Biochimica et Biophysica Acta, 524 (1978) 68—77

BBA 68398

PHOSPHORYLATION OF RAT KIDNEY PYRUVATE KINASE TYPE L BY CYCLIC 3′,5′-AMP-DEPENDENT PROTEIN KINASE

LARS BERGLUND *

Institute of Medical and Physiological Chemistry, Biomedical Center, University of Uppsala, Box 575, S-751 23 Uppsala (Sweden)

(Received September 21st, 1977)

Summary

Pyruvate kinase (ATP:pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40) type L was partly purified from rat kidney. During the last two purification steps, the incorporation of [^{32}P]phosphate into protein on incubation with [^{32}P]ATP and cyclic 3′,5′-AMP-dependent protein kinase was found to parallel the pyruvate kinase activity. After phosphorylation of the enzyme, a major radioactive band with a molecular weight of 57 000 was found on polyacrylamide gel electrophoresis. [^{32}P]Phosphorylserine was isolated from the kidney pyruvate kinase. Immunological identity was found between the liver and kidney pyruvate kinases type L. By autoradiography of high-voltage electropherograms after partial acid hydrolysis of the phosphorylated rat liver and kidney pyruvate kinases type L, identical results were obtained. The affinity for phospho*enol*pyruvate was found to be decreased by phosphorylation of the enzyme with a change in the apparent K_m from 0.15 mM to 0.35 mM. After incubation of the phosphorylated kidney pyruvate kinase with phosphatase the phospho*enol*pyruvate saturation curve was found to be identical to that for the unphosphorylated enzyme. Thus, the activity of the rat kidney pyruvate kinase type L is with all probability regulated by a reversible phosphorylation-dephosphorylation reaction, thereby indicating that hormonal regulation of gluconeogenesis via cyclic AMP may be of importance in the renal cortex.

Introduction

The occurrence of several isozymes of pyruvate kinase (ATP:pyruvate 2-*O*-phosphotransferase, EC 2.3.1.40) is well established [1]. In the renal cortex

* Present Address: Division of Metabolic Disease, Department of Medicine, University of California, La Jolla, Calif. U.S.A.

Abbreviations: cyclic AMP, adenosine 3′,5′-monophosphate; Fru-1,6-P_2 = fructose-1,6-bisphosphate, *P-enol*pyruvate = phospho*enol*pyruvate; SDS, sodium dodecyl sulphate.

two such isozymes have been described [2]. The dominating form is the A type isozyme, which is present in most other tissues as well [1]. The L type occurs as the minor form of pyruvate kinase in the renal cortex, and is also present in hepatocytes and the small intestine [1—4]. This isozyme is an allosterically regulated enzyme, and the activity of the L-type isozyme from rat and pig liver has been shown to be regulated by a cyclic AMP-dependent phosphorylation reaction [5,6]. Upon phosphorylation the activity of the enzyme is decreased, and the enzyme is rendered more sensitive to allosteric inhibition [5—8]. The A-type isozyme from pig renal cortex is apparently not phosphorylated in a cyclic AMP-dependent reaction [9], and its kinetic properties also differ from those of the corresponding L-type liver enzyme [8]. It is known that the synthesis of the L-type pyruvate kinase from liver and kidney is stimulated by a fructose-rich diet, while this is not the case for the A-type pyruvate kinases from these tissues [10]. Since the liver and renal cortex are the major gluconeogenic organs in mammals, it seems to be of importance to investigate the regulation of the L-type pyruvate kinase from kidney.

Experimental procedure

Materials

Rat liver pyruvate kinase (type L) was purified by the method of Titanji et al. [11]. The catalytic subunit of rat liver cyclic AMP-dependent protein kinase was purified as described elsewhere [12] and assayed essentially according to the method of Corbin and Reimann [13]. Phosphoprotein phosphatase was prepared and assayed as described by Titanji [14]. [γ-^{32}P]ATP was prepared as described by Engstrom [15] with the modifications of Mardh [16]. Radioactivity was analyzed as described elsewhere [16].

The DEAE-cellulose and phosphocellulose used were Whatman DE-52 and P-11, respectively. Dowex 50 and hydroxyapatite were purchased from Bio-Rad Lab., Richmond, Calif., U.S.A. Sephadex G-50 was a product of Pharmacia, Uppsala, Sweden. NADH and lactate dehydrogenase were manufactured by Boehringer Mannheim, Germany and bovine serum albumin was a product of Sigma Chemical Co., St. Louis, Mo., U.S.A. All other chemicals were of the highest quality commercially available, and used without further purification.

Purification of rat kidney pyruvate kinase type L

The rat kidney pyruvate kinase was purified essentially as described for the rat liver enzyme by Titanji et al. [11]. In order to increase the amounts of L-type pyruvate kinase, the rats were fed with a fructose-rich diet prior to use [10]. Kidneys from male Sprague-Dawley rats were homogenized in a Potter-Elvehjem homogenizer with 4 vols. of 15 mM Tris · HCl (pH 7.5)/0.25 M sucrose/5 mM EDTA/1 mM dithiothreitol. The homogenate was centrifuged at 16 000 × *g* for 20 min in a Sorvall RC-2B centrifuge. The supernatant was collected and recentrifuged for 60 min at 100 000 × *g* in a MSE 65 centrifuge, after filtration through a glass-wool filter. This supernatant was chromatographed on a 2-l Sephadex G-50 column equilibrated and eluted with 5 mM potassium phosphate (pH 7.0)/30% (v/v) glycerol/0.1 mM Fru-1,6-P_2/0.1 mM

dithiothreitol. These additions were made in these concentrations to all buffers used in the purification.

The void volume from the Sephadex G-50 filtration was transferred to a DEAE-cellulose column (4.0 × 20.3 cm) equilibrated with the same buffer and eluted with a linear gradient (Fig. 1). The active fractions were pooled and diluted with 2 vols. of 30% glycerol/0.1 mM Fru-1,6-P_2/0.1 mM dithiothreitol. After adjustment to pH 5.5 with 2M H_3PO_4, the preparation was applied to a phosphocellulose column (3 × 11 cm) equilibrated with 20 mM potassium phosphate (pH 5.5). Elution was performed with a linear gradient from 20—300 mM potassium phosphate (pH 5.5). The enzyme was eluted at about 120 mM potassium phosphate. The active fractions were pooled, concentrated and adjusted to pH 7.0 with 1 M NaOH. The material was put on a Sephadex G-50 column (3.1 × 38.5 cm) equilibrated and eluted with 1 mM potassium phosphate (pH 7.0). To this buffer, and to the following buffers used during the purification, 50 mM KCl was added in order to stabilize the enzyme (Engstrom et al., unpublished data). The fractions eluting at the void volume were chromatographed on a hydroxyapatite column (2.0 × 3.9 cm) (Fig. 2A). Elution was performed with a linear gradient and the active fractions concentrated. The results of the purification are summarized in Table I.

Assay of pyruvate kinase activity

During the purification procedure the enzyme was assayed by the technique in ref. 5. In the kinetic experiments the enzyme was assayed spectrophotomet-

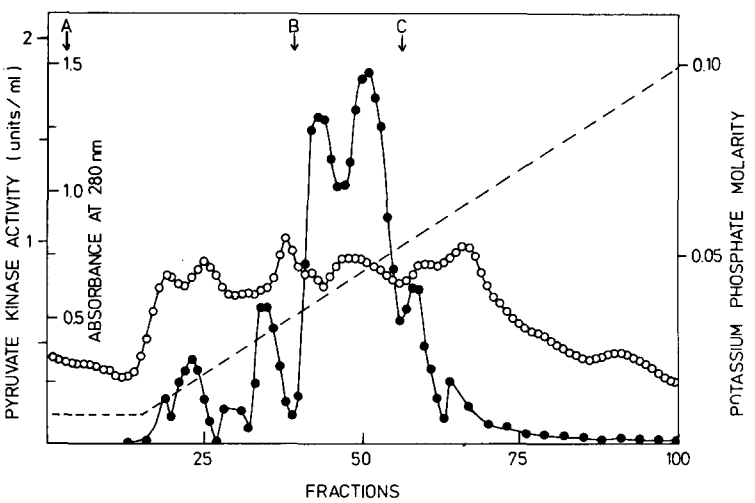


Fig. 1. Chromatography of rat kidney cell sap on DEAE-cellulose after desalting on Sephadex G-50. The column (4.0 × 20.3) was equilibrated as described in the text. After loading of the sample, the column was washed with two volumes of the equilibration buffer. Elution was performed with a linear gradient from 5 mM to 100 mM potassium phosphate (pH 7.0)/30% glycerol/0.1 mM Fru-1,6-P_2/0.1 mM dithiothreitol in a total volume of 2 l. The start of the gradient is marked A. The majority of pyruvate kinase activity (1077 units) did not attach to the column and was collected in the washing fraction, constituting the A-type pyruvate kinase. The fraction indicated by the arrows B and C were pooled (499 units) in a total volume of 375 ml. This pool constituted the rat kidney pyruvate kinase type L, and was further processed as described in the text. ●———●, Pyruvate kinase activity, ○———○, absorbance at 280 nm; - - - - - -, molarity of potassium phosphate as analyzed by conductometry.

TABLE I

PARTIAL PURIFICATION OF RAT KIDNEY PYRUVATE KINASE TYPE L

Purification step	Total pyruvate kinase activity (units)	Total protein (mg)	Specific activity (units/mg)
100 000 × *g* supernatant	2471	3221	0.8
DEAE-cellulose chromatography			
Fraction A	1077		
Fraction L	499	237	1.3
Phosphocellulose chromatography	332	31	10.7
Hydroxyapatite chromatography	122	2.8	44

rically in a coupled assay with lactate dehydrogenase [7]. Before the kinetic experiments the enzyme was dialyzed extensively against 20 mM potassium phosphate (pH 7.0)/30% glycerol/0.1 mM dithiothreitol, in order to remove Fru-1,6-P_2.

Polyacrylamide gel electrophoresis experiments

SDS polyacrylamide gel electrophoresis was performed by the method previously described [17]. In the experiments with ^{32}P-labeled pyruvate kinase, some gels containing the enzyme were sliced and the radioactivity of the slices measured.

Immunological experiments

Antibodies to rat liver pyruvate kinase type L were induced in New Zealand white rabbits and processed [18]. Immunodiffusion in 1% agarose in 75 mM veronal buffer (pH 8.6) was carried out by the method of Ouchterlony [19].

Phosphorylation experiments

0.1 ml of the final enzyme fraction was incubated with 0.25 mM [^{32}P]ATP and protein kinase in the presence of 2.25 mM magnesium acetate. The specific activity of the [^{32}P]ATP used was about 100 000 cpm/nmol. The reaction was started by the addition of 19 units of the catalytic subunit from rat liver cyclic AMP-dependent protein kinase, giving a final incubation volume of 0.2 ml. The reaction was allowed to proceed for 20 min at 30°C, and was then stopped with 2 ml cold 10% trichloroacetic acid, +0.5 mg bovine serum albumin. The samples were treated as described earlier [17] and the radioactivity was measured.

Dephosphorylation of pyruvate kinase

Rat kidney pyruvate kinase was incubated with [^{32}P]ATP and cyclic AMP-dependent protein kinase as described above. The incubation was stopped after 20 min at 30°C by filtration of the incubation mixture on a 1.5 ml Sephadex G-50 column in a Pasteur pipette. The column was equilibrated and eluted with 25 mM Tris · HCl (pH 7.5)/1 mM dithiothreitol/30% glycerol/0.4 mg/ml of bovine serum albumin (0.2 ml fractions). The position of the ^{32}P-labeled enzyme was located by measuring the radioactivity of the fractions. Following

this procedure, 0.1 ml of the enzyme was incubated for 45 min at room temperature with 2 units of phosphoprotein phosphatase, giving a total volume of 0.125 ml. The incubations were stopped with 20 vols. of 20 mM potassium phosphate (pH 7.5)/30% glycerol/0.1 mM dithiothreitol. After dilution of the samples, the enzyme activity was analyzed as described above.

Partial acid hydrolysis of pyruvate kinase and isolation of [^{32}P]phosphorylserine

After phosphorylation of pyruvate kinase as described above, the enzyme was subjected to partial acid hydrolysis in 6 M HCl for 30 min at 100° C. The hydrolysis procedure and the following high-voltage electrophoresis experiments were performed as described by Engstrom [15]. [^{32}P]Phosphorylserine was isolated from the phosphorylated rat kidney pyruvate kinase after hydrolysis in 2 M HCl for 20 h at 100° C by chromatography on Dowex 50 as described elsewhere [20].

Results

Purification of rat kidney pyruvate kinase

On hydroxyapatite chromatography, [^{32}P]phosphate was incorporated into protein in parallel to the pyruvate kinase activity (Fig. 2A). This was also the case when the eluted fractions in the phosphocellulose chromatography were treated in the same way (unpublished data). In some preparations, 2 fractions corresponding to L-type pyruvate kinase activity were found after DEAE-cellulose chromatography. These fractions were purified separately, and in the subsequent purification procedure their positions coincided with each other. They were investigated separately in the experiments described below, but no differences between these two L-type fractions were detected.

In parallel to the purification of the rat kidney pyruvate kinase, purification of the rat liver pyruvate kinase type L from the same rats was performed. During the purification procedure the chromatographic behaviors of the two enzymes were alike. This indicated that the two L-type rat pyruvate kinases were, at least, very similar. In the following experiments the concentrated pools from the respective hydroxyapatite chromatography were used to represent the liver and kidney pyruvate kinase type L.

Polyacrylamide gel electrophoresis

Several protein bands could be detected on SDS polyacrylamide gel electrophoresis of the rat kidney pyruvate kinase (Fig. 2B). A major band corresponding to a molecular weight of 57 000 was found which corresponded to rat liver pyruvate kinase type L investigated in parallel experiments. After phosphorylation of the pyruvate kinases, one radioactive peak was found in the sliced gels. The position of this peak corresponded to the major band in the rat kidney pyruvate kinase pool. When purified liver pyruvate kinase was used, the radioactive material was located at a position corresponding to the protein band. Gels containing only protein kinase showed no radioactive material, and no protein band was detected. These findings indicated that the major band in the kidney pool comprised the rat kidney pyruvate kinase type L, and that this

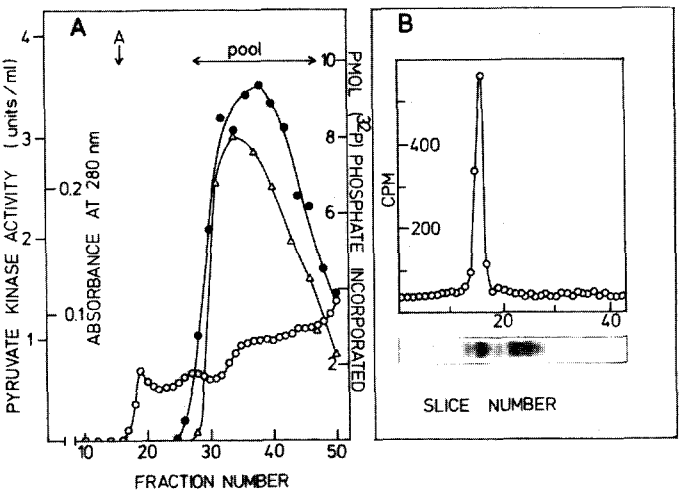


Fig. 2(A). Chromatography on hydroxyapatite of rat kidney pyruvate kinase type L. The concentrated pool from the phosphocellulose chromatography was transferred to a column of hydroxyapatite (2.0 X 3.9 cm) after being desalted on Sephadex G-50 as outlined in the text. The column was washed with 15 ml of 1 mM potassium phosphate (pH 7.0) containing the additives as described in the text. This buffer was also used for equilibration. After washing of the column with 15 ml of 4 mM potassium phosphate (pH 7.0) the enzyme was eluted with a linear gradient from 4 mM to 20 mM of the same buffer (50 + 50 ml). Samples of the collected fraction were incubated with ATP and cyclic AMP-dependent protein kinase, as described under Experimental. Fraction of about 2.5 ml were collected. ●———●, pyruvate kinase activity, ○———○, absorbance at 280 nm, and △———△, protein-bound radioactivity in the fraction samples. The start of the gradient is marked by A. (B) Polyacrylamide gel electrophoresis in detergent of rat kidney pyruvate kinase. Radioactivity measured from gel slices is given in the upper part of the figure. In the lower part, a gel containing 13 μg of protein from the final enzyme fraction is shown. The gel was stained with Coomassie Blue for detection of the protein bands present. The radioactivity was measured in an identical gel from the same experiment. The position of the major band, which contained the radioactive material, corresponded to a molecular weight of 57 000.

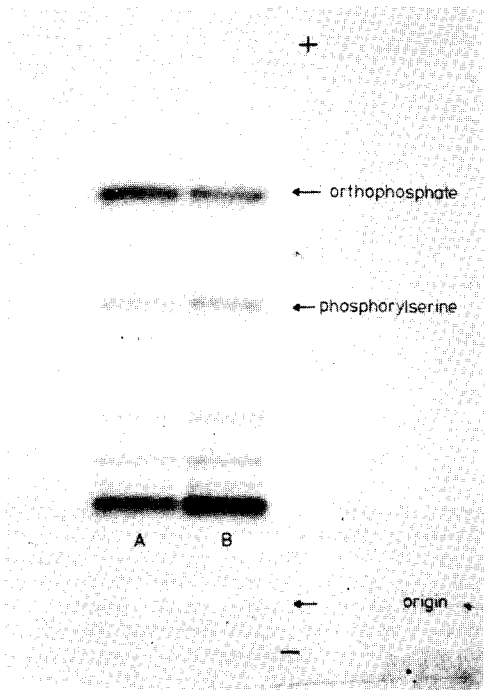
enzyme was phosphorylated on incubation with ATP and cyclic AMP-dependent protein kinase. The amount of radioactivity present in the major band from the kidney pool corresponded to about 0.7 mol [^{32}P]phosphate/mol of pyruvate kinase subunit, assuming that the specific activities of the rat liver and kidney pyruvate kinases type L were the same [5].

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

In order to establish that the rat kidney pyruvate kinase was phosphorylated on a seryl residue, the enzyme was hydrolyzed after phosphorylation as outlined above. From the acid hydrolysates [^{32}P]phosphorylserine was isolated by chromatography on Dowex 50. The molar amount of [^{32}P]phosphate bound to serine residues of the enzyme was found to be about 0.2 mol/mol of enzyme subunit, calculated on the same assumptions as before [20].

[^{32}P]Phosphopeptide pattern after partial acid hydrolysis

^{32}P-labeled rat kidney pyruvate kinase type L was subjected to high-voltage electrophoresis in 0.05 M pyridine/HAc (pH 3.5) after partial acid hydrolysis in 6 M HCl for 30 min. The type-L rat liver enzyme was investigated by the same technique in parallel experiments. Fig. 3 shows the result obtained by auto-



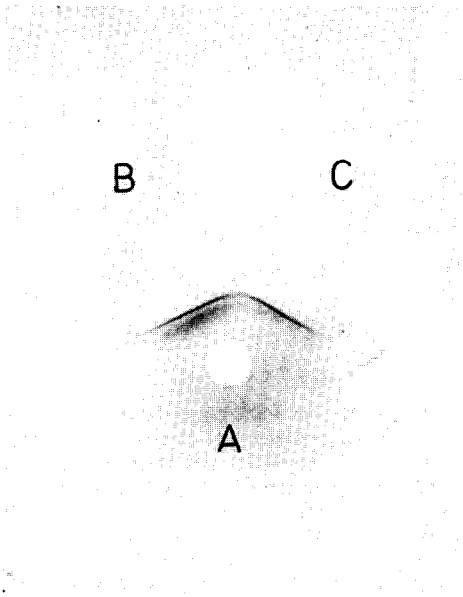


Fig. 3. Radioautograph of electropherogram of partial acid hydrolysates of rat kidney (A) and rat liver (B) pyruvate kinases type L. The enzymes were incubated with ATP and cyclic AMP-dependent protein kinase as described in the text.

Fig. 4. Immunodiffusion analysis of rat liver and rat kidney pyruvate kinases type L. Antibodies to the liver enzyme were placed in well A, and the kidney and liver enzymes in wells B and C, respectively.

radiography of the electropherogram. It is seen that the same radioactive bands were found for both pyruvate kinases. This indicates that the amino acid sequences around the seryl residue in the two enzymes are identical. It has been shown prevously that no radioactive bands are present when cyclic AMP-dependent protein kinase is treated as described above in the absence of pyruvate kinase [11], which implies that the radioactive bands did not originate from the protein kinase.

Immunological studies

In immunodiffusion experiments a precipitation line was seen between pyruvate kinase type L from rat kidney and antibodies to the rat liver pyruvate kinase, indicating a cross-reaction. Furthermore, immunological identity was seen between the rat liver and rat kidney type-L pyruvate kinases (Fig. 4).

Effect of phosphorylation and dephosphorylation on pyruvate kinase activity

As shown in Fig. 5, rat kidney pyruvate kinase type L exerted a weak cooperative effect with the substrate *P-enol*pyruvate. By phosphorylation this effect was enhanced and the activity of the kidney enzyme was inhibited at lower P-enolpyruvate concentrations. The apparent K_m values for *P-enol*-pyruvate were 0.15 mM and 0.35 mM for the unphosphorylated and the phos-

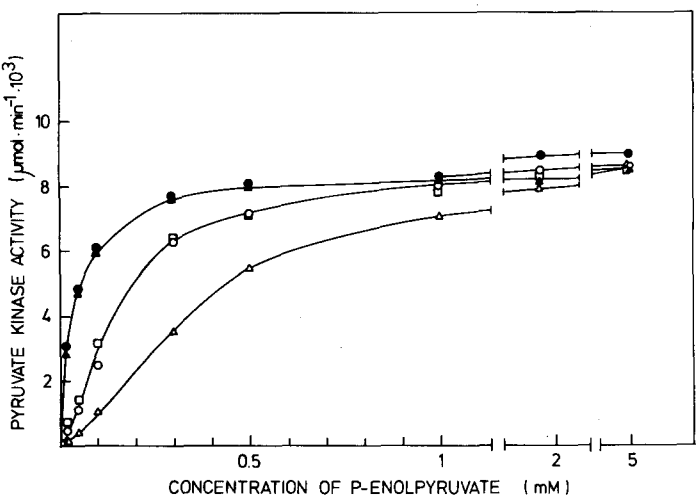


Fig. 5. Effect of phosphorylation and dephosphorylation on the activity of rat kidney pyruvate kinase type L. 2.4 units of pyruvate kinase was phosphorylated as described in the text, and the reaction was interrupted by dilution in 20 vol of ice-cold 20 mM potassium phosphate (pH 7.0)/30% glycerol/0.1 mM dithiothreitol. Prior to the assay the enzyme was diluted another four times in the same buffer. The unphosphorylated pyruvate kinase was treated identically except that ATP was omitted during the incubation. Some samples were treated with phosphatase as described in the text. (○———○) activity of unphosphorylated pyruvate kinase; (△———△) activity of phosphorylated pyruvate kinase in the absence, and (□———□) in the presence of phosphatase. Filled symbols represent the corresponding activities in the presence of 5 μM Fru-1,6-P_2.

phorylated enzymes, respectively. The Hill constant increased from 1.4 to 1.6 on phosphorylation of the enzyme. In the presence of 5 μM Fru-1,6-P_2, both enzyme forms showed hyperbolic relationships to *P-enol*pyruvate, with apparent K_m values of 0.05 mM. The Hill constant was calculated to be 1.0 in both cases. The effect of phosphorylation on the activity of the kidney pyruvate kinase was thus fully counteracted by 5 μM Fru-1,6-P_2.

For the unphosphorylated enzyme used in these experiments either ATP or cyclic AMP-dependent protein kinase was omitted during the phosphorylation reaction. The dependence of the rate of enzyme activity on the *P-enol*pyruvate concentration was similar in these two cases. The apparent K_m value for this substrate was thus found to be clearly difference from that of the phosphorylated pyruvate kinase.

In order to investigate the kinetic effect of the dephosphorylation of rat kidney pyruvate kinase, the enzyme was phosphorylated and then incubated with phosphoprotein phosphatase as described. The kinetic properties of the phosphatase-treated phosphoenzyme were very similar to those of the unphosphorylated enzyme (Fig. 5). Samples were taken from the phosphorylated pyruvate kinase that was incubated in the presence or absence of phosphatase, precipitated with trichloroacetic acid, washed [17] and the radioactivity measured. On addition of phosphatase, virtually no radioactivity was found in the samples (3% remaining, compared to the samples incubated in the absence of this enzyme), indicating a dephosphorylation of pyruvate kinase. These results are thus in keeping with the concept that the activity of L-type pyruvate

kinase from rat kidney is regulated by a reversible phosphorylation-dephosphorylation reaction.

Discussion

The aim of the present investigation was to study whether L-type kidney pyruvate kinase belongs to the group of enzymes which are phosphorylated on incubation with ATP and cyclic AMP-dependent protein kinase. Previous studies in this laboratory have revealed that the L-type pyruvate kinases from rat or pig liver belongs to this group [5,6]. It is also known that some L-type pyruvate kinases cross-react in immunological experiments [21,22].

A homogeneous preparation of the catalytic subunit from rat liver cyclic AMP-dependent protein kinase was used in the phosphorylation experiments. The ability of a certain protein kinase to phosphorylate different proteins from several tissues is well documented [23], and the use of a liver protein kinase in these experiments therefore seemed appropriate.

During the purification procedure, in the last two chromatographies the activity of L-type kidney pyruvate kinase paralleled the incorporation of [^{32}P]phosphate into protein. Furthermore, in the polyacrylamide gel electrophoresis experiments, the major band with the same molecular weight as rat pyruvate kinase was shown to contain radioactive material, indicating a phosphorylation of the kidney pyruvate kinase.

The activity of kidney type L pyruvate kinase was influenced by phosphorylation, and an inhibition was seen at lower, physiological *P-enol*pyruvate concentrations [24]. The change in activity of the phosphorylated kidney pyruvate kinase on incubation with phosphatase, showed that the activity of this enzyme in vitro was regulated by a reversible phosphorylation-dephosphorylation reaction.

In earlier studies we have shown that the A-type pyruvate kinase from pig kidney is not a substrate for cyclic AMP-dependent protein kinase [9,25]. Since the L-type kidney pyruvate kinase in this study was shown to be such a substrate, hormonal control of the enzyme activity is conceivable. The results thus indicates that regulation of enzyme activity during gluconeogenesis via cyclic AMP is of importance also in renal cortex, and that pyruvate kinase seems to be a target enzyme for this type of regulation. In view of this, the distribution of pyruvate kinase isozymes to different types of kidney cells seems probable. The appearance of the L-type pyruvate kinase in cells connected with gluconeogenesis would seem to be in agreement with the findings regarding the phosphorylation of the enzyme by cyclic AMP-dependent protein kinase. Further studies are necessary, however, to prove this hypothesis.

Acknowledgements

This project was supported by the Swedish Medical Research Council (Project 13X-50), Magnus Bergvalls Stiftelse, Nordisk insulinfond, and Svenska Diabetesforbundet. The excellent technical assistance of Miss Jill Anderson is gratefully acknowledged. Antibodies to rat liver pyruvate kinase were a generous gift of Drs. O. Ljungstrom and P. Ekman, and the phosphoprotein

phosphatase was kindly donated by Dr. V. Titanji. I also wish to thank Drs. L. Engstrom and G. Hjelmquist for valuable discussions.

References

1 Imamura, K. and Tanaka, T. (1972) J. Biochem. (Tokyo) 71, 1043—1051
2 Imamura, K. Taniuchi, T. and Tanaka, T. (1972) J. Biochem. (Tokyo) 72, 1001—1015
3 Van Berkel, T.J.C., Koster, J.F. and Hulsmann, W.C. (1972) Biochim. Biophys. Acta 276, 425—429
4 Osterman, J. and Fritz, P.J. (1974) Biochemistry 13, 1731—1736
5 Ljungstrom, O., Hjelmquist, G. and Engstrom, L. (1974) Biochim. Biophys. Acta 358, 289—298
6 Engstrom, L., Berglund, L., Bergstrom, G., Hjelmquist, G. and Ljungstrom, O. (1974) in Lipmann Symposium: Energy, Biosynthesis and Regulation in Molecular Biology, (Richter, D., ed.), pp. 192—204, Walter de Gruyter, Berlin
7 Ekman, P., Dahlqvist, U., Humble, E. and Engstrom, L. (1976) Biochim. Biophys. Acta 429, 374—382
8 Ljungstrom, O., Berglund, L. and Engstrom, L. (1976) Eur. J. Biochem. 68, 497—506
9 Berglund, L., Ljungstrom, O. and Engstrom, L. (1977) J. Biol. Chem. 252, 6108—6111
10 Sandoval, I.V. and Carbonell, J. (1973) Biochim. Biophys. Acta 315, 343—346
11 Titanji, V.P.K., Zetterqvist, O. and Engstrom, L. (1976) Biochim. Biophys. Acta 422, 98—108
12 Zetterqvist, O., Ragnarsson, U., Humble, E., Berglund, L. and Engstrom, L. (1976) Biochem. Biophys. Res. Commun. 70, 696—703
13 Corbin, J.D. and Reiman, E.M. (1974) in Methods in Enzymology (Hardman, J.G. and O'Malley, B.W., eds.), Vol. 38, pp. 287—290, Academic Press, New York
14 Titanji, P.V.K. (1977) Biochim. Biophys. Acta 481, 140—151
15 Engstrom, L. (1962) Ark. Kem. 19, 129—140
16 Mardh, S. (1975) Biochim. Biophys. Acta 391, 448—463
17 Ljungstrom, O., Berglund, L., Hjelmquist, G., Humble, E. and Engstrom, L. (1974) Uppsala J. Med. Sci. 79, 129—137
18 Ljungstrom, O. and Ekman, P. (1977) Biochem. Biophys. Res. Commun. 78, 1147—1155
19 Ouchterlony, O. (1958) in Progress in Allergy (Kallos, P. and Waksman, B.H. eds.), Vol. 5, pp. 1—78, Karger, Basel
20 Ljungstrom, O. and Engstrom, L. (1974) Biochim. Biophys. Acta 336, 140—150
21 Lincoln, D.R., Black, J.A. and Rittenberg, M.B. (1975) Biochim. Biophys. Acta 410, 279—284
22 Marie, J., Kahn, A. and Boivin, P. (1977) Biochim. Biophys. Acta 481, 96—104
23 Walsh, D.A. and Krebs, E.G. (1973) in The Enzymes (Boyer, P.D., ed.), Vol. 8, 3rd edn., pp. 555—581, Academic Press, New York
24 Flory, W., Peczon, B.D., Koeppe, R.E. and Spivey, H.O. (1974) Biochem. J. 141, 127—131
25 Humble, E., Berglund, L., Titanji, V., Ljungstrom, O., Edlund, B., Zetterqvist, O. and Engstrom, L. (1975) Biochem. Biophys. Res. Commun. 66, 614—621