase) phosphatase of heart muscle, Li and co-workers [13,39]. Since the demetallating procedures effective for the type 1 enzymes failed to change the metal requirements of the type 2 enzyme, type 2 might not contain metal. Alternatively, since magnesium satisfied the absolute metal

requirements of the isolated enzyme, the original native enzyme could have contained loosely bound magnesium which was removed during chromatography.

Acknowledgements

We wish to acknowledge the excellent technical assistance of Charles J. Antony, and Faye H. Fang. This work was supported in part by NIH Grant No. 13319 and in part by a research grant (RR-400) from the General Clinical Research Centers Program of the Division of Research Resources, National Institutes of Health.

References

1 Kobayashi, M., Kato, K. and Sato, S. (1975) Biochim. Biophys. Acta 377, 343—355
2 Pinna, L.A., Donella, A., Uari, G. and Moret, V. (1976) Biochem. Biophys. Res. Commun. 70, 1308—1315
3 Titanji, V.P.K. and Pahlman, S. (1978) Biochim. Biophys. Acta 525, 380—391
4 Kato, K. and Sato, S. (1974) Biochim. Biophys. Acta 358, 299—307
5 Antoniw, J.F., Nimmo, H.G., Yeaman, S.J. and Cohen, P. (1977) Biochem. J. 162, 423—433
6 Maeno, H. and Greengard, P. (1972) J. Biol. Chem. 247, 3269—3277
7 Knight, B.L. and Skala, J.P. (1979) J. Biol. Chem. 254, 1319—1325
8 Brandt, H., Killilea, S.D. and Lee, E.Y.C. (1974) Biochem. Biophys. Res. Commun. 61, 598—604
9 Lee, E.Y.C., Brandt, H., Capulong, Z.L. and Killilea, S.D. (1976) in Advances in Enzyme Regulation (Weber, G., ed.), Vol. 14, pp. 467—490, Pergamon Press, Oxford.
10 Imazu, M., Imaoka, T., Usui, H. and Takeda, M. (1978) Biochem. Biophys. Res. Commun. 84, 777—785
11 Brandt, H., Lee, E.Y.C. and Killilea, S.D. (1975) Biochem. Biophys. Res. Commun. 63, 950—956
12 Chou, C.-K., Alfano, J. and Rosen, O.M. (1977) J. Biol. Chem. 252, 2855—2859
13 Li, H.-C., Hsiao, K.-J. and Chan, W.W.S. (1978) Eur. J. Biochem. 84, 215—225
14 Kato, K., Kobayashi, M. and Sato, S. (1974) Biochim. Biophys. Acta 371, 89—101
15 Baba, T. and Tsuiki, S. (1974) Biochim. Biophys. Acta 370, 419—430
16 Huang, F.L. and Glinsmann, W.H. (1976) Eur. J. Biochem. 70, 419—426
17 Cohen, P., Nimmo, G.A. and Antoniw, J.F. (1977) Biochem. J. 162, 435—444
18 Khandelwal, R.L. and Zinman, S.M. (1978) J. Biol. Chem. 253, 560—565
19 Cohen, P., Rylatt, D.B. and Nimmo, G.A. (1977) FEBS Lett. 76, 182—186
20 Huang, F.L., Tao, S. and Glinsmann, W.H. (1977) Biochem. Biophys. Res. Commun. 78, 615—623
21 Goris, J., Vandenheede, J.R. and Merlevede, W. (1978) Eur. J. Biochem. 91, 457—464
22 Brandt, H., Capulong, Z.L. and Lee, E.Y.C. (1975) J. Biol. Chem. 250, 8038—8044
23 Yan, S.C.B. and Graves, D.J. (1978) Fed. Proc. 37, 1425 (Abstr. 857)
24 Bulbulian, G.J., Bantle, G.A. and Bishop, J.S. (1976) Fed. Proc. 35, 1410 (Abstr. 269)
25 Khatra, B.S. and Soderling, T.R. (1978) Biochem. Biophys. Res. Commun. 85, 647—654
26 Torres, H.N. and Chelala, C.A. (1970) Biochim. Biophys. Acta 198, 495—503
27 Fischer, E.H. and Krebs, E.G. (1958) J. Biol. Chem. 231, 65—71
28 Krebs, E.G., Love, D.S., Bratvold, G.E., Trayser, K.A., Meyer, W.L. and Fischer, E.H. (1964) Biochemistry 3, 1022—1033
29 Glynn, I.M. and Chappell, J.B. (1964) Biochem. J. 90, 147—149
30 Smith, C.H., Villar-Palasi, C., Brown, N.E., Schlender, K.K., Rosenkrans, A.M. and Larner, J. (1972) Methods Enzymol. 28, 530—539
31 Bishop, J.S. and Larner, J. (1969) Biochim. Biophys. Acta 171, 374—377
32 Kikuchi, K., Tamura, S., Hiraga, A. and Tsuiki, S. (1977) Biochem. Biophys. Res. Commun. 75, 29—37
33 Kato, K. and Bishop, J.S. (1972) J. Biol. Chem. 247, 7420—7429
34 Antoniw, J.F. and Cohen, P. (1976) Eur. J. Biochem. 68, 45—54
35 Najjar, V.A. (1948) J. Biol. Chem. 175, 281—290
36 Hsiao, K.-J., Sandberg, A.R. and Li, H.-C. (1978) J. Biol. Chem. 253, 6901—6907
37 Anderson, R.A., Bosron, W.K., Kennedy, F.S. and Vallee, B.L. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 2989—2993
38 Nakai, C. and Thomas, J.A. (1975) J. Biol. Chem. 250, 4081—4086
39 Binstock, J.F. and Li, H.-C. (1979) Biochem. Biophys. Res. Commun. 87, 1226—1234