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PYRUVATE KINASE TYPE M_2 IS PHOSPHORYLATED IN THE INTACT CHICKEN LIVER CELL

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SUMMARY: Isolated hepatocytes from 24 h starved chicks were depleted of phosphate and incubated in the presence of ^{32}P-orthophosphate. Pyruvate kinase type M_2 from crude cell extracts was partially purified by chromatography on DEAE-Sephacel and hydroxylapatite. SDS slab gel electrophoresis of the fractions containing the enzyme and immunoprecipitation with antisera showed the phosphorylation of pyruvate kinase type M_2. Phosphoamino acid analysis identified serine as phosphate acceptor.

Pyruvate kinase (EC 2.7.1.40) isoenzyme type L from mammalian liver is inactivated in vitro via phosphorylation by a *cAMP dependent* protein kinase (for rev. see ref. 1). This has been shown principally relevant also in vivo (2-4). In contrast to type L, pyruvate kinase type M_2 from chicken liver, where it represents the predominant amount of pyruvate kinase activity (5,6), is inactivated and phosphorylated in vitro by a *cAMP independent* protein kinase (7), probably at serine (8).

In this paper we demonstrate for the first time (a) the phosphorylation of chicken liver pyruvate kinase type M_2 in the intact cell and (b) that this phosphorylation is found at serine.

MATERIALS and METHODS

Materials: Biochemicals were from Boehringer Mannheim (Mannheim, FRG), Nonidet NP40 from Fluka (Neu Ulm), DEAE-Sephacel and protein A-Sepharose from Pharmacia (Freiburg), hydroxylapatite (DNA grade) from Bio-Rad Laboratories (München), $^{32}P_i$ from Amersham-Buchler (Braunschweig). All other reagents were of analytical grade and purchased from E. Merck (Darmstadt) and Serva Feinbiochemica (Heidelberg). Animals were male Lohmann-White Leghorn chicks from a cockerel farm near Giessen, fed on 13-B-Junghennenalleinfutter,

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Raiffeisen Hauptgenossenschaft (Frankfurt/Main). Prior to the experiments the animals (3-9 weeks of age) were starved for 24 h.

Isolation and incubation of liver cells: Isolation of liver cells was performed in principle as described previously (9). For washing the cells instead of Krebs-Henseleit bicarbonate medium the following solution (medium A) was used: 118.1 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4 \cdot 7\ H_2O$, 25.0 mM $NaHCO_3$, 10 mM HEPES/KOH, pH 7.4. After washing the cells were resuspended in excess medium A + 2 % (w/v) bovine serum albumin (= medium B). All steps of incubation were carried out under gassing with 95 % O_2 + 5 % CO_2 and shaking in a Dubnoff shaker at 37°C with 100 Osz./min. After 30 min the cell suspension was centrifuged (50 g, 30 to 60 seconds), the cells were resuspended in excess medium B and incubated for another period of 30 min. After centrifugation (50 g, 30 to 60 seconds) the cells were resuspended in as much medium B + 20 mM dihydroxyacetone that a concentration of about $135 \cdot 10^6$ unstained cells per ml resulted. 3 ml of this cell suspension was incubated with 1 ml $^{32}P_i$ in diluted HCl (3 mCi/ml) + 0.017 ml 1 M Tris/Cl (pH 7.5). After 120 min the incubation was stopped by rapid centrifugation for 30 seconds in an 'Eppendorf Zentrifuge 3200' at maximal speed and freezing the pellet after removal of the supernatant in liquid nitrogen.

Partial purification of ^{32}P-pyruvate kinase: To the frozen pellet 1 ml buffer A (10) per ml original cell suspension was added (buffer A: 10 mM Tris/Cl, 1 mM EDTA-Na_2, 1 mM 2-mercaptoethanol, 0.05 % (v/v) Nonidet NP40, pH 7.2). The pellet was vigorously shaken in an 'Eppendorf Rotationsmischer 3300' at room temperature until it was completely thawed and a homogeneous suspension had resulted (about 10 min). After centrifugation the supernatant was transferred into another tube and mixed with 0.1 ml glycerol per ml supernatant (= crude cell extract).

1 ml portions of this mixture were passed at room temperature through a column (0.5 cm x 10 cm) of DEAE-Sephacel equilibrated with buffer A + 10 % (v/v) glycerol. The column was washed with 4 ml equilibration buffer; the outflow containing pyruvate kinase type M_2 fell on top of a column (0.5 cm x 10 cm) of hydroxylapatite that had been equilibrated with the same buffer. Elution was done by 20 ml of a linear gradient of 0 - 500 mM potassium phosphate in 1 mM EDTA-Na_2, 1 mM 2-mercaptoethanol, 10 % (v/v) glycerol at pH 7.2.

Pyruvate kinase activity was determined by the coupled optical test described by Eigenbrodt and Schoner (6). SDS gel electrophoresis was carried out in discontinuous vertical slab gels (8.1 % (w/v) polyacrylamide, 0.22 % bisacrylamide rsp. 10.0 % polyacrylamide, 0.26 % bisacrylamide) with the buffer system described by Laemmli (11). For protein staining Serva Blau R was used.

Analysis of ^{32}P-amino acid: After staining/destaining the SDS gel band corresponding to pyruvate kinase was cut off. Protein was extracted by shaking the gel slices overnight at 37°C in a solution of 50 mM NH_4HCO_3, 0.1 % (w/v) SDS, 5 % (v/v) 2-mercaptoethanol, pH 8.5, and precipitated by 5 % (w/v) ice-cold trichloroacetic acid. The sediment was washed with 96 % (v/v) ethanol (-20°C) and thereafter with diethyl ether : ethanol (v:v = 1:1) (-20°C). Then the protein was partly hydrolysed (6 N HCl, 110°C, 2 hours) and subjected to two-dimensional analysis of phosphoamino acids as described in (12,13). Radioactivity was detected by autoradiography using Kodak safety film for an exposition time of 1 week.

Immunoprecipitation of pyruvate kinase: 4 mg protein A-Sepharose was swollen for 15 min in 0.5 ml buffer B (50 mM Tris/Cl, 0.1 %

(v/v) Triton X-100, 100 mM 2-mercaptoethanol, 10 mM EDTA-Na_2, 100 mM NaF, pH 7.5). The gel was washed and blotted dry. 10 µl rabbit antiserum against chicken liver pyruvate kinase type M_2 rsp. L (obtained as described in (14)) was added and adsorbed for 15 min at 4°C onto the protein A-Sepharose. The IgG-protein A-Sepharose complexes were washed three times with 1 ml ice-cold buffer B. 20 µl hydroxylapatite fraction was incubated with the antiserum in solid phase for 60 min at 4°C. The immune complexes were washed with 1 ml buffer C (50 mM sodium phosphate, 40 mM NaF, 10 mM EDTA-Na_2, 1 mg/ml bovine serum albumin, 1 mM phenylmethylsulfonylfluoride) + 1 M NaCl, 1 ml buffer C + 1 % (v/v) Triton X-100 and 1 ml H_2O. After addition of 50 µl buffer D (125 mM Tris/Cl, 10 % (w/v) SDS, 1.4 M 2-mercaptoethanol, 20 % (v/v) glycerol, 6 M urea, pH 6.8) the mixture was heated for 5 min at 96°C. The supernatant was applied to SDS slab gel electrophoresis. Radioactivity was detected by autoradiography.

RESULTS

The crude cell extract of the ^{32}P-orthophosphate labeled liver cells was passed through DEAE-Sephacel and chromatographed on hydroxylapatite (Fig. 1A). Aliquots of the fractions 3 - 6 were subjected to SDS slab gel electrophoresis: the protein staining is shown in fig. 1B; the radioactivity of the resulting bands was visualized by autoradiography (Fig. 1C). In the fractions 5 and 6 there was only one protein band with a molecular mass somewhat smaller than 60,000. This corresponds to the molecular mass of pure pyruvate kinase type M_2 (M_r = 58,000).

To ensure the identity of this protein to the isoenzyme type M_2 of chicken liver pyruvate kinase, immunoprecipitations in solid phase were carried out with aliquots of the hydroxylapatite fractions. The precipitates were subjected to SDS slab gel electrophoresis and subsequently to autoradiography (Fig. 2). No phosphorylated protein could be precipitated by antiserum against type L pyruvate kinase. Antiserum against type M_2, however, precipitated in fraction 5 and 6 a protein that was phosphorylated and had a molecular mass of 58,000.

The result of the phosphoamino acid analysis is given in fig. 3. P-serine was identified as ^{32}P-radioactive amino acid resulting from the hydrolysis of phosphorylated pyruvate kinase.

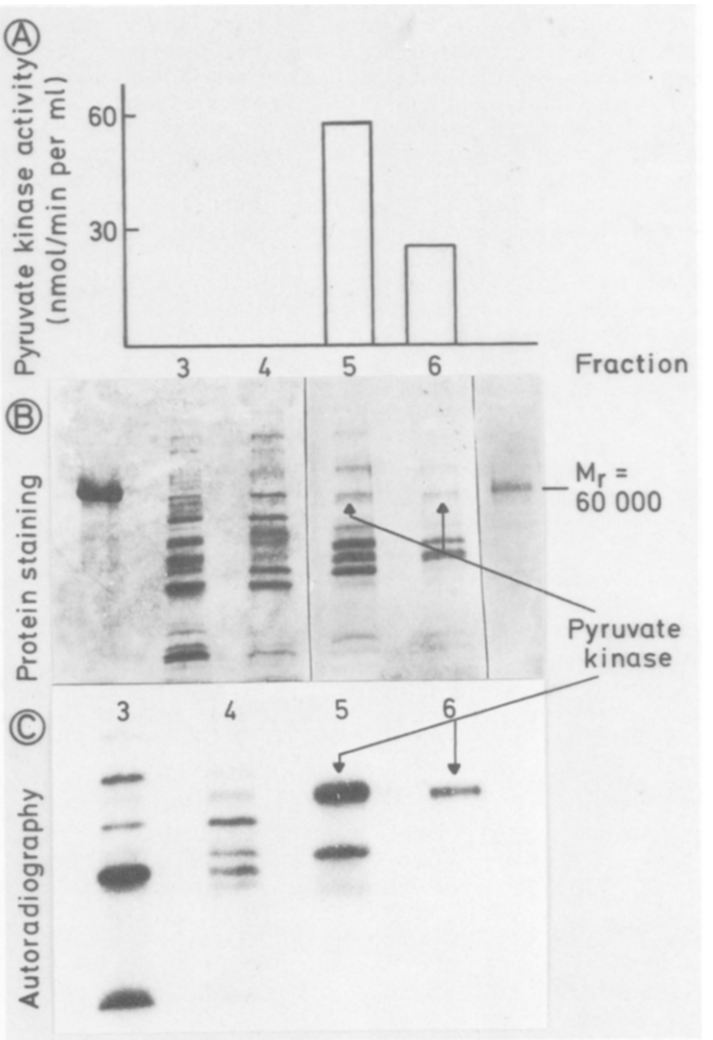

Fig. 1: SDS gel electrophoresis of hydroxylapatite fractions. Pyruvate kinase from chicken liver cells incubated in the presence of ^{32}P-orthophosphate was partially purified as described in the Materials and Methods section. The resulting hydroxylapatite fractions 3-6 were subjected to SDS slab gel electrophoresis. Part A: pyruvate kinase activity. Part B: protein staining of the gel. Part C: autoradiography of the stained gel. Catalase was used as marker for the molecular mass of 60,000. The volume per fraction was 2 ml.

DISCUSSION

Eigenbrodt et al. (7) demonstrated in vitro the cAMP independent phosphorylation of pyruvate kinase type M_2 from chicken liver. The phosphate acceptor is a serine residue in an acidic environment (8). The experiments presented in this paper confirm these findings and

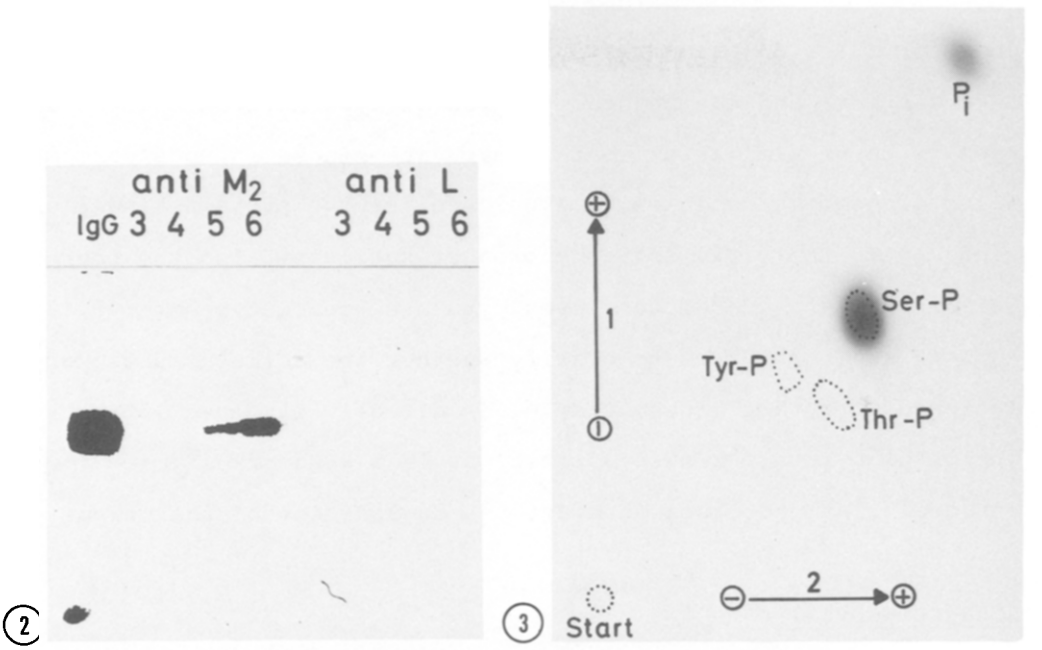

Fig. 2: SDS gel electrophoresis of immunoprecipitated pyruvate kinase phosphorylated in vivo.

In the hydroxylapatite fractions 3-6 of fig. 1 immunoprecipitations with antisera against type M_2 or type L of pyruvate kinase were carried out. The precipitates were subjected to SDS slab gel electrophoresis and the radioactivity was visualized by autoradiography. ^{32}P-labeled IgG was used as marker (M_r = 58,000).

Fig. 3: ^{32}P-amino acid analysis of pyruvate kinase phosphorylated in vivo.

Phosphorylated pyruvate kinase protein was eluted from the stained gel (see fig. 1), hydrolysed, and amino acids were separated by high voltage electrophoresis. The radioactive spots visualized by autoradiography are shown. The positions of the standard phosphoamino acids are given by the dotted lines.

prove their validity for the intact chicken liver cell: pyruvate kinase type M_2 is phosphorylated in the isolated cell; the reaction needs no added cAMP; the phosphorylation takes place at serine. Since only acid-stable phosphoamino acids can be analysed by the method used, it cannot be excluded that also other amino acids may be phosphorylated as well, but undergo degradation during acid hydrolysis.

In rat liver type L pyruvate kinase is the predominant isoenzyme (15). Its well known phosphorylation mechanism seems to be very different from that of type M_2, because it is cAMP dependent and

phosphorylates a serine residue in a basic environment (1,16,17). On the other hand the phosphorylation of type M_2 from chicken liver appears to be similar to that of pyruvate kinase in Rous sarcoma virus transformed chicken embryonic cells; the protein kinase associated with the viral src-gene product catalyzes also the phosphorylation in vitro of chicken liver pyruvate kinase type M_2 (18,19).

Work is in progress to clarify whether the cellular phosphorylation of pyruvate kinase type M_2 is directly involved in the regulation of pyruvate kinase activity or is a kind of signal for intracellular proteases to start the degradation of the enzyme protein.

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