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PHOSPHORYLATION OF PURIFIED RAT LIVER PYRUVATE KINASE BY CYCLIC 3′, 5′-AMP-STIMULATED PROTEIN KINASE

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SUMMARY

1. Rat liver pyruvate kinase (type L) was purified by precipitation at pH 5 and $(NH_4)_2SO_4$ fractionation, followed by chromatography on DEAE-cellulose, hydroxylapatite and Sepharose 6B in the presence of glycerol in order to stabilize the enzyme. The enzyme preparation was nearly homogeneous as judged by Sepharose 6B chromatography and polyacrylamide gel electrophoresis in the presence of detergent.

2. The enzyme was shown to be phosphorylated by [^{32}P]ATP and cyclic 3′,5′-AMP-stimulated protein kinase. In preliminary experiments, the activity of the enzyme was decreased by phosphorylation, especially at low phosphoenolpyruvate concentrations. The results suggest that the L type of rat liver pyruvate kinase belongs to the enzymes whose activity is regulated by phosphorylation–dephosphorylation reactions.

INTRODUCTION

Many hormones are known to influence the intracellular level of cyclic 3′,5′-AMP [1]. The main known effect of this cyclic nucleotide in mammals is to stimulate one type of protein kinase activity which acts on certain enzymes and other proteins [2, 3]. The protein phosphorylation is reversible, due to the action of phosphoprotein phosphatase activity [2, 4]. In the cases so far studied the activity of the enzymes that are reversibly phosphorylated in these reactions is markedly influenced by the phosphorylation–dephosphorylation reactions.

Since cyclic 3′,5′-AMP has many diverse effects on metabolism, it seems reasonable to assume that more enzymes than those known today should be subjected to regulatory phosphorylation under the influence of the cyclic nucleotide. This view is supported by the widespread occurrence of cyclic 3′,5′-AMP-stimulated protein kinases in different tissues [5] and in the case of rat liver, from the fact that the phosphorylation of cell-sap proteins on incubation with [^{32}P]ATP in vitro is considerably stimulated by cyclic 3′,5′-AMP [6, 7].

In a recent paper, we reported the presence in rat liver cell sap of three main polypeptide components which were phosphorylated under the influence of cyclic 3′,5′-AMP [7]. Evidence was obtained that none of the components derived from active phosphorylase kinase (EC 2.7.1.38), phosphorylase (EC 2.4.1.1.) or glycogen

synthetase (EC 2.4.1.11), i.e. the enzymes which are definitely known to be phosphorylated in a controlled manner in rat liver.

Since cyclic 3′,5′-AMP stimulates gluconeogenesis in liver tissue [8], we considered the possibility that one of the three phosphorylatable polypeptides might be derived from liver pyruvate kinase (ATP:pyruvate phosphotransferase; EC 2.7.1.40), an enzyme which could be envisaged to be inhibited by a cyclic 3′,5′-AMP-stimulated phosphorylation. In preliminary experiments we found that the main isoenzyme (L type [9]) of pyruvate kinase in rat liver was copurified with phosphate-incorporating activity on precipitation at pH 5 ,$(NH_4)_2SO_4$ fractionation and DEAE-cellulose chromatography.

We, therefore, decided to purify the L type of rat liver pyruvate kinase in order to look for a regulatory phosphorylation of the enzyme. In the present paper an account is given of this purification. The enzyme is shown to be phosphorylated by cyclic 3′,5′-AMP-stimulated protein kinase from rat liver and from bovine muscle, with a concomitant decrease in enzyme activity especially at low substrate concentrations.

EXPERIMENTAL

Materials

[γ-^{32}P]ATP was prepared as previously described [10] but isolated by chromatography on a DEAE-cellulose column, eluted with a linear gradient of 0.1 to 0.25 M Tris–HCl buffer (pH 7.5) (Mårdh, S., unpublished).

The DEAE-cellulose used was Whatman DE-52. Sepharose 6B was purchased from Pharmacia. Hydroxylapatite was prepared according to Tiselius et al. [11]. Collodion Bags SM 13200 were products of Sartorius-Membranfilter GmBH, Göttingen, Germany. Rabbit muscle lactate dehydrogenase (EC 1.1.1.27) and yeast hexokinase (EC 2.7.1.1) were obtained from Boehringer.

Rat liver protein kinase was prepared by chromatography of unfractionated cell sap on Sephadex G-50 and DEAE-cellulose, using the methods described earlier [7]. The material eluted with 0.1 M sodium acetate was analyzed for protein and protein kinase activity by the method for histone kinase activity described previously [7]. The fraction used contained 4 mg protein/ml and had a protein kinase activity of 0.6 units/mg [7].

Bovine muscle protein kinase was prepared according to the routine method of Gilman [12]. The material eluted with 300 mM potassium phosphate on DEAE-cellulose was further purified on calcium phosphate gel according to Miyamoto et al. [13]. After elution and dialysis the kinase-containing fraction was chromatographed on a DEAE-cellulose column equilibrated with 5 mM Tris–HCl buffer (pH 7.5), 1 mM EDTA. The column was eluted with a linear gradient consisting of this buffer to the same buffer containing 500 mM potassium phosphate (pH 7.5). The fraction used for phosphorylation experiments contained 0.085 mg protein/ml and had a specific activity of 3 units/mg.

Analytical methods

The radioactivity of ^{32}P-labeled phosphate was measured by determining its

Cerenkov irradiation in aqueous solutions [14], using an Intertechnique SL 30 liquid scintillation spectrometer. Ninhydrin analyses were performed according to Moore and Stein [15].

Protein concentration was estimated by the biuret reaction [16], using bovine serum albumin as standard, or by measuring the ultraviolet absorbance at 280 nm in a Zeiss PMQ II spectrophotometer.

During enzyme purification the enzyme activity was estimated according to Kimberg and Yielding [17] with some modifications. The enzyme was diluted with 20 mM potassium phosphate buffer (pH 7.0) in 30% (v/v) glycerol–0.1 mM Fru-1,6-P_2–0.1 mM dithiothreitol and left at 20 °C for 10–15 min before the assays. The substrate solution contained 67 mM Tris–HCl buffer (pH 7.5), 10 mM magnesium acetate, 2.67 mM ADP, 2.67 mM trisodium phosphoenolpyruvate (Sigma), 0.133 mM Fru-1,6-P_2 and 67 mM KCl. 0.15 ml of the substrate solution was incubated with 0.05 ml of enzyme for 10 min at 30 °C. The enzyme reaction was interrupted by adding 0.2 ml of 0.125% dinitrophenylhydrazine in 2 M HCl and the incubation mixtures were left at 20 °C for 5 min. 0.2 ml of 10 M NaOH and 2 ml of ethanol were then added. The samples were centrifuged at 2000 × *g* for 5 min and the absorbance at 520 nm was measured. In standard samples known amounts of sodium pyruvate were incubated in the same way with the substrate solution. One unit of pyruvate kinase activity is defined as that amount of enzyme which transforms one μmole of substrate per min under the conditions used.

Pyruvate kinase activity was also assayed by following the decrease in absorbance at 340 nm in a reaction coupled with lactate dehydrogenase, using a Zeiss PMQ II spectrophotometer. The assay was performed at 28 °C, and the assay mixtures contained 17 mM imidazole–HCl buffer (pH 7.5), 1.7 mM $MgCl_2$, 33 mM KCl, 0.15 mM NADH, 1 mM ADP, 0.1 mM dithiothreitol, 0.4 or 10 μM Fru-1,6-P_2, 2 mM glucose, 1.5 units of lactate dehydrogenase and 0.5 unit of yeast hexokinase. After addition of pyruvate kinase (up to 0.008 unit) the mixture was incubated for 3 min at 28 °C, and the reaction was started by the addition of phosphoenolpyruvate, giving a final volume of 1.0 ml.

Phosphorylation and polyacrylamide gel electrophoresis in sodium dodecylsulfate of purified enzyme

Polyacrylamide gel electrophoresis in dodecylsulfate was performed on purified pyruvate kinase and a protein kinase-containing cell-sap fraction prepared by precipitation at pH 5.5, $(NH_4)_2SO_4$ fractionation and DEAE-cellulose chromatography [7]. The material eluted with 0.1 M sodium acetate was used for the experiments. This preparation also contained endogenous protein kinase substrates as described earlier [7]. 20 μg of purified pyruvate kinase were incubated for 5 min at 30 °C with 10 mM magnesium acetate, 0.15 mM cyclic 3′,5′-AMP, 0.15 mM [^{32}P]ATP and 11 μg of the cell-sap fraction in a total volume of 90 μl. Incubations were also performed without addition of the cell-sap fraction, and with 11 and 110 μg of this fraction, respectively, in the absence of pyruvate kinase. The reactions were interrupted with 150 μl of 8 M urea containing 2% (v/v) 2-mercaptoethanol and 2% (w/v) sodium dodecylsulfate. The mixtures were then heated to 45 °C for 45 min. Polyacrylamide gel electrophoresis in dodecylsulfate was performed for 6 h with a current of 6 mA/gel as described earlier [7]. After staining and destaining, the gels were dehydrated in 45% methanol–9%

acetic acid. They were cut into halves longitudinally and dried on Whatman 3 mM chromatography paper at 70 °C.

Isolation of [^{32}P]phosphorylserine from ^{32}P-labeled enzyme

6 μg of pyruvate kinase were incubated with 4.1 μg of bovine muscle protein kinase and 24 nmoles of [^{32}P]ATP (57 000 cpm/nmole) for 20 min at 30 °C in the presence of 20 mM potassium phosphate buffer (pH 7.0), 5 mM magnesium acetate and 0.01 mM cyclic 3′,5′-AMP. The final volume was 0.25 ml. The reaction was interrupted by adding 1 mg of bovine serum albumin followed immediately by 1 ml of 10% (w/v) trichloroacetic acid. The precipitate was collected by centrifugation, washed with 2 ml of 10% trichloroacetic acid and dissolved in 0.5 ml of 0.2 M NaOH, and subsequently reprecipitated with 2 ml of 10% trichloroacetic acid. After washing with 2 ml of acetone and twice with 2 ml of diethyl ether the precipitate was hydrolyzed with 2 M HCl together with 30 μmoles of unlabeled phosphorylserine and 15 μmoles of phosphorylthreonine, as recently described [6]. The dried hydrolysate was chromatographed on Dowex 50 and Dowex 1 [6]. The fractions were analyzed for radioactivity and ninhydrin-reacting material.

RESULTS

Purification of the enzyme

All the steps were carried out at 4 °C. The centrifugations were performed in a Sorvall RC-2B centrifuge. It was found that the enzyme was more stable in phosphate buffers than in Tris buffers. Phosphate buffers were, therefore, used throughout the purification. In addition, glycerol and Fru-1,6-P_2 were used to stabilize the enzyme.

Homogenization

Livers from 20 250–350-g male Sprague–Dawley rats fed a standard laboratory diet ad libitum were homogenized with 4 vol. (v/w) of 0.25 M sucrose–0.1 M KCl–0.1 mM dithiothreitol. The homogenates were centrifuged at 10 000 × *g* for 10 min.

pH precipitation

1 M acetic acid was added to the supernatant at 0 °C until pH 5 was reached. After 5 min the precipitate was removed by centrifugation at 15 000 × *g* for 15 min. The pH of the supernatant was raised to 7.0 by adding 1 M potassium phosphate buffer (pH 7.2) in an amount corresponding to five times the amount of 1 M acetic acid used.

$(NH_4)_2SO_4$ fractionation

Solid $(NH_4)_2SO_4$ was added to 30% saturation (0.176 g/ml). After 20 min the material was centrifuged at 15 000 × *g* for 15 min. The precipitate was discarded. Solid $(NH_4)_2SO_4$ was then added to the supernatant to 40% saturation (0.062 g/ml of 30% supernatant). After centrifugation the precipitate was dissolved in 20 ml of 20 mM potassium phosphate buffer (pH 7.2)–30% (v/v) glycerol–0.1 mM dithiothreitol. The material was then chromatographed on a 3.1 cm × 40 cm Sephadex G-50 column in equilibrium with the same buffer.

Chromatography on DEAE-cellulose

The enzyme solution was chromatographed on a DEAE-cellulose column which was eluted with a linear increase of phosphate buffer, as described in Fig. 1A. The enzyme was eluted at a phosphate concentration of about 50 mM.

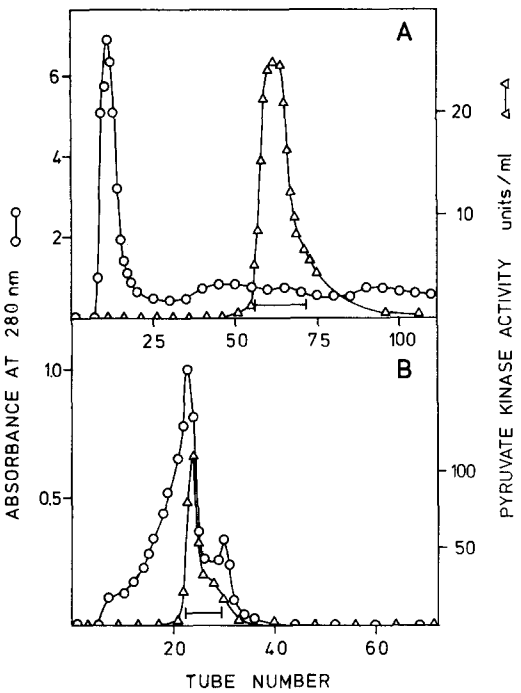

Fig. 1. DEAE-cellulose chromatographies of pyruvate kinase. Pooled material from the Sephadex G-50 chromatography containing 2800 units of pyruvate kinase was chromatographed on a 150-ml 3.1 cm × 20 cm DEAE-cellulose column equilibrated with 20 mM potassium phosphate buffer (pH 7.2), containing 30% glycerol and 0.1 mM dithiothreitol (Fig. 1A). The column was eluted with a linear gradient consisting of 500 ml of the above buffer to 500 ml of 80 mM potassium phosphate buffer with the same additions. 10-ml fractions were collected every 5 min and analyzed for their protein content and pyruvate kinase activity. The fractions indicated were pooled, and contained a total of 2700 enzyme units. This material was diluted with 2 vol. of a solution containing 30% glycerol, 0.1 mM dithiothreitol and 1 mM Fru-1,6-P_2. It was then applied to a second DEAE-cellulose column (1.8 cm × 19 cm) equilibrated with 10 mM potassium phosphate buffer (pH 7.2), containing 30% glycerol, 0.1 mM dithiothreitol and 5 mM Fru-1,6-P_2 (Fig. 1B). The column was eluted with a linear gradient of 300 ml of the above buffer to 300 ml of 50 mM potassium phosphate buffer (pH 7.2) with the above additions. 5.5-ml fractions were pooled and analyzed as above. The material indicated was pooled and contained a total of 1900 units of enzyme.

Rechromatography on DEAE-cellulose

The pooled enzyme fractions were rechromatographed on a DEAE-cellulose column in the presence of 5 mM Fru-1,6-P_2, as described in Fig. 1B. Some impurities were eluted in front of the enzyme.

Chromatography on hydroxylapatite

The pooled enzyme fractions were chromatographed on a (3.1 cm × 33 cm) Sephadex G-50 column in equilibrium with 1 mM potassium phosphate buffer (pH 7.0)–30% glycerol–0.1 mM Fru-1,6-P_2, and then applied to a (2 cm × 3.2 cm) column of hydroxylapatite. After washing the column with 10 ml of the same buffer the enzyme was eluted with 4 mM potassium phosphate buffer (pH 7.0)–30% glycerol–0.1 mM Fru-1,6-P_2. The pooled fraction (17 ml) was concentrated to 1.1 ml, using a collodion bag.

Chromatography on Sepharose 6B

The enzyme was finally chromatographed on a 100-ml Sepharose 6B column, as described in the legend to Fig. 2. The enzyme was eluted in parallel with the major part of the protein, indicating a fairly high degree of purity.

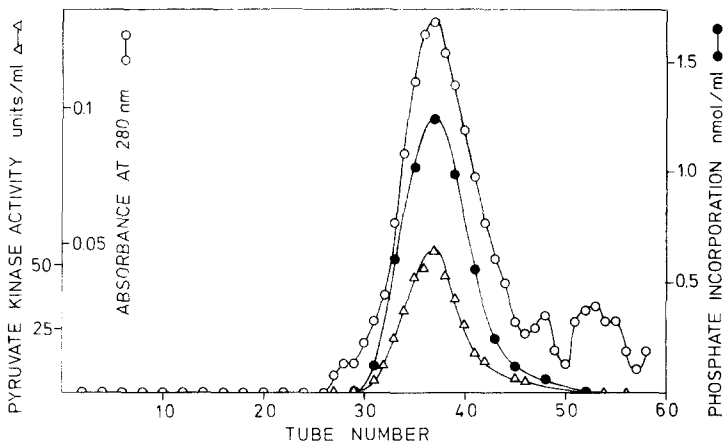

Fig. 2. Chromatography of rat liver pyruvate kinase on Sepharose 6B. The column (1.8 cm × 40 cm) was equilibrated and eluted with 100 mM potassium phosphate buffer (pH 7.0) containing 30% glycerol, 0.1 mM Fru-1,6-P_2 and 0.1 mM dithiothreitol. The column was loaded with 760 units of enzyme in 1 ml of buffer. 1.4-ml fractions were collected every 20 min and analyzed for their protein content and pyruvate kinase activity. Phosphate incorporation was determined by incubating 50 μl of the fractions at 30 °C with 4 μg of bovine muscle protein kinase and 0.25 mM [^{32}P]ATP in the presence of 5 mM magnesium acetate and 0.01 mM cyclic 3′,5′-AMP in a total volume of 250 μl. After 30 min the reaction was interrupted with trichloroacetic acid and the protein-bound [^{32}P]phosphate was determined as described earlier [7].

Table I gives the result of the purification. The specific enzyme activity of the final enzyme preparation was about 450 units/mg.

Phosphorylation of the purified enzyme with protein kinase and [^{32}P]ATP

Fractions from the Sepharose 6B chromatography were incubated with [^{32}P]ATP, cyclic 3′,5′-AMP and bovine muscle protein kinase, as described in the legend to Fig. 2. A protein phosphorylation proportionate to the enzyme activity was obtained. Under the assumption that 1 mg of enzyme/ml correspond to an absorbance of 1.0 at 280 nm the phosphorylation was 1 mole of phosphate/10^5 g of protein. This

corresponds to 0.6 mole of phosphate/mole of enzyme subunit, provided that the enzyme consists of subunits with a molecular weight of 62 000, as further discussed below. In other experiments using rat liver protein kinase a value of 1.2 moles of phosphate/mole of subunit was obtained. When cyclic 3',5'-AMP was omitted, the corresponding figure was 0.2 mole of phosphate/mole of subunit. The results suggest that under optimal conditions the enzyme might be phosphorylated to an extent of one phosphoryl group per enzyme subunit.

TABLE I

PURIFICATION OF RAT LIVER PYRUVATE KINASE

The preparation was started with 257 g of rat liver. In the early steps protein was measured according to the biuret method and in the chromatographic steps by determining $A_{280\ nm}$.

Purification step	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification factor	Recovery (%)
10 000 × *g* supernatant	3400	21 300	0.16	–	100
pH 5 supernatant	3350	12 150	0.28	1.8	99
30–40% $(NH_4)_2SO_4$ precipitate	2800	1 430	1.9	12	82
First DEAE-cellulose	2700	110	25	160	79
Second DEAE-cellulose	1880	23	82	520	55
Hydroxylapatite	990	2.3	430	2700	29
Sepharose 6B	580	1.3	450	2800	17

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis in detergent of the purified enzyme resulted in only one band after staining with Coomasie blue (Fig. 3A), also indicating a fairly high degree of purity. The molecular weight of this polypeptide, determined with the same reference proteins as described earlier [7], was found to be 61 000. Since the molecular weight of the native enzyme has been reported to be in the order of 200 000 [18, 19] the enzyme seems to be composed of presumably four subunits of equal size. Recently, the preparation of a cell-sap fraction which contained both protein kinase and three phosphorylatable unidentified components was described [7]. On incubation of pyruvate kinase with a catalytic amount of this fraction in the presence of [^{32}P]ATP and cyclic 3',5'-AMP the pyruvate kinase subunit was shown to be phosphorylated (Fig. 3D), whereas omission of the protein kinase fraction resulted in no detectable phosphorylation (Fig. 3B). When the same catalytic amount of the protein kinase-containing fraction was phosphorylated in the absence of pyruvate kinase, hardly any phosphorylated material could be detected (Fig. 3C), thus excluding the possibility that the main phosphorylatable material was derived from the protein kinase fraction. When a larger amount of this fraction was phosphorylated (Fig. 3E) it was found that the phosphorylated component with the highest molecular weight had the same migration velocity on the gel as the pyruvate kinase subunit. The similarity of the molecular weights, of about 61 000–62 000, strongly indicates that they are identical.

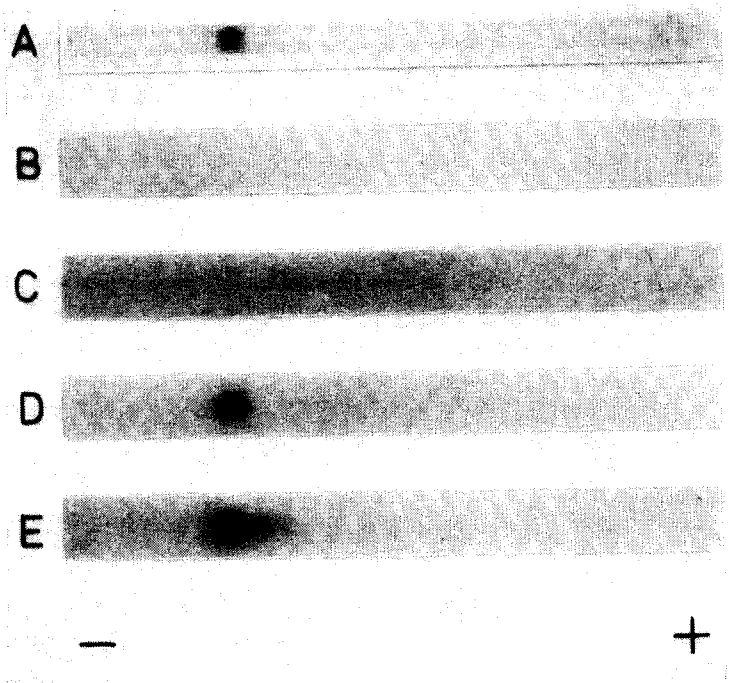

Fig. 3. Polyacrylamide gel electrophoresis in detergent of pyruvate kinase and a rat liver cell-sap fraction containing protein kinase and phosphorylatable endogenous protein. The experiment was performed as described under Experimental. The values given refer to the amount of protein applied to each gel. (A) Electrophoretogram of 2 μg of pyruvate kinase preparation stained with Coomasie blue. (B–E) Radioautographies of electrophoretograms of: B and D, 2 μg of pyruvate kinase phosphorylated in the absence and presence of 1 μg of cell-sap fractions, respectively; C and E, 1 and 10 μg of cell-sap fraction phosphorylated in the absence of pyruvate kinase.

Isolation of [^{32}P]phosphorylserine from ^{32}P-labeled enzyme

From an acid hydrolysate of ^{32}P-labeled enzyme, [^{32}P]phosphorylserine but no [^{32}P]phosphorylthreonine was isolated by chromatography on Dowex 50 and Dowex 1. From the specific radioactivity of the [^{32}P]ATP used and of the [^{32}P]phosphorylserine isolated, as well as the amount of unlabeled phosphorylserine added, the molar amount of [^{32}P]phosphate bound to the serine residues of the enzyme was calculated to be 0.7 mole per mole of enzyme subunit. The calculation was based on the assumption that all enzyme subunits are phosphorylatable and have a molecular weight of 61 000 and that the recovery of [^{32}P]phosphorylserine from the enzyme was the same as that of the added unlabeled phosphorylserine.

Thus, within experimental errors, all the protein-bound phosphate was serine-bound. This finding is in agreement with the regulatory phosphorylation of serine residues alone as first described for rabbit muscle phosphorylase [20].

Effect of phosphorylation on the activity of pyruvate kinase

Some preliminary experiments were performed to examine the effect of phosphorylation on the activity of the enzyme. As can be seen in Fig. 4, the activity of the phosphorylated enzyme was markedly less than that of the unphosphorylated enzyme,

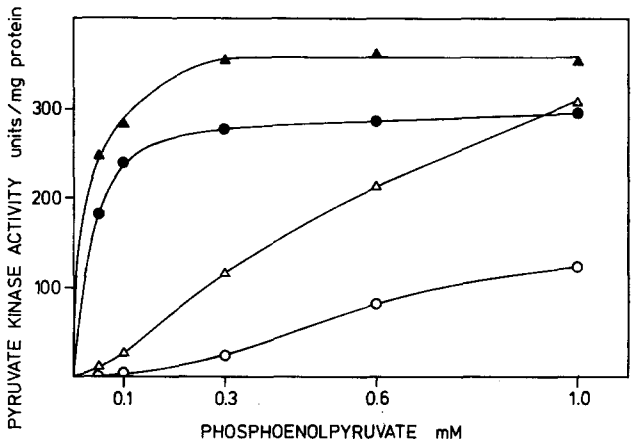

Fig. 4. The effect of phosphorylation on the activity of pyruvate kinase. 2.5 μg of purified enzyme were incubated for 15 min at 30 °C with 30 μg of rat liver protein kinase and 0.5 mM ATP in the presence of 25 mM potassium phosphate buffer (pH 7), 5 mM magnesium acetate, 7.5% glycerol and 0.05 mM cyclic 3',5'-AMP in a total volume of 100 μl. Controls without ATP and/or protein kinase were run in parallel. The incubations were terminated by diluting the samples with 400 μl of a cold solution consisting of 50 mM imidazole–HCl buffer (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$ and 0.1 mM dithiothreitol. Pyruvate kinase activity was determined spectrophotometrically in the coupled assay in the presence of $4 \cdot 10^{-7}$ M Fru-1,6-P_2, unless otherwise stated. Enzyme incubated with: ○—○, protein kinase + ATP; △—△, protein kinase; ▲—▲, no additions; ●—●, protein kinase + ATP, and assayed with 0.01 mM Fru-1,6-P_2 in the substrate solution.

especially at low concentrations of phosphoenolpyruvate. It can also be seen that an increased concentration of Fru-1,6-P_2 mainly overcame the effect of the phosphorylation, almost restoring full activity at a concentration of 0.01 mM. This seems to exclude the possibility that the enzyme had been irreversibly inactivated during incubation with ATP and protein kinase, e.g. by proteolytic degradation. The rat liver protein kinase preparation used in these experiments was shown to inhibit the pyruvate kinase activity when incubated in the abscence of ATP. The nature of this inhibition has not been further studied.

DISCUSSION

Liver tissue contains two different pyruvate kinases [9]. The amount of enzyme obtained in the present investigation, and its behaviour during $(NH_4)_2SO_4$ fractionation and DEAE-cellulose chromatography, were as expected for the L type enzyme [9]. It was purified to a fairly high degree, as judged from its chromatographic and polyacrylamide gel electrophoretic behaviour. Its specific activity was about the same as that reported for a crystalline enzyme preparation [18].

From the results it is apparent that the enzyme was phosphorylated on serine residues by cyclic 3',5'-AMP-stimulated protein kinase. The molar phosphate incorporation has not been definitely determined so far, but the data obtained suggests that each subunit of the enzyme might be phosphorylated. It seems reasonable to assume that the enzyme contains four subunits, as is the case for the same enzyme from other species [21, 22].

The experiments so far performed on the effect of cyclic 3′,5′-AMP-dependent phosphorylation on pyruvate kinase indicate that the enzyme activity was decreased, especially at low phosphoenolpyruvate concentrations. This would be consistent with the effect of cyclic 3′,5′-AMP on gluconeogenesis [8], when the activity of a key glycolytic enzyme should be suppressed [23].

The results suggest that the L-type of rat liver pyruvate kinase belongs to the enzymes whose activity is regulated by phosphorylation–dephosphorylation reactions. These reactions and their influences on the pyruvate kinase activity will be studied further.

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