Biochimica et Biophysica Acta, 422 (1976) 98—108

BBA 67689

REGULATION IN VITRO OF RAT LIVER PYRUVATE KINASE BY PHOSPHORYLATION-DEPHOSPHORYLATION REACTIONS, CATALYZED BY CYCLIC-AMP DEPENDENT PROTEIN KINASES AND A HISTONE PHOSPHATASE

VINCENT P.K. TITANJI, ÖRJAN ZETTERQVIST and LORENTZ ENGSTRÖM

Institute of Medical and Physiological Chemistry, Biomedical Centre, University of Uppsala (Sweden)

(Received July 10th, 1975)

Summary

1. Cyclic-AMP dependent protein kinases, resolved by chromatography on DEAE-cellulose and hydroxylapatite, catalysed the phosphorylation of rat liver pyruvate kinase and calf thymus histones by [γ^{32}P]ATP. [^{32}P]phosphopeptides, from acid hydrolysates of pyruvate kinase phosphorylated by the different protein kinase fractions, displayed identical electrophoretic patterns. Phosphorylation inhibited pyruvate kinase activity.

2. Full activity was restored when phosphorylated pyruvate kinase was dephosphorylated by a histone phosphatase from the soluble fraction of rat liver. These results are consistent with the hypothesis that pyruvate kinase is regulated by phosphorylation-dephosphorylation reactions.

Introduction

Previous communications from this laboratory [1,2] have shown that rat and pig liver pyruvate kinases (type L) (E.C. 2.7.1.40) are phosphorylated by adenosine 3',5'-monophosphate dependent protein kinase (E.C. 2.7.1.37). As this phosphorylation inhibited pyruvate kinase activity, it was proposed that the activity of pyruvate kinase is regulated by phosphorylation-dephosphorylation reactions. The proposed mechanism requires that the effect of cyclic-AMP dependent phosphorylation can be reversed by a phosphoprotein phosphatase from the same source [3]. The aim of the present investigation was to see if this condition would be fulfilled with respect to pyruvate kinase. Pyruvate kinase was also shown to be a substrate of the different protein kinase fractions.

Experimental

[γ^{32}P]ATP was prepared as described by Engström [4] with the modifications of Mårdh [5]. Whatman DEAE-cellulose (DE-52) and phosphocellulose were used. Hydroxyapatite was obtained from Bio-Rad and Sephadex G-25, G-50 and Sepharose 6B, from Pharmacia Fine Chemicals. Calf thymus histones (type II A) were bought from Sigma. Ultrafiltration cell, model 202, and Diaflo PM-10 filters were from Amicon.

Rabbit muscle lactate dehydrogenase (E.C. 1.1.1.27) and yeast hexokinase (E.C. 2.7.1.1) were bought from Boehringer, Mannheim, and dialysed against 25 mM imidazol/HCl, pH 7.5, containing 0.1 mM dithiothreitol before use.

Enzyme preparations

Rat liver pyruvate kinase was prepared by a modification of earlier procedures [1,2]. 30 male Sprague-Dawley rats were fasted for 48 h and then fed with ordinary laboratory chow, Anticimex 210, supplemented with fructose: 2 parts of ground chow to 3 parts of fructose [6,7] for another 72 h. The relative concentration (%, w/w) of the ingredients in the supplemented chow was as follows: protein, 8.3; fat, 1.5; ash, 3.0; water 3.4; and carbohydrate 81.5. This treatment raised the level of pyruvate kinase from 30 to 100 units/g wet weight of liver.

Pyruvate kinase was purified in potassium phosphate buffer containing 30% glycerol (w/v) and 0.1 mM dithiothreitol. After the DEAE-cellulose step 0.1 mM fructose 1,6-diphosphate was added to all buffers to stabilize the enzyme. Henceforth, we shall only refer to the potassium phosphate concentration since glycerol and fructose 1,6-diphosphate were routinely included in the buffers at the specified concentrations.

The rats were killed by cervical fracture, bled and their livers excised. Subsequent steps were at 0—4° C. The livers were rinsed, minced in cold homogenizing solution containing 250 mM sucrose, 1 mM EDTA and 0.1 mM dithiothreitol. Within 10—15 min livers from 5 animals were successively removed, pooled and homogenized in 4 vol. (v/w) of the homogenizing solution in a Waring blender at full speed for 20 s. The homogenates were centrifuged at 12 000 × *g* for 10 min and the supernatant was recentrifuged at 100 000 × *g* for 1 h.

Thereafter, DEAE-cellulose (2 ml packed bed/g of liver), equilibrated with 20 mM potassium phosphate, pH 7.2, was added to the supernatant and stirred for 1—2 h until more than 90% of the pyruvate kinase activity was bound to the ion-exchanger. The ion-exchanger with bound enzyme was poured into a column, 5 cm in diameter, layered with 100 ml of packed DEAE-cellulose, also equilibrated with 20 mM potassium phosphate, pH 7.2. After packing, the column was washed with 5—6 l of the starting buffer, until the absorbance of the eluate at 280 nm was less than 0.4 (light path = 1 cm). The enzyme was eluted with 60 mM potassium phosphate, pH 6.0. The fractions, containing pyruvate kinase activity, were pooled and adjusted to pH 5.5 with 1 M KH_2PO_4 or, preferably, by dropwise addition of cold concentrated H_3PO_4 with constant stirring. The enzyme solution was diluted threefold with 30% glycerol in 0.1 mM dithiothreitol and 0.1 mM fructose 1,6-diphosphate,

and applied to a phosphocellulose column (3.2 cm × 40 cm), previously equilibrated with 20 mM potassium phosphate, pH 5.5. Elution was performed at pH 5.5 with a 2000 ml linear potassium phosphate gradient (20—600 mM). 10 ml fractions were collected every 10 min and those with pyruvate kinase activity were concentrated with a Diaflo PM-10 filter to about 100 ml and chromatographed on a Sephadex G-50 column (3.2 cm × 40 cm), equilibrated with 1 mM potassium phosphate, pH 7.0, in 50 mM KCl. The enzyme was then applied to a hydroxyapatite column (3.5 cm × 5 cm), equilibrated with the same buffer. The column was washed with 100 ml of 4 mM potassium phosphate (pH 7.0) in 50 mM KCl and the enzyme then eluted with 10 mM potassium phosphate buffer, also containing 50 mM KCl.

After chromatography on Sepharose 6B [1], the enzyme was essentially homogeneous when analysed by polyacrylamide gel electrophoresis in sodium dodecyl sulphate. The specific activity of pyruvate kinase was 5, 50, 250 and 450 units per mg protein (A_{280nm}) after the DEAE-cellulose, phosphocellulose, hydroxyapatite and Sepharose 6B chromatography, respectively. The overall yield, 25 mg of protein, represented 20% of the initial enzymic activity.

Histone phosphatase was prepared from 150 g of liver from male Sprague-Dawley rats maintained on ordinary laboratory chow. 50 g portions of the liver were homogenised in 3 vol. of a buffer, containing 50 mM imidazol/HCl, pH 7.5, 5 mM EDTA, 0.5 mM dithiothreitol and 0.1 M NaCl in a Waring blender at full speed for 50 s. This and subsequent steps were performed at 0—4°C. The combined homogenates were centrifuged at 16 000 × *g* for 20 min and the debris discarded. The supernatant, containing 2400 units was diluted twofold with the homogenising buffer and brought to 70% saturation by slow addition of solid ammonium sulphate with constant stirring. After 15 min the precipitate was collected by centrifugation at 16 000 × *g* for 20 min, dissolved in 150 ml 50 mM imidazol/HCl, pH 6.0, containing 1 mM dithiothreitol and recentrifuged at 50 000 × *g* for 2 h. The supernatant was frozen overnight at −25°C. After thawing at 4°C, the enzyme solution was centrifuged at 16 000 × *g* for 20 min to remove precipitated protein, and the supernatant then dialysed for 8 h against 50 mM imidazol/HCl, pH 6.0, containing 1 mM dithiothreitol. The dialysis was repeated once, and the sample was applied to a DEAE-cellulose column (2.5 cm × 30 cm) equilibrated with 50 mM imidazol/HCl, pH 6.0, 1 mM dithiothreitol. The enzyme was eluted with a 1500 ml linear gradient (0—0.5 M NaCl) in the starting buffer. Fractions of 8 ml were collected every 10 min. A minor histone phosphatase peak appeared with 20—40% of the gradient volume, whereas most of the activity was eluted with 42—86% of the gradient. The latter peak was pooled and precipitated with ammonium sulphate up to 70% saturation. The precipitate, collected by centrifugation at 50 000 × *g* for 20 min, was dissolved in 5 ml of 50 mM imidazol/HCl, pH 6.0, containing 1 mM dithiothreitol and concentrated to 1 ml by ultrafiltration in a collodion bag (Sartorius-Membranfilter GmbH, Göttingen). The enzyme was further chromatographed on a Sepharose 6B column (2 cm × 40 cm) equilibrated and eluted with 50 mM imidazol/HCl, pH 6.0, 0.1 M NaCl, 0.5 mM dithiothreitol and 0.1 mM EDTA. Samples of 1 ml were collected every 20 min. The enzyme which appeared with 0.8 column volume was dialysed against 50 mM imidazol/HCl, pH 6.0, 1 mM dithiothreitol and rechromatographed on a DEAE-cellulose

column (2.5 cm × 15 cm) equilibrated with the same buffer. The enzyme was eluted with an 800 ml linear gradient of 0—0.5 M NaCl in the starting buffer. Fractions of 5.5 ml were collected every 5 min. Histone phosphatase now appeared with 70—86% of the gradient. The enzyme fractions eluted between 79—86% of the gradient had the highest specific activity. They were pooled, concentrated by collodion bag filtration, dialysed against 50 mM imidazol/HCl, pH 6.0, 1 mM dithiothreitol and stored at −25°C in 1-ml portions which were thawed once, suitably diluted and used. The enzyme was stable for at least 1 week. The overall yields were 17, 8.8 and 5.3% of the initial activity (2400 units) after the first DEAE-cellulose, Sepharose 6B and the second DEAE-cellulose chromatography respectively. The final preparation had a specific activity of 20 units per mg protein (A_{280nm}) when tested as described below.

Cyclic-AMP dependent protein-kinases were prepared from livers of male Sprague-Dawley rats fed with ordinary laboratory chow ad libitum. All manipulations were at 0—4°C. After excision, the livers were immediately homogenised in 4 vols. of 250 mM sucrose, 1 mM EDTA and 1 mM dithiothreitol with four strokes at 960 rmp in a Potter Elvehjem glass homogeniser, fitted with a Teflon pestle. The homogenates of four livers were pooled and centrifuged at 10 000 × *g* for 10 min. The supernatant was further centrifuged at 100 000 × *g* for 60 min. Half of the 100 000 × *g* supernatant (i.e. 120 ml, corresponding to 30 g of liver) was passed through a Sephadex G-50 column (5 cm × 20 cm) equilibrated with 5 mM potassium phosphate buffer, pH 7.25, containing 30% glycerol and 0.5 mM dithiothreitol. The filtrate was applied to a DEAE-cellulose column (2.6 cm × 18 cm). The column was washed overnight with 1 l of 5 mM potassium phosphate, pH 7.25, in 30% glycerol and 0.5 mM dithiothreitol. The enzymes were eluted at pH 7.25 with a 1000 ml linear gradient of potassium phosphate buffer (5—500 mM) containing 30% glycerol and 0.5 mM dithiothreitol. 5-ml samples were collected every 15 min.

The active fractions I—IV (see results Fig. 1A) were pooled separately and diluted with 30% glycerol and 0.5 mM dithiothreitol to give approximately the same conductance as that of the starting buffer at 4°C. The enzyme solution (containing not more than 20—30 mg of protein) was loaded into a hydroxyapatite column (2.5 cm × 5 cm), equilibrated with the same buffer as was the DEAE-cellulose, and eluted with a (200 ml + 200 ml) linear potassium phosphate gradient (5—250 mM), pH 7.25, with the same additions as were used for DEAE-cellulose chromatography.

Because of its apparently greater yield, protein kinase I (Fig. 1A) was chosen for further studies. Except when otherwise stated, all studies were performed with protein kinase I which had been purified through the hydroxyapatite step.

Enzyme activity measurements

Pyruvate kinase activity was assayed as described earlier [1], or using a Beckman DU spectrophotometer with an Optilab multianalog-201, coupled to a Perkin-Elmer linear recorder. The reaction medium contained 20 mM imidazol, pH 7.5, 2 mM magnesium acetate, 33 mM KCl, 1 mM ADP, 0.15 mM NADH, 2 mM glucose, 2 units of lactate dehydrogenase and 1 unit of hexokinase in a final volume of 1 ml. Pyruvate kinase was diluted in 50 mM imi-

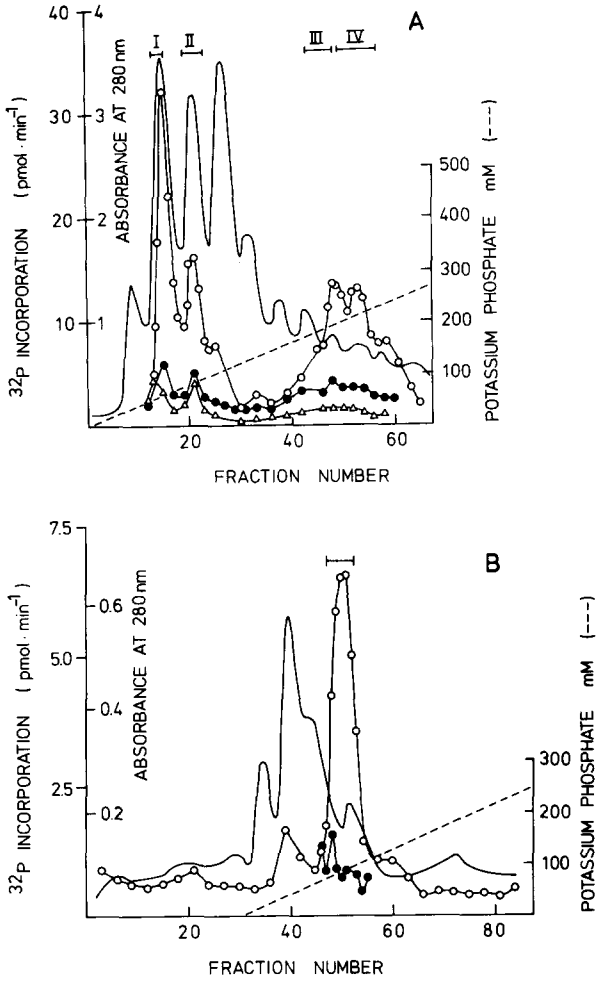

Fig. 1. (A) Chromatography of cell sap on DEAE-cellulose. The column (2.6 cm × 18 cm) was equilibrated and eluted as described under Experimental. 5-ml fractions were collected every 15 min. 25 µl aliquots were assayed for protein kinase activity with histones as substrate in the presence (○———○) or absence (●———●) of cyclic AMP. Endogenous phosphorylation (△———△) was measured in the presence of cyclic AMP. Unbroken line refers to ultra violet absorbance at 280 nm. Broken line represents the calculated concentration of phosphate on the top of the column at the moment the indicated tube was filled. (B) Chromatography of protein kinase I on hydroxyapatite. The column (2.5 cm × 5 cm) was equilibrated as described under Experimental. 5-ml fractions were collected every 30 min. Protein kinase was assayed with histones as substrate in 25 µl aliquots in the presence (○———○) or absence (●———●) of cyclic AMP. Unbroken and broken lines have the same meaning as above.

dazol, pH 7.5, containing 5 mM magnesium acetate, 50 mM KCl and 0.1 mM dithiothreitol. After preincubating the enzyme for 3 min at 30° C in the incubation medium, phosphoenol pyruvate (50 µl) was added and the oxidation of NADH was followed by the decrease of absorbance at 340 nm. In the kinetic studies no fructose 1,6-diphosphate was added to the assays, although approximately 0.25×10^{-8} M of the effector was present in the tests, since pyruvate kinase was stored in phosphate buffer containing 0.1 mM fructose 1,6-diphosphate. 1 unit of pyruvate kinase is that amount of enzyme which converts

1 μmol of phosphoenol pyruvate per min at specified conditions [1].

Histone phosphatase was measured in the presence of 10 mM $MgCl_2$ as described by Meisler and Langan [8], using [^{32}P]phosphohistone (mixed) prepared as described by them, with the exceptions that the protein kinase was peak I from the DEAE-cellulose step (see above) and 5 μM cyclic AMP was included in the histone phosphorylation medium. After extracting the [^{32}P]-phosphate released [8,10] an aliquot of the organic phase was mixed with 10 ml of 0.5 M NaOH and counted in an Intertechnique Liquid Scintillation Spectrometer [5]. 1 unit of phosphatase was that amount of enzyme which released 1 nmol [^{32}P]phosphate from [^{32}P]phosphohistone per min at 30°C (assuming the same specific radioactivity as that of the [^{32}P]ATP used in the preparation of [^{32}P]phosphohistone).

The dephosphorylation of pyruvate kinase was followed by measuring the release of [^{32}P]phosphate from the phosphorylated enzyme prepared as follows: 2 mg of pyruvate kinase were incubated with 0.5 mg of protein kinase I in a medium (5 ml) containing 0.1 mM [^{32}P]ATP, 20 mM potassium phosphate (pH 7.5), 10 mM magnesium acetate, 4 μM cyclic AMP, 1 mM dithiothreitol, 7.5% glycerol (w/w) and 0.01 mM fructose-1,6-diphosphate at 30°C for 1 h. 4.1 mol of [^{32}P]phosphate were incorporated per mol of pyruvate kinase. It was assumed that pyruvate kinase has a mol. weight of 250 000 [1], and a specific activity of 450 units per mg ($A_{280\,nm}$). The incubation mixture was chromatographed at 4°C on a Sephadex G-25 column (2.0 cm × 30 cm), equilibrated with 1 mM potassium phosphate buffer, pH 7.0, in 30% glycerol, 50 mM KCl, 0.1 mM fructose 1,6-diphosphate and 1 mM dithiothreitol. The phosphorylated pyruvate kinase, which appeared with the void volume, was applied to a hydroxyapatite column, equilibrated with the same buffer. The phosphoenzyme was eluted with a linear gradient (100 ml + 100 ml) of potassium phosphate, pH 7.0, (5—50 mM) containing the same additions as the starting buffer. 1 ml fractions were collected every 5 min. The main radioactive peak coincided with pyruvate kinase activity (assayed with the colour test of ref. 1). This material was pooled, dialysed against 500 vol. of the starting buffer, and stored at −25°C where the enzymic activity of phosphorylated pyruvate kinase (measured in the presence of fructose 1,6-diphosphate) was found to be stable. 50% of the original enzymatic activity was recovered.

Protein kinase activity was tested according to the method of Kumon et al. [9] with slight modifications. The incubation medium, 150 μl, contained 20 mM potassium phosphate buffer (pH 7.5), 20 mM magnesium acetate, 0.1 mM [^{32}P]ATP (20 000—80 000 cpm/nmol), 4 μM cyclic AMP, 60 μg mixed calf thymus histones, or a suitable amount of pyruvate kinase. After stopping the phosphorylation of pyruvate kinase with trichloroacetic acid, 100 μg of bovine serum albumin were added within 10 sec before the isolation of ^{32}P-labelled protein. The filters were washed with 10% trichloroacetic acid in 10 mM KH_2PO_4, using a Millipore 3025 sampling manifold, folded into polyethylene scintillation vials and their Cerenkov radiation measured in an Intertechnique SL liquid scintillation spectrometer.

Adenosine triphosphatase activity was estimated by analysing an aliquot of the protein kinase test mixture for [^{32}P]orthophosphate released. After extraction by the method of Martin and Doty [10] as modified by Meisler and

Langan [8], an aliquot of the organic phase was added to 10 ml of 0.5 M NaOH in a polyethylene scintillation vial and counted [5].

Other methods

Partial acid hydrolysis of ^{32}P-labelled protein was performed according to the method of Engström [4]. 10 μg of pyruvate kinase were phosphorylated with 10 μg of each protein kinase fraction in the presence of 10 mM magnesium acetate at 30° C for 30 min. All other conditions were as described above. Prior to termination of the reaction, 500 μg of bovine serum albumin were added to each reaction tube followed by trichloroacetic acid. The precipitates were collected by centrifugation at 2500 × *g* for 10 min and dissolved in 0.5 ml of a 0.5 M NaOH solution. This was followed immediately by trichloroacetic acid precipitation. The washing procedure was repeated once. The final pellet was dried in acetone and partial hydrolysis performed in 6 M HCl on a boiling water bath for 30 min. The hydrolysis products, dried over KOH crystals in a vacuum dessicator, were dissolved in 0.1 ml of water and 0.025 ml aliquots were applied to Whatman-3 paper. Electrophoresis was then performed in 50 mM pyridine acetate buffer, pH 3.5, at 1500 V for 2 h. The electrophoretogram was dried and placed on Kodak X-ray films for radioautography.

Protein was determined according to Lowry et al. [11] with bovine serum albumin as standard, or from the absorbance at 280 nm where 1 mg/ml of protein was assumed to have an absorbance of 1.0 (light path = 1 cm).

Results

Purification and properties of cyclic-AMP dependent protein kinase

On DEAE-cellulose the cyclic-AMP dependent protein kinases were resolved into four fractions, designated I—IV in order of elution (Fig. 1A). Peaks I and II appeared in repeated experiments, but the resolution of peak III from IV was variable.

During elution from hydroxyapatite, protein kinase I appeared after the main protein peak (Fig. 1B). Protein kinase II was similarly eluted but protein kinases III and IV were eluted over a broad range with low recovery.

Protein kinases I and II, purified through the hydroxyapatite step, contained neither detectable adenosine triphosphatase, nor histone phosphatase activity; they were stable at −25° C for at least 2 months during which freezing and thawing were well tolerated with little loss in activity. As can be seen on Fig. 1A, all the kinase fractions were stimulated by cyclic AMP.

Phosphorylation and inhibition of pyruvate kinase

The experiments illustrated in Fig. 2A and B showed that, like calf thymus histones, pyruvate kinase was a substrate of the respective protein kinases I—IV. Generally up to 4 mol of [^{32}P] phosphate were incorporated per mol of pyruvate kinase (see Experimental). To study the specificity of the protein kinases, the acid hydrolysis products of phosphorylated pyruvate kinase were analysed by paper electrophoresis (Fig. 3). Irrespective of the protein kinase used, these products displayed the same electrophoretic pattern. This suggested that the protein kinases phosphorylate the same sites on pyruvate kinase.

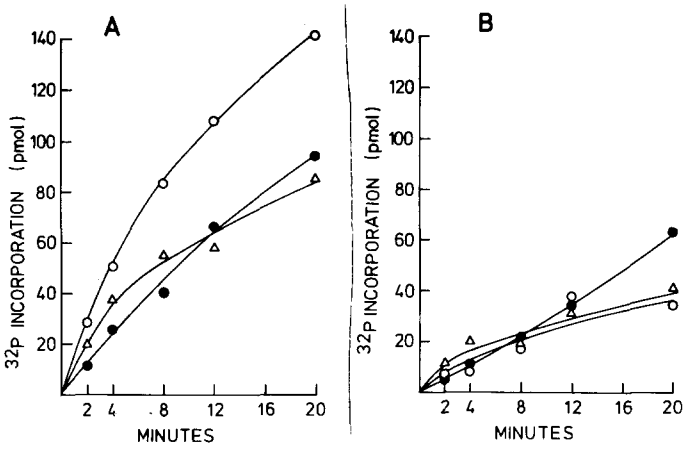

Fig. 2. Time course of phosphorylation. The incubation medium contained 60 μg of pyruvate kinase (A) or 240 μg of mixed calf thymus histones (B) in a final volume of 600 μl (for other details see Experimental). Incubations were at 30°C. 100-μl aliquots were withdrawn at times indicated and added to 2 ml of trichloroacetic acid for determinations of [^{32}P]phosphate incorporation. 50, 20 and 21 μg of protein kinases I, II and III + IV from the DEAE-cellulose step were used in respective experiments. ●———●, protein kinase I; △———△, protein kinase II; ○———○, protein kinase III + IV.

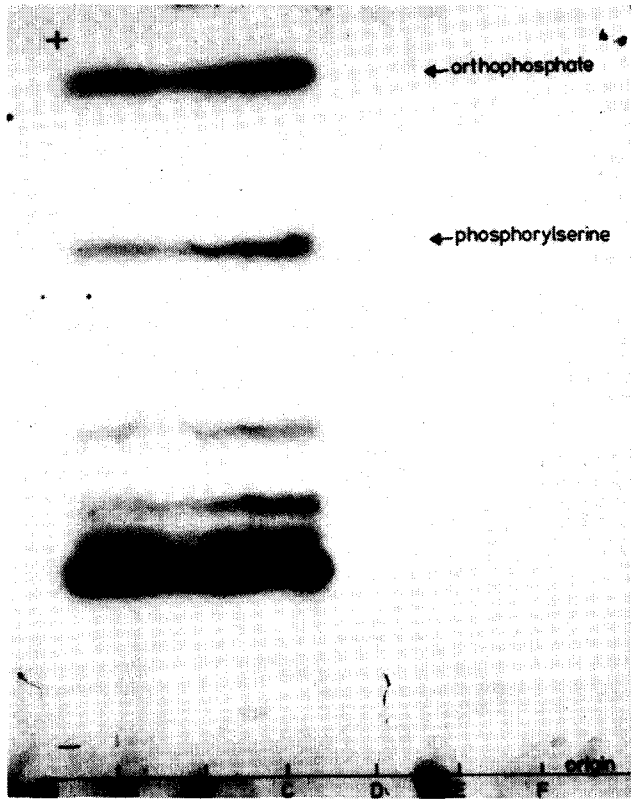

Fig. 3. Radioautograph of an electrophoretogram of partial acid hydrolysates of phosphorylated pyruvate kinase. Pyruvate kinase was phosphorylated (see Experimental) by (A) Protein kinase I, (B) Protein kinase II, (C) Protein kinases III + IV from the DEAE-cellulose step. In experiments D, E and F the respective kinases were incubated in the absence of pyruvate kinase.

Incorporation of 1.5—2 mol of phosphate per mol of pyruvate kinase was sufficient to inhibit over 90% of the enzymatic activity at 0.15 mM phosphoenol pyruvate (Fig. 4). This indicates that saturation of the available phosphorylation sites was not required for over 90% inhibition of the enzyme activity.

In other experiments, not illustrated, phosphorylation by protein kinase II was found to inhibit pyruvate kinase. However, the effect was less pronounced with protein kinases III + IV. Of interest is the finding that protein kinases I and II, purified through hydroxyapatite, had no inhibitory effect on pyruvate kinase in the absence of ATP, in contrast to the crude fractions used earlier [1].

Dephosphorylation and reactivation of pyruvate kinase

The effect of dephosphorylation was studied using a histone phosphatase preparation from the soluble fraction of rat liver. Usually an aliquot of the

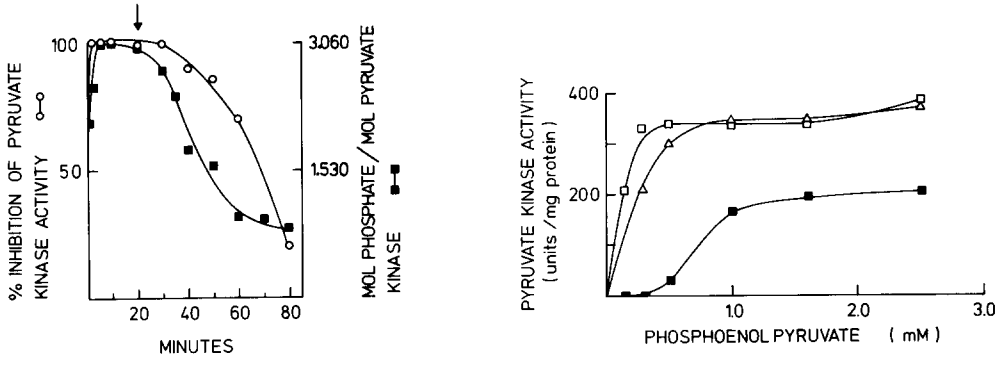

Fig. 4. Reversible inactivation of pyruvate kinase. 29 units of pyruvate kinase were incubated at 30°C for 5 min in a 400 µl phosphorylation medium. The reaction was started by addition of 10 µg of protein kinase I, previously dialysed against 20 mM Tris · HCl, pH 7.5, and 0.1 mM dithiothreitol. The complete incubation medium (500 µl), contained in addition 10 mM magnesium acetate, 40 mM imidazol/HCl, pH 7.5, 5 µM cyclic AMP, 0.1 mM [^{32}P]ATP (sp. activity 50 000 cpm/nmol), 50 µg of bovine serum albumin and 0.2 mM dithiothreitol. 3 identical incubations were run in parallel. At several intervals, 50 µl aliquots were withdrawn from one of the reaction solutions, diluted 10-fold with ice-cold 40 mM imidazol, pH 7.5, in 30% glycerol, 0.1 mM dithiothreitol, 20 mM NaF, 2 mM EDTA and 50 µg of bovine serum albumin per ml, immediately sealed and stored at —15°C. Corresponding 50-µl aliquots from the other 2 reaction solutions were precipitated with 2 ml of trichloroacetic acid and kept on ice for the determination of [^{32}P]phosphate incorporation. After 20 min (arrow), 200 µl of each incubation solution were combined with 600 µl of 40 mM imidazol/HCl, pH 7.5, containing 400 µg of bovine serum albumin, 5 mM magnesium acetate, 3 mM glucose, 2 units of hexokinase and 20 µg of protein phosphatase at 30°C. At indicated times, 100-µl samples were withdrawn, diluted 5-fold and stored or precipitated with trichloroacetic acid, as described above. At —15°C pyruvate kinase was stable for at least 3 days; the protein kinase and phosphatase reactions were completely stopped after the incubation. The entire experiment was completed in about 6—8 h. Pyruvate kinase activity was assayed at 0.15 mM phosphoenol pyruvate. The activity of the enzyme before phosphorylation was taken to be 100%.

Fig. 5. Dephosphorylation and reactivation of pyruvate kinase. 29 units of pyruvate kinase were phosphorylated for 20 min (see Fig. 4A) until 1 mol of the enzyme contained 3.1 mol of [^{32}P]phosphate. 200 µl of this sample were subsequently incubated for 40 min with 20 µg of the phosphatase until only 0.9 mol of [^{32}P]phosphate remained bound per mol of pyruvate kinase. After 20 and 60 min incubation samples were diluted and stored for pyruvate kinase activity measurements as described in the legend of Fig. 4. □———□, untreated enzyme; △———△, dephosphorylated pyruvate kinase; ■———■, phosphorylated pyruvate kinase.

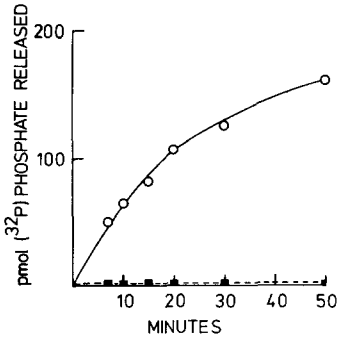

Fig. 6. Dephosphorylation of pyruvate kinase. The incubation medium (0.8 ml) contained 222 pmol of purified phosphopyruvate kinase, 50 mM imidazol/HCl, pH 7.5, 50 mM KCl, 5 mM magnesium acetate, 0.1 mM dithiothreitol, 50 μg bovine serum albumin, and 7.5% glycerol. The reaction was started by addition of 20 μg protein phosphatase. Duplicate samples were incubated in parallel. 100-μl portions were withdrawn at different intervals and precipitated with 2 ml of 5 mM silicotungstic acid in 5 mM H_2SO_4. Orthophosphate released was extracted [8,10] and an aliquot of the organic phase combined with 10 ml of 0.5 M NaOH [5] and the Cerenkov radiation measured (see Experimental). ○———○, phosphopyruvate kinase + phosphatase; ■———■, phosphopyruvate kinase alone.

phosphorylated pyruvate kinase was incubated with the histone phosphatase without prior removal of other components of the phosphorylation medium. After the reaction the protein kinase and protein phosphatase were inhibited respectively by 5—10 fold dilution with 2 mM EDTA and 20 mM NaF, pending the measurement of pyruvate kinase activity. The extent of dephosphorylation was assumed to be the difference between the initial content of ^{32}P-label on pyruvate kinase and that which remained after the phosphatase reaction.

As can be seen in Fig. 4, the incubation of the phosphorylated pyruvate kinase with the histone phosphatase resulted in both a loss of ^{32}P-label and a reactivation of pyruvate kinase. However, the reactivation was not perfectly parallel to the loss of ^{32}P-label, suggesting that the process of reactivation was complex. Since previous studies [1,2] had shown that the kinetic differences between unphosphorylated and phosphorylated pyruvate kinase depend, in part, on the concentration of phosphoenol pyruvate, the activities of phosphorylated and dephosphorylated pyruvate kinase were compared at different phosphoenol pyruvate concentrations. In Fig. 5 it is shown that upon dephosphorylation pyruvate kinase activity was fully restored.

To rule out the possibility that the loss of ^{32}P-label in the experiments of Figs. 4 and 5 could have resulted from the action of a protease in the phosphatase or other enzymes used, an equivalent amount of purified phosphopyruvate kinase was incubated for 30 min in the same medium as was used in experiments of Figs. 4 and 5. [^{32}P]phosphate released was determined after extraction according to Martin and Doty [10] or calculated from the amount of ^{32}P-label which remained bound to pyruvate kinase. All the loss in ^{32}P-label (which was about 50% of the total radioactivity) could be accounted for by the isobutanol benzene extractable orthophosphate. Therefore, it was assumed that the loss of ^{32}P-label by phosphorylated pyruvate kinase was due to its dephosphorylation and not to proteolysis. Furthermore purified phospho-pyruvate kinase was a substrate of the histone phosphatase (Fig. 6).

Discussion

The resolution of cyclic-AMP dependent protein kinases into several fractions (Fig. 1A) is in agreement with published results [9,12,13], suggesting the multiplicity of these kinases in rat liver cystosol. However, further resolution of the early peak into two fractions (Fig. 1A) has not been described before.

The present investigations revealed reversible changes in the activity of pyruvate kinase, caused by phosphorylation and dephosphorylation of the enzyme. Earlier studies [1,2] demonstrated that the inhibition of pyruvate kinase by phosphorylation could be removed by fructose 1,6-diphosphate. Glycogen synthetase D, a well-studied phosphoenzyme [14] is physiologically inactive but regains full activity upon dephosphorylation, or in the presence of glucose 6-phosphate [15]. Pyruvate kinase, like glycogen D synthetase, is inhibited in the phosphorylated form (Fig. 4). Upon dephosphorylation full enzymatic activity is regained (Fig. 5).

The observation that only partial saturation of the phosphorylation sites is required to completely inhibit pyruvate kinase (Fig. 4) suggests cooperative response of these sites. However, conclusions about the extent of phosphorylation needed to cause complete inhibition of pyruvate kinase must be drawn cautiously, since the intrinsic phosphate content of the enzyme was unknown.

Protein kinases and a phosphatase, used in regulating pyruvate kinase, were isolated from the same cell fraction as pyruvate kinase. It is proposed that these enzymes may effect the regulation of pyruvate kinase activity in vivo by phosphorylation-dephosphorylation reactions.

Acknowledgements

The present investigation was supported by the Swedish Medical Research Council (Project 13X-50 and 13X-4485). The skilful technical assistance of Miss Elvy Netzel is gratefully acknowledged.

References

1 Ljungström, O., Hjelmquist, G., Engström, L. (1974) Biochim. Biophys. Acta 358, 289–298
2 Engström, L., Berglund, L., Bergström, G., Hjelmquist, G., Ljungström, O. (1974) in Lipmann Symposium: Energy, Regulation and Biosynthesis in Molecular Biology, pp. 192–204, Walter de Gruyter Inc., Berlin
3 Krebs, E.G. (1973) in Endocrinology, Proc. of the Fourth International Congress, Excerpta Medica, pp. 17–29, Amsterdam
4 Engström, L., (1962) Ark. Kemi 19, 129–140
5 Mårdh, S. (1975) Biochim. Biophys. Acta, 391, 448–463
6 Mårdh, S., Ljungström, O., Högstedt, S., Zetterqvist, Ö. (1971) Biochim. Biophys. Acta 251, 419–426
7 Tanaka, T., Harano, Y., Sue, F., Morimura, H. (1967) J. Biochem. 62, 71–91
8 Meisler, M., Langan, T.A. (1969) J. Biol. Chem. 244, 4961–4968
9 Kumon, A., Nishiyama, K., Yamamura, H., Nishizuka, Y. (1972) J. Biol. Chem. 247, 3726–3735
10 Martin, J.B., Doty, D.M. (1949) Anal. Chem. 21, 965–967
11 Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
12 Chen, L., Walsh, D.A. (1971) Biochemistry 10, 3614–3621
13 Dastugue, B., Tichonicky, L., Kruth, J. (1973) Biochimie 55, 1021–1030
14 Soderling, T.R., Park, C.R. (1974) in Advances in Cyclic Nucleotide Research, Vol. 4, pp. 283–333, Raven Press, New York
15 Nakai, C., Thomas, J.A. (1974) J. Biol. Chem. 249, 6459–6467