A HIGHLY PURIFIED RAT-LIVER PHOSPHOPROTEIN PHOSPHATASE PREPARATION WITH ACTIVITY TOWARDS PHOSPHOPYRUVATE KINASE (TYPE L).

Vincent P. K. TITANJI+, Örjan ZETTERQVIST and Lorentz ENGSTRÖM
Institute of Medical and Physiological Chemistry, Biomedical Centre, Box 575, S-751 23 Uppsala, Sweden

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1. Introduction

The control of pyruvate kinase (type L) activity by enzymatic phosphorylation and dephosphorylation has been proposed [1–7] to be important in establishing the balance between glycolysis and gluconeogenesis in the liver. At gluconeogenetic conditions pyruvate kinase is presumably inhibited as a result of a cyclic AMP-dependent phosphorylation [2,3,7]. Whereas the mechanism of the phosphorylation of pyruvate kinase is easily understood by comparison with that of phosphorylase *b* kinase and glycogen synthetase *a*, the properties of the dephosphorylation reaction are still under investigation [3,8–11]. For example, the relationship of phosphopyruvate kinase phosphatases to other phosphoprotein phosphatases of carbohydrate metabolism is still unclear. Here, we have modified the procedure in [8] and obtained from rat liver a highly purified phosphoprotein phosphatase that is approaching protein homogeneity, as judged by gel electrophoresis. This enzyme preparation is active in vitro, not only towards phosphopyruvate kinase, but also towards phosphorylase *a*, phosphoprotamine and mixed phosphohistones.

2. Materials and methods

[^{32}P]Phosphorylase *a* was prepared essentially as in [12]. [^{32}P]Phosphopyruvate kinase, [^{32}P]phosphoprotamine, and [^{32}P]phosphohistones were prepared as in [8]. The catalytic subunit of cyclic AMP-dependent protein kinase used was prepared by method B from peak I of rabbit skeletal muscle protein kinase [13]. The specific activity of [^{32}P]phosphoproteins ranged from 20–100 cpm/pmol. Dephosphorylation of [^{32}P]phosphoproteins at concentrations indicated, was performed as in [8]. The final reaction mixture contained 50 mM Tris–HCl (pH 7.5), 1 mM dithiothreitol, 0.1 mg bovine serum albumin/ml, 2.5 mM $MnCl_2$ and the respective phosphoproteins at the concentrations in the figure legends and text. The reaction mixture for dephosphorylation of pyruvate kinase contained 0.05 mM fructose 1,6-diphosphate to stabilize the enzyme. The volumes of the reaction mixture 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 the addition of protein phosphatase and allowed to continue at 30°C for 5 min, except in fig.4 where longer incubation periods were used. The release of [^{32}P]orthophosphate was linear for ⩾10 min at the enzyme concentrations used. One unit of protein phosphatase is defined as the amount of enzyme necessary to catalyse the release of 1 nmol phosphate/min under these conditions. Substrate concentration refers to the phosphate moiety in the respective phosphoproteins. Polyacrylamide disc gel electrophoresis was done following [14]. Histone–agarose was synthesized as in [10,15], except that 1 g CNBr was used to activate 10 ml settled Sepharose 4B (Pharmacia). Sephadex G-100 was from Pharmacia, Uppsala. All other reagents were of highest purity available from Boehringer, Merck and Sigma.

+Present address: Dept. of Biochemistry, University Centre for Health Sciences, University of Yaoundé, Yaoundé, United Republic of Cameroon, West Africa

2.1. *Modified procedure for the purification of phosphoprotein phosphatase*

Liver phosphoprotein phosphatase was purified as in [8] up to and including the second DEAE-cellulose chromatography. The pooled and concentrated enzyme (~5 ml) was then chromatographed on a Sephadex G-100 column (2.4 × 47 cm) equilibrated and eluted with 50 mM imidazol/HCl (pH 6.5) containing 20% glycerol, 15 mM 2-mercaptoethanol, 0.1 M NaCl and 2.5 mM $MgCl_2$. The fraction with spec. act. >800 units/mg protein were pooled, concentrated and rechromatographed on the same column (fig.1). The most active fractions from the second Sephadex G-100 chromatography were concentrated in a collodion bag to ~2–3 ml and stored in 50% glycerol at −25°C. Under these conditions the enzyme is stable for >6 months. For further purification ~5000 units of the phosphatase were diluted 4-fold with 50 mM imidazol/HCl (pH 7.5) containing 20% sucrose, 15 mM 2-mercaptoethanol, 2.5 mM $MgCl_2$ and applied to a histone–agarose column equilibrated with the same buffer. The column was washed with ~40 ml of the starting buffer and eluted at a 20 ml/h flowrate with a linear gradient of 0.1–0.5 M NaCl (100 ml + 100 ml) in the starting buffer. The most active fractions were pooled (fig.2) and concentrated as above. The A_{280} was measured and the specific activity of the pooled and concentrated enzyme determined. 87% glycerol was added to final concn. 50% and it was ascertained that the protein was >0.2 mg/ml. The enzyme was stored at −25°C and remained active for ≥2 months.

3. Results and discussion

3.1. *Purification and properties of a rat-liver phosphoprotein phosphatase*

In [8] a procedure was developed for the partial purification of a rat-liver phosphoprotein phosphatase which was active on [^{32}P]phosphopyruvate kinase, [^{32}P]phosphohistones and [^{32}P]phosphoprotamine. Here we have modified the procedure in [8], permitting the recovery of phosphoprotein phosphatase in a highly purified form and in a final yield of 5% (table 1). The additional, effective steps used in this preparation were chromatography on Sephadex G-100, which yielded a >5-fold purification (fig.1), and histone–agarose chromatography (fig.2). Examination of 1.4 μg purified phosphatase by SDS–polyacrylamide disc gel electrophoresis revealed a single Coomassie blue-stained band (fig.3). This electrophoresis displayed a protein of mol. wt 35 000, which is in good agreement with values obtained by Sephadex G-100 chromatography of the partially purified enzyme, run in non-denaturing conditions [8].

Here we describe a reliable and relatively convenient procedure for the extensive purification of rat-liver phosphoprotein phosphatase active against phosphopyruvate kinase. The recovery at the step of precipitation with ethanol was rather constant for different preparations, i.e., ~70–100 units phosphatase/g liver. This, however, represents 15–60% of the activity of the initial extract. The reason for this variation is unknown. If the enzymatic activity

Table 1
Purification of a rat-liver phosphoprotein phosphatase

	Total units	Units/mg protein	Yield %
1. 16 000 × *g* Supernatant	325 740	2.07	100
2. 0–70% Ammonium sulphate	291 350	2.24	89
3. Extract of ethanol precipitate	47 840	4.10	14.6
4. DEAE-cellulose I	33 978	13.6	10.4
5. DEAE-cellulose II	33 565	506	10.3
6. Sephadex G-100 II	17 283	2948	5.3
7. Histone–agarose	[a]	5300	5.3

[a] The recovery at this step was nearly quantitative since values obtained from 3 separate expt were 100, 100 and 80% of the initial amount. Protein was measured according to Lowry for steps 1 and 2 and from the A_{280} assuming $E^{1\ cm}_{0.1\%} = 1$ for the other steps. Phosphatase activity was measured with 60 μM [^{32}P]-phosphoprotamine as substrate [8]

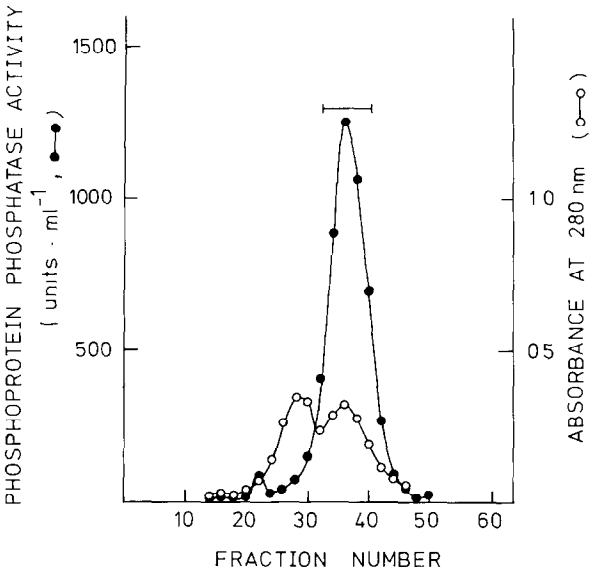
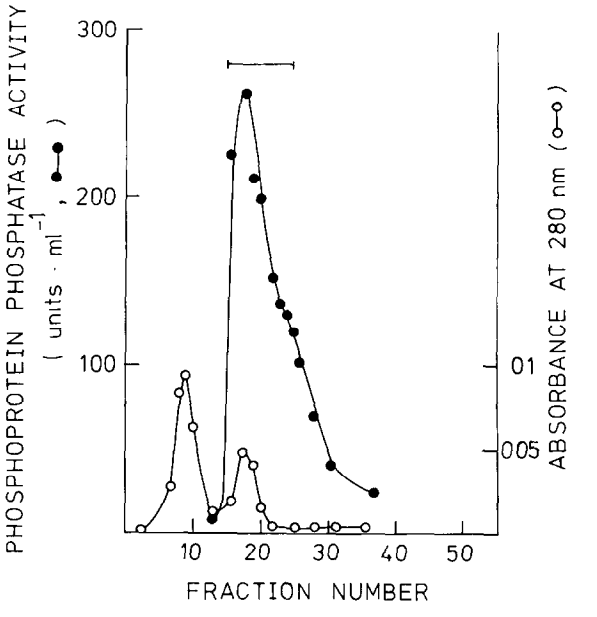


Fig.1. Sephadex G-100 chromatography of rat-liver phosphoprotein phosphatase. Protein phosphatase (33 600 units) from the DEAE-cellulose chromatography II (see table 1) was first chromatographed on a Sephadex G-100 column I equilibrated and eluted as in the text. After this first G-100 chromatography which is not illustrated, 29 100 units was recovered partially separated from most of the protein which emerged before the activity. The pooled material was concentrated and rechromatographed on the same Sephadex G-100 column, as shown in the figure. Phosphatase activity was detected with [^{32}P]phosphoprotamine (60 μM) as substrate. The horizontal bar indicates the fractions used in further experiments.



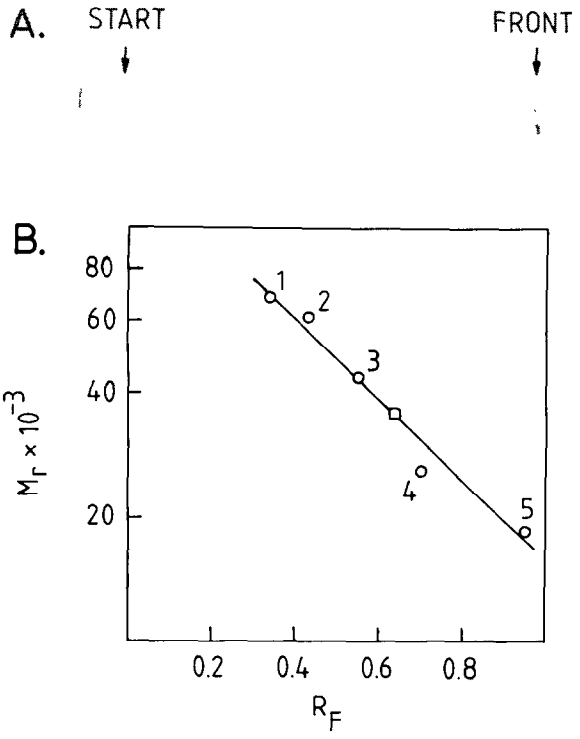


Fig.3. SDS disc gel electrophoresis of highly purified phosphoprotein phosphatase. The enzyme and reference proteins were treated essentially as in [17], i.e., with Na_2CO_3, SDS and mercaptoethanol. 60 μl, containing 1.4 μg of each protein, were applied to disc gel electrophoresis in 0 4244 M Tris and 0.0308 M HCl on gel 11.1 × 0.9 cm [14]. (A) Phosphatase; (B) Phosphatase (□) plus reference proteins (○); (1) bovine serum albumin (subunits mol. wt 68 000); (2) beef liver catalase (60 000); (3) ovalbumin (43 000); (4) bovine pancreas chymotrypsinogen (25 700); (5) β-lactoglobulin (18 400).

obtained at the precipitation with ethanol is taken to be represented by most of the phosphatase with mol. wt 35 000, a ~0.4 μmol/kg liver can be estimated.

The present enzyme preparation is similar to other phosphatases of similar size (mol. wt 30 000–35 000) in its stability to an unusual ethanol precip-

Fig.2. Histone–agarose chromatography. Phosphoprotein phosphatase (5600 units) from the Sephadex G-100 II chromatography was purified on a histone-agarose column (1.2 × 6 cm), equilibrated and eluted as in section 2. Fractions of 3 ml were collected. The horizontal bar indicates the fractions used in further experiments. Further details are in the text.

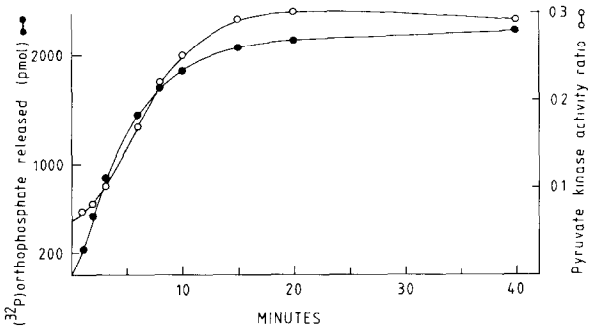


Fig.4. Effect of the purified phosphoprotein phosphatase on pyruvate kinase activity. [^{32}P]Phosphopyruvate kinase, 2000 pmol in terms of [^{32}P]phosphate, was incubated at 30°C with 2 units purified phosphoprotein phosphatase, in a final mixture (200 μl) containing 0.050 mM fructose-1,6-diphosphate, 15% glycerol, 25 mM KCl, 2.5 mM $MnCl_2$, 50 mM $MnCl_2$, 50 mM Tris–HCl, 0.5 mM potassium phosphate (pH 7.5), 0.1 mg bovine serum albumin/ml and 1 mM dithiothreitol. Reactions were run in triplicate. At times indicated, 20 μl were withdrawn from 2 of the mixtures for determination of [^{32}P]orthophosphate released [8]. 20 μl were withdrawn from the third incubation mixture and diluted with 100 μl ice-cold, 20 mM potassium phosphate (pH 7.0) containing 30% glycerol, 0.1 mg bovine serum albumin/ml and 1 mM dithiothreitol. These samples were stored at −25°C until thawed once and assayed for pyruvate kinase activity at 0.15 mM and 5 mM phosphoenolpyruvate [2]. The other ingredients in the pyruvate kinase test were 0.15 mM NADH, 5 mM $MgCl_2$, 5 mM potassium phosphate (pH 7.5), 2 units lactate dehydrogenase, 1 unit hexokinase, 2 mM glucose, 0.1 mg bovine serum albumin/ml and 0.1 mM dithiothreitol. The sample of [^{32}P]phosphopyruvate kinase used contained 0.73 mol phosphate/mol subunit, assuming subunit mol. wt 62 500 and $E^{1\,cm}_{0.1\%}$ = 0.68 at 280 nm. During the entire experiment total pyruvate kinase as measured at 5 mM phosphoenolpyruvate was stable. The activity ratio of pyruvate kinase prior to phosphorylation was 0.3. (The activity ratio is defined as the ratio of pyruvate kinase activity at 0.15 mM phosphoenolpyruvate to that at 5 mM phosphoenolpyruvate.)

itation step at 20°C, and its broad specificity in vitro. The enzyme of the present preparation catalysed the dephosphorylation of [^{32}P]phosphoprotamine, [^{32}P]phosphorylase *a* [^{32}P]phosphopyruvate kinase and [^{32}P]phosphohistones. In a typical experiment at 5 μM substrate, the rates of dephosphorylation of the aforementioned substrates were similar, i.e., 475, 550, 515 and 500 units/mg protein phosphatase, respectively.

However, in contrast to other preparations, the present one tends to be destabilized when stored in EDTA and has so far proved to be insensitive to inhibition by heat-stable inhibitors 1 and 2 prepared from rabbit skeletal muscle [21,24]. No effect of these inhibitors was obtained at 5 μM [^{32}P]phosphopyruvate kinase, [^{32}P]phosphoprotamine and [^{32}P]phosphorylase *a*. These types of inhibitors are present in several tissues and are known to inhibit phosphorylase *a* phosphatase activity [21,22]. The cause of the lack of inhibition is not known at the present, but the findings suggest that not all enzymes with phosphorylase *a* phosphatase activity are inhibited by these inhibitors.

In [2] it was shown that the dephosphorylation of [^{32}P]phosphopyruvate kinase by a partially purified preparation of rat-liver phosphoprotein phosphatase, reactivated the enzyme. Fig.4 shows that the reactivation of rat-liver pyruvate kinase (type L) is accomplished also by the highly purified enzyme of this report. Thus, this preparation may very well be responsible for dephosphorylation of phosphopyruvate kinase in vivo. However it remains to be determined whether this low molecular weight enzyme represents all of the phosphopyruvate kinase phosphatase activity. Multiple, high molecular forms of phosphopyruvate kinase phosphatase were demonstrated in rat-liver extracts prepared under mild conditions [10, 23].

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References

[1] Ljungstrom, O., Hjelmquist, G. and Engström, L. (1974) Biochim. Biophys. Acta 358, 289–298.
[2] Titanji, V. P. K., Zetterqvist, O. and Engström, L. (1976) Biochim. Biophys Acta 422, 98–108.
[3] Felieu, J. E., Hue, L. and Hers, H.-G. (1976) Proc. Natl. Acad. Sci. USA 73, 2762–2766.
[4] Engström, L. (1978) in: Regulatory Mechanisms of Carbohydrate Metabolism, Proc. 11 FEBS Meet. (Esmann, V. ed) pp. 53–60, Pergamon Press, New York.
[5] Riou, I. P., Claus, T. H. and Pilkis, S. I. (1977) J. Biol. Chem. 253, 656–659.
[6] Eigenbrodt, E., Mostafa, A. F. M. and Schoner, W. (1977) Hoppe-Seyler's Z. Physiol. Chem. 358, 1047–1055.

[7] Blair, J. B., Cimbala, M. A., Foster, I. L. and Morgan, R. A. (1976) J. Biol. Chem. 251, 3756–3762.
[8] Titanji, V. P. K. (1977) Biochim. Biophys. Acta 481, 140–151.
[9] Titanji, V. P. K. (1977) Acta Univ. Uppsal. 284, 8–17.
[10] Titanji, V. P. K. and Påhlman, S. (1978) Biochim. Biophys. Acta 525, 380–391.
[11] Eigenbrodt, E. and Schoner, W. (1977) Hoppe-Seyler's Z. Physiol. Chem. 358, 1057–1067.
[12] Fischer, E. H. and Krebs, E. G. (1962) Methods Enzymol. 5, 373–376.
[13] Beavo, J. A. Bechtel, P. J. and Krebs, E. G. (1974) Methods Enzymol. 38, 299–308.
[14] Neville, Jr., D. M. (1971) J. Biol. Chem. 246, 6328–6334.
[15] Tsung, P. K., Sakamoto, T. and Weissmann, G. (1974) Biochem. J. 145, 437–448.
[16] Khandelwal, R. L., Vandenheede, J. R. and Krebs, E. G. (1976) J. Biol. Chem. 251, 4850–4858.
[17] Neville, D. M., jr (1971) J. Biol. Chem. 246, 6335–6338.
[18] Lee, E. Y. C., Brandt, H., Capulong, Z. and Killilea, S. D. (1976) Adv. Enz. Reg. 14, 467–488.
[19] Chou, C. K., Alfano, I. and Rosen, O. M. (1977) J. Biol. Chem. 252, 2855–2859.
[20] Li, H-C., Hsiao, K-J. and Chan, W. W. S. (1978) Eur. J. Biochem. 84, 215–225.
[21] Cohen, P., Nimmo, G. A. and Antoniw, J. F. (1977) Biochem. J. 162, 435–444.
[22] Huang, F. L., Tao, S. and Glinsmann, W. H. (1977) Biochem. Biophys. Res. Commun. 78, 615–623.
[23] Titanji, V. P. K. (1978) Uppsala, J. Med. Sci. 83, 129–134.
[24] Nimmo, G. A. and Cohen, P. (1978), Eur. J. Biochem. 87, 341–351.