Upsala J Med Sci 83: 129–134, 1978

Separation of Rat-Liver Phosphoprotein Phosphatases Active on Phosphorylated Pyruvate Kinase (Type L)

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ABSTRACT

The substrate specificity of rat liver phosphoprotein phosphatases has been investigated. The enzymes were resolved into three fractions, termed A, B and C, on elution from DEAE-cellulose with apparent molecular weights, as determined by Sephadex G-200 chromatography, of approximately 250 000, 250 000 and 140 000, respectively. All fractions catalyzed the dephosphorylation of calf-thymus phosphohistones, salmon phosphoprotamine and rabbit skeletal muscle phosphorylase *a*. The major portion of the activity towards these substrates was found in fraction B. The activity towards rat liver phosphopyruvate kinase (type L) resided almost exclusively in fractions B and C. It is concluded that rat liver contains multiple forms of phosphoprotein phosphatases and that phosphatases of fraction B and C are the major activities towards phosphopyruvate kinase.

INTRODUCTION

The mechanism of hormonal regulation of carbohydrate metabolism in the liver has been studied extensively at the level of pyruvate kinase (type L) (for recent review see ref. 1). Experiments from this (1–3) and other laboratories (4–6) have established that pyruvate kinase (type L) is inhibited as a result of cyclic-AMP dependent phosphorylation. Whereas the cyclic-AMP dependent protein kinases which catalyse the phosphorylation of pyruvate kinase (type L) and other proteins have been investigated in great detail (for recent review and further references see 6), the phosphoprotein phosphatases which reverse the phosphorylation are still under intensive research (see 7 for further references).

After the discovery that a histone phosphatase preparation counterbalances the cyclic-AMP dependent phosphorylation of pyruvate kinase (type L) (8), attempts were made to purify the enzyme(s) and although an extensively purified preparation was obtained, severe losses in enzymatic activity were noted at the initial stages of the procedure (9). Since this inactivation was incompatible with the known stability of the final phosphatase preparation, it was postulated that labile enzyme forms were removed by the procedure.

The aims of this investigation were to separate the phosphoprotein phosphatases of rat liver cytosol and to compare the specificity displayed towards phosphopyruvate kinase with that observed towards selected phosphoproteins from other organs.

EXPERIMENTAL

Materials. Recrystallized bovine serum albumin and phenylmethylsulfonyl fluoride were bought from Sigma. Sephadex G-200 was from Pharmacia Fine Chemicals, Uppsala. "Buffer A" containing 10 mM imidazol/HCl, pH 7.5, 15 mM mercaptoethanol and 2.5 mM $MgCl_2$ was used as indicated.

(^{32}P)Phosphoproteins. Rat-liver pyruvate kinase was isolated and phosphorylated as described earlier (8). Sedimentation equilibrium experiments with pyruvate kinase were performed by the meniscus-depletion technique of Yphantis (10, 11). A Beckman Model E analytical ultracentrifuge equipped with an RTIC temperature control unit and an electronic control was used. All measurements were conducted in standard 12 mm double sector cells with sapphire windows. The centrifuge was run at 20°C for 18 h at 10 000 rpm. Pyruvate kinase was dialysed against 20 mM potassium phosphate, pH 7.0, containing 0.1 mM fructose 1,6-diphosphate and 0.1 mM dithiothreitol. Assuming a partial specific volume, $\bar{v}$, of 0.72 the molecular weight of pyruvate kinase was calculated to be 250 000. Assuming $E^{1\,cm}_{0.1\%}=0.68$ at 280 nm the maximal degree of phosphorylation was found to lie between 3.2 and 3.6 mol (^{32}P)phosphate per mol pyruvate kinase tetramer. Analysis of cold phosphate on the unlabelled enzyme was not performed, although the different maximal levels of phosphorylation obtained with different enzyme preparations suggests the presence of

some phosphate on the unlabelled enzyme. Prior to use, phosphopyruvate kinase was chromatographed on a Sephadex G-50 column equilibrated and eluted with 5 mM imidazol-HCl, pH 7.5, containing 10% glycerol, 50 mM KCl, 0.1 mM fructose-1,6-diphosphate and 0.1 mM dithiothreitol. (^{32})Phosphohistones (Sigma Type IIA) and (^{32}P)phosphoprotamine were prepared and the alkali-labile phosphate content determined as described previously (9, 12).

(^{32}P)Phosphorylase *a* was prepared using 5–10 mg of rabbit skeletal muscle phosphorylase *b* (Boehringer) and 0.3 mg phosphorylase *b* kinase (Sigma) essentially according to the method of Fischer & Krebs (13). Before use, (^{32}P)phosphorylase *a*, which contained about 4 nmol phosphate per mg protein, was dialyzed against 5 mM imidazol/HCl, pH 7.5, containing 1.0 mM dithiothreitol.

Enzyme assays and other methods. Phosphoprotein phosphatase activity was assayed according to a previous method (9). The final reaction mixture contained 50 mM tris/HCl, pH 7.5, 1 mM dithiothreitol, 0.1 mg per ml bovine serum albumin, 2.5 mM $MnCl_2$ and the respective phosphoproteins: 5 μM phosphopyruvate kinase, 7 μM (^{32}P)phosphorylase *a*, 20 μM (^{32}P)phosphohistones or 60 μM (^{32}P)phosphoprotamine. The reaction mixture for the dephosphorylation of pyruvate kinase contained, in addition, 5% glycerol and 0.05 mM fructose-1,6-diphosphate to stabilize the enzyme. The volumes of the reaction mixtures were 40 μl for the dephosphorylation of (^{32}P)-phosphopyruvate kinase and (^{32}P)phosphorylase *a* and 100 μl for the other substrates. The reaction was started by addition of the protein phosphatase and allowed to continue at 30°C for 5–10 min. The release of (^{32}P)-orthophosphate was linear for at least 15 min at the enzyme concentrations used. One unit of protein phosphatase is defined as the amount of enzyme which catalyses the release of 1 nmol of orthophosphate per min under these conditions. The substrate is expressed as the concentration of the (^{32}P)phosphate moiety in the respective phosphoproteins. The specific radioactivity of the phosphoproteins ranged from 20–100 cpm·pmol^{-1}. Cyclic AMP-dependent protein kinase activity was determined in "cell-sap" prepared as described earlier. One unit of protein kinase activity is defined as the amount of enzyme necessary to catalyze the incorporation of 1 pmol (^{32}P)-phosphate into calf-thymus histones under the specified conditions (8). Protein in crude fractions was assayed according to the method of Lowry et al. (14) and in purified fractions from the absorbance at 280 nm assuming $E^{1\,cm}_{0.1\%} = 1$.

Separation of protein phosphatases on DEAE-cellulose. Freshly excised livers (60 g) from male Sprague Dawley rats weighing 300–350 g were homogenized in 3 vol of 250 mM sucrose, containing 15 mM β-mercapteoethanol, 1 mM EDTA (pH 7.0, NaOH) and 0.1 mM phenylmethylsulfonyl fluoride. A Potter-Elvehjem glass-homogenizer fitted with a teflon pestle was used and homogenization was performed in 30 sec with six strokes at 940 rpm. This and other steps were performed at 0–4°C. The homogenate was centrifuged at 16 000×*g* for 20 min and the supernatant obtained was further spun at 46 000×*g* for 2 h. The postmicrosomal supernatant was filtered through glass wool to remove floating fat. The supernatant was applied to a DEAE-cellulose column (Whatman DE-52) (3.2×20 cm) equilibrated and eluted with buffer A containing 40 mM NaCl. The column was washed at a flow-rate of 200 ml/h for 5–6 h and the enzymes were eluted at a flow rate of 60 ml/h using a (500+500 ml) linear gradient of 40–350 mM NaCl in buffer A. Fractions of 10 ml were collected and assayed for protein phosphatase activity (Fig. 1)

Sephadex G-200 chromatography. To each of the pooled fractions in Fig. 1 (about 50 mg protein) solid ammonium sulphate to 70% saturation was added with constant stirring. After 15 min each sample was centrifuged at 16 000×*g* for 20 min and the precipitate dissolved in a minimal volume of buffer A and dialyzed against the same buffer containing 20% sucrose and 40 mM NaCl. The enzyme (about 2 ml) was then chromatographed on a Sephadex G-200 column (2.4×57 cm) equilibrated and eluted with buffer A containing 0.1 mM NaCl at a flow rate of 10–12 ml/h; fractions of 2.4–2.7 ml were collected and assayed for phosphoprotein phosphatase activity. In order to estimate apparent molecular weights of the phosphatase fractions, the column was calibrated with catalase (240 000), aldolase (158 000), bovine serum albumin (67 000) and hen's egg albumin (45 000) (results not illustrated).

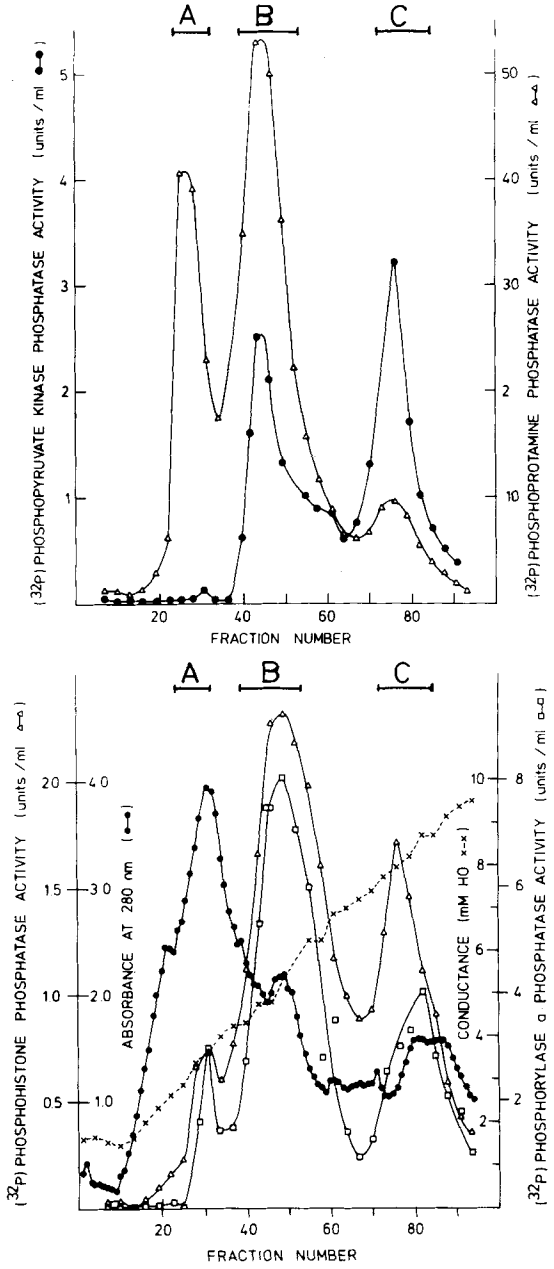
After the chromatography on Sephadex G-200, the enzymes were concentrated by ammonium sulphate precipitation (fractions A and B) or by ultrafiltration in a collodion bag (fraction C), and dialyzed at 1–2°C against buffer A containing 20% sucrose and 40 mM NaCl. The fractions were stored frozen in 0.1 ml portions and thawed only once prior to use. Little loss of activity occurred after 1 month. Ethanol precipitation at 20°C of phosphatase fractions was performed according to the method of Brandt et al. (15), except that 2–4 mg protein per ml was added to 5 ml 95% ethanol. The precipitate was collected at 10 000×*g* for 5 min at 4°C, extracted with buffer A and dialysed against 1 l of the same buffer for 8–12 h prior to assay.

RESULTS AND DISCUSSION

Separation of rat-liver phosphoprotein phosphatases active on phosphorylated pyruvate kinase (type L)

Experiments were carried out in order to compare the elution profiles of phosphoprotein phosphatases active towards (^{32}P)phosphopyruvate kinase with those active towards other phosphoproteins. Three fractions, termed A, B and C, were distinguished in order of elution from DEAE-cellulose (Fig. 1). Fractions A, B and C were eluted between 0.1–0.15, 0.17–0.25 and 0.27–0.3 M NaCl respectively. The total recovery of (^{32}P)phosphoprotamine phosphatase was usually about 50–70% of the initial activity (Table I).

Almost all the activity on (^{32}P)phosphorylated pyruvate kinase was recovered in fractions B and



Figs. 1. Separation of phosphoprotein phosphatase on DEAE-cellulose. The two panels to the same experiment. Details are given in the text and in the legend to Table I. The horizontal bars indicate the fractions that were used for further experiments.

C (Fig. 1). Fraction A showed only trace activity on this substrate. (^{32}P)Phosphoprotamine, (^{32}P)-phosphohistones and (^{32}P)phosphorylase *a* of rabbit skeletal muscle were the better substrates for phosphatase fraction A.

The relative ratio of (^{32}P)phosphoprotamine/(^{32}P)phosphopyruvate kinase phosphatase activities was found to be constant in different preparations of the postmicrosomal supernatant, but different for the separated fractions A, B and C (Fig. 1, Table I). Fraction C showed the lowest ratio of phosphoprotamine/phosphopyruvate kinase phosphatase activity, indicating increased preference for the latter substrate.

In order to further compare the phosphatases, their physical properties were examined by chromatography on a calibrated Sephadex G-200 column. Phosphatase fractions A and B had a similar apparent molecular weight, approximately 250 000 (based on the mean of two determinations for each fraction). Fraction C had an apparent molecular weight of 140 000. Analysis of samples from the different regions of the Sephadex G-200 chromatograms revealed no further resolution of phosphoprotamine phosphatase from phosphopyruvate kinase phosphatase activity (results not illustrated).

The apparent molecular weights of the fractions are higher than those reported by Kobayashi et al. (see footnote in ref. 16, p. 353). The reason for this difference is not known. The conditions used for the preparation and chromatography of the extracts in the present studies are different from those used by Kobayashi et al. It has been suggested that multiple forms of liver phosphoprotein phosphatases might be produced by the action of proteases, released from lysosomes during vigourous homogenisation of the tissue (17). To minimize the effects of proteolysis, homogenisation was performed under mild conditions and in the presence of phenylmethylsofonyl fluoride. Although complete inhibition of proteolysis could not be guaranteed, its effects were considered to be small, because fractions with apparent molecular weights lower than reported above could not be detected even after storage of the cytosol fraction for 6 h before further purification. This observation, taken together with the differences in kinetic properties elaborated below, supports the possibility that fraction A, B and C probably serve different functions.

Stability of the phosphatases

Under the conditions chosen for chromatography and upon storage at 2°C phosphoprotein phosphatase, fraction B seemed to be the most stable,

Table I. *Purification of phosphoprotein phosphatases from rat-liver cell-sap*

Phosphoprotamine and phosphopyruvate kinase phosphatase activities varied widely between 0.12–0.4 and 0.01–0.03 μmol (^{32}P)phosphate released per g wet weight of liver and min respectively (based on recoveries from cell-sap). The recovery of protein in the same fractions was about 100 mg per g of liver. In this particular experiment 14400 units of phosphoprotamine phosphatase and 1200 units of phosphopyruvate kinase phosphatase were recovered in the cell-sap from 60 g of liver. The calculation of the recovery of enzyme activity after the Sephadex column is based on the values obtained after chromatography of a portion of the activity from the DEAE-cellulose step. The slight activation of phosphatase B was not always obtained. The activity ratio is defined as the rate of dephosphorylation of (^{32}P)phosphoprotamine (60 μM) divided by the rate of the dephosphorylation of (^{32}P)phosphopyruvate kinase (5 μM)

Step	Phosphoprotamine (u/mg protein)	Phosphatase yield (%)	Phosphopyruvate kinase phosphatase		Activity ratio[a] ^{32}P-protamine ^{32}P-pyruvate kinase
			u/mg protein	% yield	
Cell-sap	3.0	100	0.3	100	11.6±0.6
DEAE-cellulose					
A	11.9	17.0	[b]	0.8	[b]
B	25.2	44.6	0.9	19.7	16.1±2.6
C	7.4	7.1	1.7	19.2	4.3±1.5
Sephadex G-200					
A	43.5	17.8	[b]	[b]	[b]
B	114.0	59.6	3.5	22.7	31.2
C	13.0	2.8	2.2	7.5	5.9

[a] Activity ratio: mean±S.D. of four preparations are given for cell-sap and DEAE-cellulose fractions; mean values of two preparations are given for the Sephadex G-200 fractions.
[b] In most cases the rates of the dephosphorylation of (^{32}P)phosphopyruvate kinase were too low for reliable estimates to be made.

followed by fraction A. Phosphatase fraction C was the least stable; enzymatic activity decreased during freezing and thawing in the absence of sucrose. Under similar conditions, phosphatases A and B maintained full enzymatic activity. As can be seen in Table I, only about 30% of the phosphatase C activity applied to the Sephadex G-200 column was recovered, whereas the other two fractions were almost quantitatively recovered.

Earlier, a phosphoprotein phosphatase (M. W. 32000) active towards phosphorylated pyruvate kinase was purified through an unusual ethanol precipitation step at 20°C as described by Brandt et al. (9, 15). The stability of the phosphatase fractions A, B and C, was also investigated using the ethanol treatment. We found that the enzymatic activity of fractions A and B towards (^{32}P)phosphorylase *a* were increased by 50–100% after ethanol treatment (15). However, the activity of the same fraction A towards (^{32}P)phosphoprotamine and (^{32}P)phosphopyruvate kinase decreased to about 30% of the initial values. With phosphatase fraction B, the decrease was to about 70% of the initial activity. Regardless of the substrate used, ethanol treatment either completely inactivated phosphatase C or reduced the activity to less than 30–50% of the initial values. Whereas the precipitation of crude extracts with ethanol at 20°C facilitates the further purification of a phosphoprotein phosphatase, the same treatment reduces the activity of the DEAE-cellulose fractions towards all the other substrates studied except phosphorylase *a*. These observations might serve to explain the losses in enzymatic activity observed during our earlier attempts (9) to purify a phosphoprotamine plus phosphopyruvate kinase phosphatase through the unusual ethanol precipitation step of Brandt et al. (15).

The available data do not permit us to identify the fraction yielding the low molecular weight phosphoprotein phosphatase which was purified earlier in this laboratory (9). In order to resolve this question, further purification of the enzymes would be required. The different stability properties support the existence of different phosphoprotein phosphatases, perhaps with overlapping specificities.

Kinetic results

In view of the differences in molecular size and stability properties described above, some kinetic experiments were made to further compare the frac-

Table II. *Effectors of phosphoprotein phosphatases B and C*

The standard reaction medium containing 5 μM (^{32}P)-phosphopyruvate kinase was used. Divalent cations were not included in the tests, except where specifically indicated. Phosphatase B (6.78 μg per test) and phosphatase C (4.22 μg per test) were from the DEAE-cellulose step. The mean±S.D. of data from four determinations are shown. Other conditions were described in the Experimental section

Effector	pmol released/5 min	
	Phosphatase B	Phosphatase C
1. No additions	5.6±0.2	29.1±1.3
2. 2.5 mM EDTA	2.2±0.1	20.9±1.9
3. 2.5 mM $MgCl_2$	48.1±1.1	55.5±0
4. 2.5 mM $MnCl_2$	28.1±0.08	46.0±2.8
5. 5 mM potassium phosphate pH 7.5	3.02±0.4	13.2±0.9
6. 25 mM NaF	2.25±0.3	10.0±0.6
7. 1 mM ATP	2.42±1.5	8.7±0.3
8. 1 mM ATP+2.5 mM $MgCl_2$	46.5±2.5	46.8±0

tions B and C which showed the highest recovery of enzymatic activity towards pyruvate kinase. Mn^{2+} and Mg^{2+} stabilized or stimulated the activity of both fractions. The extent of stimulation observed was greater with phosphatase B than with phosphatase C. Orthophosphate, ATP and NaF were inhibitory (Table II). Partial inhibition of enzymatic activity by EDTA indicates that divalent cations are not absolutely required.

The amount of phosphoprotein phosphatase activity towards phosphopyruvate kinase found in the post-microsomal supernatant (Table 1) was estimated to be about 30 units per g wet weight of liver. This would be sufficient to completely dephosphorylate in about 10 secs all the pyruvate kinase present in normally fed rats (about 1 μM subunit). In other experiments, the total recovery of cyclic AMP-dependent protein kinase in a post-microsomal fraction was found to be 30000 units per g wet weight of liver. Since histones were phosphorylated at about 50% of the rate of pyruvate kinase (Ref. 8, Fig. 2), it was estimated that complete phosphorylation of pyruvate kinase in the livers of normally fed rats would take about 1 sec. These findings, together with the identification of fractions B and C as the main phosphopyruvate kinase phosphatase *in vitro* give further support for the physiological relevance of the reversible dephosphorylation.

ACKNOWLEDGEMENTS

This investigation was supported by grants from the Swedish Medical Research Council 13X-4485 and the Medical Faculty, the University of Uppsala. Ultracentrifugation analyses were kindly performed by Dr H. Pertoft at this Institute.

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Received May 30, 1978

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