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L-TYPE PYRUVATE KINASE FROM HUMAN LIVER

PURIFICATION BY DOUBLE AFFINITY ELUTION, ELECTROFOCUSING AND IMMUNOLOGICAL STUDIES

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Summary

L-type pyruvate kinase (ATP:pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40) was highly purified from adult human liver. This purification included ammonium sulphate fractionation, DEAE-Sephadex batchwise absorption and two CM-Sephadex chromatographies with selective elution by ligands; in the former chromatography pyruvate kinase was eluted by ATP, in the latter one by phospho*enol*pyruvate and fructose 1,6-diphosphate.

The last step of the purification procedure involved a hydroxyapatite column chromatography. This purification procedure allowed us to obtain 3.6 mg of protein with a specific activity 190 I.U./mg, i.e. a 1200-fold purification with an overall yield of about 8%.

This preparation was homogeneous as judged by immunodiffusion, acrylamide and sodium dodecyl sulphate acrylamide gel electrophoresis.

Anti L-type pyruvate kinase antibodies were obtained from rabbits and the antigenic properties of L-type pyruvate kinase were studied.

The enzyme appeared to be a tetramer (molecular weight 220 000—240 000) with subunits of similar molecular weight about 60 000).

Two interconvertible major forms were found by isoelectrofocusing in a sucrose gradient and in an acrylamide slab gel: one had an isoelectric point of 5.85 ± 0.09 and was the major enzymatic form after incubation with fructose 1,6-diphosphate or high concentrations or SH reagents.

The other form (isoelectric point 6.28 ± 0.03) was the major form of L-type pyruvate kinase in liver crude extract, and after incubation of purified enzyme with a proteic fraction isolated from liver extract by ammonium sulphate precipitation.

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Introduction

L-type pyruvate kinase (ATP:pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40) has been purified from livers of various mammals [1—4]. Human L-type liver pyruvate kinase, perhaps because of its instability post mortem [5] and during the purification procedure [1,2,4,6,7] has never been totally purified.

The present paper reports the purification of L-type pyruvate kinase from adult human liver by double elective elution with ATP on the one hand and phospho*enol*pyruvate associated to fructose 1,6-diphosphate on the other.

Up to now the relationship between erythrocyte and L-type pyruvate kinase remain an object of discussion.

The enzymes are immunologically [1,5,8] and kinetically [9,10] related. Moreover several cases with erythrocyte and liver pyruvate kinase deficiency have been reported [10—13].

Erythrocyte and liver pyruvate kinase, however, can be electrophoretically distinguished [10,14].

Some authors have suggested that erythrocyte pyruvate kinase might be a hybrid of L-type and M2-type pyruvate kinases [10].

This hypothesis was recently supported by the results of Peterson et al. [15]. These authors showed that sodium dodecyl sulphate acrylamide gel electrophoresis of purified erythrocyte pyruvate kinase gave two distinct bands with similar mobilities. Furthermore fingerprinting of the cyanogen bromide peptides showed considerable similarities between the M1 and erythrocyte subunits. Conclusive data about this hypothesis, however, require the isolation of the parental species (i.e. hypothetically, L-type on the one hand and M2 or M1 type on the other).

Consequently the aim of this work was to purify human L-type pyruvate kinase and to study its subunit structure and its immunological properties in order to elucidate the similarities and the differences between erythrocyte and L-type pyruvate kinase.

Moreover, the L-type subunit of erythrocyte pyruvate kinase seems to be the subunit specifically involved in the erythrocyte pyruvate kinase deficiency since the deficiency was found in liver but not in leukocytes or in muscle of the deficient patients [10].

Thus the study of human L-type pyruvate kinase might allow a better understanding of the molecular mechanism of erythrocyte pyruvate kinase deficiency.

Materials and Methods

Materials

Ion exchangers (DEAE Sephadex A 50, CM Sephadex C 50 and Sephadex G 200) were purchased from Pharmacia, hydroxyapatite gel from Clarckson CC.

The substrates for the enzymatic reactions and intermediate enzyme were supplied by Boehringer Mannheim, Coomassie Blue R 250 came from Sigma CC. Acrylamide, bisacrylamide and sodium dodecyl sulphate were furnished by Eastmann Kodak, agarose by "l'Industrie Biologique Française" and ampholines by LKB.

Enzymatic activity was measured in a Zeiss PM Q2 spectrophotometer connected to a Servogor Recorder. The column eluates were monitored at 280 nm with a Beckman DBG spectrophotometer also coupled to a recorder. The conductivity of the buffers was measured with a Biolyon conductivity meter.

Electrofocusing was performed in an Isco model 212 column.

Methods

All the steps of purification were carried out at +4°C. Unless otherwise indicated the buffers contained 1 mM EDTA, 1 mM ϵ-aminocaproic acid and 10 mM β-mercaptoethanol.

Assays. The activity of L-type pyruvate kinase was measured according to the method of Blume [16] with 1.5 mM ADP and 5 mM phospho*enol*pyruvate. The protein concentration was measured according to the method of Lowry et al. [17] against a standard of crystallized bovine albumin.

Criteria of purity. The purity of the enzymatic preparation was appraised by three methods.

(a) Immunological methods: Two anti L-type sera were obtained from rabbits: the former with pyruvate kinase partially purified until step 4, the latter with the highly purified enzyme (final preparation). The polyvalent antiserum was obtained from a previous purification experiment and was further used to measure the ratio of L-type pyruvate kinase in the liver crude extracts.

The animals received a total of 2.6 mg of partially purified enzyme and 0.8 mg of highly purified enzyme emulsified with complete Freund adjuvant in intradermal injection every ten days for one month, then intramuscularly on the 45th day. A week after this injection the rabbits were killed and the blood was collected. The serum was heated for 30 min at 60°C, then stored frozen with 0.02% (w/v) sodium azide.

Immunological titrations were studied as described in ref. 18.

The polyvalent antiserum was tested by double immunodiffusion against the highly purified preparation, and the antiserum prepared against highly purified pyruvate kinase was tested against the liver crude extracts.

(b) Polyacrylamide gel electrophoresis: The gel was 7.5% (w/v) polyacrylamide in 40 mM Tris/glycine buffer pH 8.7. The electrophoresis was performed for 1.5 h at 2.5 mA per gel. The proteins were stained with Coomassie blue [20].

(c) Acrylamide sodium dodecyl sulphate electrophoresis: Acrylamide sodium dodecyl sulphate electrophoresis was performed according to the method of Weber and Osborn [21]. The proteins were stained with Coomassie blue.

Determination of the molecular weight by gel filtration. Purified L-type pyruvate kinase was subjected to Sephadex G-200 chromatography in 50 mM Tris/chloride buffer pH 7.5. 0.5 ml of a dilution containing 2 I.U. of pyruvate kinase was applied to the top of a column of 70 × 1.5 cm (flow rate 6 ml/h) and 1 ml fractions were collected and assayed for pyruvate kinase activity. Bovine serum albumin, hemoglobin, aldolase from rabbit muscle, γ-globulin and catalase from beef liver were used as reference compounds. The void volume was determined with blue dextran. Linear plots of V_e/V_o versus log molecular weight were obtained.

Subunit molecular weight. This was estimated by sodium dodecyl sulphate

acrylamide gel electrophoresis [21]. Dissociated bovine serum albumin, albumin from hen egg, aldolase from rabbit muscle and catalase from beef liver were used as standards.

Linear plots of log molecular weight versus relative electrophoretic mobility were obtained.

Electrofocusing in polyacrylamide ampholine gel [13]. Pyruvate was focused in thin layer acrylamide ampholine gel. Gel (2 × 100 × 200 mm) was 3% (w/v) acrylamide, 0.08% (w/v) bisacrylamide and contained 1.33% (w/v) ampholines of pH range 5—8, 0.66% (w/v) ampholines of pH range 7—9, 10% (w/v) sucrose, 0.02% (w/v) *N,N,N',N'*-tetramethylethylenediamine and 0.03% (w/v) ammonium persulphate. Persulphate in excess was eliminated by prefocusing for 1 h; then 5 µl samples (of total enzymatic activity $5—20 \cdot 10^{-3}$ I.U.) were applied from small pieces of cellulose acetate (cellogel). The isoelectrofocusing ran for 4 h at a final voltage of 1000 V at 4°C. The enzymatic activity was revealed by defluorescence under ultraviolet, by pouring on to the gel the following solution in 1% agarose: 100 mM Tris/chloride buffer (pH 8), 100 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM NADH, 1.5 mM ADP, 5 mM phospho*enol*pyruvate and 1 I.U./ml lacticdehydrogenase. Photographs were taken with No. 57 polaroid films through combined 2 B and 4 Wratten filters.

Studies on influence of ligands and liver extract fractions on the electrofocusing pattern of purified L-type pyruvate kinase. The highly purified L-type enzyme was extensively dialysed in a 50 mM Tris/chloride buffer pH 7.3, containing 100 mM KCl, 4 mM $MgCl_2$, 1 mM EDTA and 500 mM sucrose (36 h dialysis with at least 6 buffer changes). It was incubated for 16 h at the room temperature in the presence of various ligands or SH reagents: either 1 mM fructose 1,6-diphosphate, or 5 mM ATP, or 5 mM phospho*enol*pyruvate, or 10 mM pyruvate or 10 mM L(+)-Alanine, or 50 mM dithiothreitol.

Liver extract was fractionated by ammonium sulphate precipitation at pH 7. 23 g of solid ammonium sulphate were added to 100 ml of liver extract (see below), and the precipitate (containing all the L-type pyruvate kinase) was discarded. Then 14 g per 100 ml of ammonium sulphate were added to the supernatant, and the precipitate was collected by centrifugation (fraction P1). A second fraction (P2) was obtained by adding 15 g of ammonium sulphate to 100 ml of the latter supernatant. These fractions were extensively dialysed against the 50 mM Tris/chloride buffer pH 7.3, described above, then incubated as previously indicated with the purified enzyme.

In some experiments these fractions were previously subjected to trypsin proteolysis (incubation for 4 h at 37°C in the presence of 0.5 mg/ml trypsin in the buffer containing 10 mM $CaCl_2$ and devoid of EDTA). Then pH was adjusted to 7.3, $CaCl_2$ neutralized by 20 mM EDTA and trypsin by 10 mM diisopropylfluorophosphate, and the extracts were incubated with purified pyruvate kinase as indicated above.

Electrofocusing in sucrose gradient. Electrofocusing of either purified enzyme or liver crude extract was performed in continuous sucrose gradient 5—35% w/v) containing 2% (w/v) ampholines of pH range 5—8 and 2 mM β-mercaptoethanol as previously described [18].

0.5-ml fractions were collected. pH values were measured at 0°C, the pH meter being standardized with standard pH buffer at the same temperature.

Results

Purification procedure

Unless otherwise indication all the steps of the purification were carried out at +4° C.

Step 1, extraction. Human liver was removed from a corpse 4 h after death and stored frozen at −80° C until use. The liver was homogenized in a Waring blendor with 4 volumes of 100 mM phosphate buffer, pH 7, containing 100 mM KCl, 1 mM diisopropylfluorophosphate and 10 mM $MgCl_2$. Then digitonin was added up to saturation and the extract was gently stirred for 30 min in a cold room. The cell debris were eliminated by centrifugation for 45 min at 18 000 × *g*. Gold toluene was added up to a concentration of about 10% (v/v) and vigorously emulsified with the extract. After centrifugation for 40 min at 10 000 × *g*, the upper fat layer was sucked off and the clear extract was decanted.

Step 2, ammonium sulphate fractionation. The extract was precipitated by adding 23 g of solid ammonium sulphate per 100 ml. The precipitate was collected by centrifugation for 45 min at 18 000 × *g* and washed once with 1 l of 50 mM phosphate buffer (pH 6) containing 0.01 mM fructose 1,6-diphosphate, 300 mM sucrose and 20 g ammonium sulphate per 100 ml. Then the precipitate was collected again by centrifugation and extensively dialysed against 5 mM phosphate buffer pH 7.2 containing 0.01 mM fructose 1,6-diphosphate and 300 mM sucrose.

Step 3, elution from DEAE-Sephadex (pH 7.2). DEAE Sephadex A 50, previously equilibrated with the dialysis buffer was added to the extract and gently stirred for 30 min at 4° C. The ion exchanger was gathered in a Buchner funnel and washed at room temperature with the dialysis buffer, then with 5 mM phosphate buffer (pH 7.2) containing 40 mM NaCl until the absorbance at 280 nm of the eluate was almost nil.

Pyruvate kinase was eluted with 5 mM phosphate buffer pH 7.2 containing 300 mM sucrose and 40 mM $MgSO_4$.

The eluate was precipitated by adding 31 g of solid ammonium sulphate per 100 ml.

The precipitate was collected by centrifugation for 30 min at 18 000 × *g* and extensively dialysed against a 10 mM phosphate buffer pH 6.1 with 0.01 mM fructose 1,6-diphosphate, 1 mM $MgCl_2$ and 500 mM sucrose.

Step 4, CM-Sephadex chromatography with selective elution by ATP. The preparation (1.257 mg of protein) was deposited at the top of a CM-Sephadex C-50 column (2 × 20 cm) equilibrated with 10 mM phosphate buffer (pH 6.1) without fructose 1,6-diphosphate or $MgCl_2$. The column was washed with the same buffer at a flow rate of 25 ml/h, then with a 50 mM phosphate buffer (pH 6.1) until all absorbance at 280 nm disappeared. The elution was promoted by adding to the 50 mM phosphate buffer pH 6.1, 4 mM ATP and 0.1 mM fructose 1,6-diphosphate. To avoid changes in the ionic strength, ATP and fructose 1,6-diphosphate were added in a solution of the same conductivity and the same pH as the washing buffer. Active fractions were collected and concentrated by vacuum dialysis against a 10 mM phosphate buffer pH 6.1, containing 1 M sucrose.

Step 5, CM-Sephadex C-50 chromatography column with elution by phosphoenolpyruvate and fructose 1,6-diphosphate. The preparation (9.85 mg of protein) was applied to a CM-Sephadex column (2 × 10 cm) equilibrated with the same buffer. The column was washed (flow rate 36 ml/h) with this buffer then with a 50 mM phosphate buffer, pH 6.1. The elution was caused by adding to the 50 mM phosphate buffer pH 6.1, 2 mM phospho*enol*pyruvate and 2 mM fructose 1,6-diphosphate. Active fractions were pooled and precipitated by solid ammonium sulphate (31 g per 100 ml). After centrifugation the precipitate was collected and dialysed against a 10 mM phosphate buffer, pH 7.2, with 0.1 mM fructose 1,6-diphosphate, 1 mM $MgCl_2$ and 1 M sucrose.

Step 6: hydroxyapatite column separation. The preparation was deposited at the top of a hydroxyapatite column (10 × 1 cm) previously equilibrated with the 10 mM phosphate buffer (pH 7.2) used in the previous dialysis. L-type pyruvate kinase was not bound to the column and was readily washed off with the equilibrating buffer. The enzyme was precipitated by adding solid ammonium sulphate (31 g per 100 ml). The precipitate was collected and the enzyme was stored in 50 mM Tris/chloride buffer, pH 8, containing 10 mM dithiothreitol, 10 mM $MgCl_2$, 100 mM KCl, 1 M sucrose and 31 g per 100 ml of ammonium sulphate. This purification procedure allowed us to obtain a purified enzyme with a specific activity of 190 I.U./mg of protein, i.e. a purification of about 1200—fold with an overall yield of 8%.

Table I summarizes the various steps of the purification procedure and their results.

Criteria of purity

The immunodiffusion experiments showed a single precipitation line be-

TABLE I

STEPS OF THE PURIFICATION PROCEDURE

	Activity (I.U.)	Proteins (mg)	Specific activity (I.U./mg)	Accumulation purification	Yield (%)
Liver crude extract	12 000		0.208		
L-type pyruvate kinase *	9 000	57 700	0.156	1	100
Ammonium sulphate precipitation	7 427				82.5
Dialysis	7 000	21 800	0.321	2	78
Elution from DEAE-Sephadex	6 113				68
Dialysis	4 400	1 257	3.5	22	49.8
CM-Sephadex chromatography with selective elution by ATP	2 869	52.5	54.5	349	32
CM-Sephadex chromatography with elution by phospho*enol*pyruvate and fructose 1,6-diphosphate	1 400	9.85	142	910	15.5
Dialysis	807				9
Hydroxyapatite chromatography	695	3.6	190	1 217	7.7

* L-type pyruvate kinase activity of the liver extract was determined by measuring the ratio of the total pyruvate kinase activity inhibited by anti L-type serum.

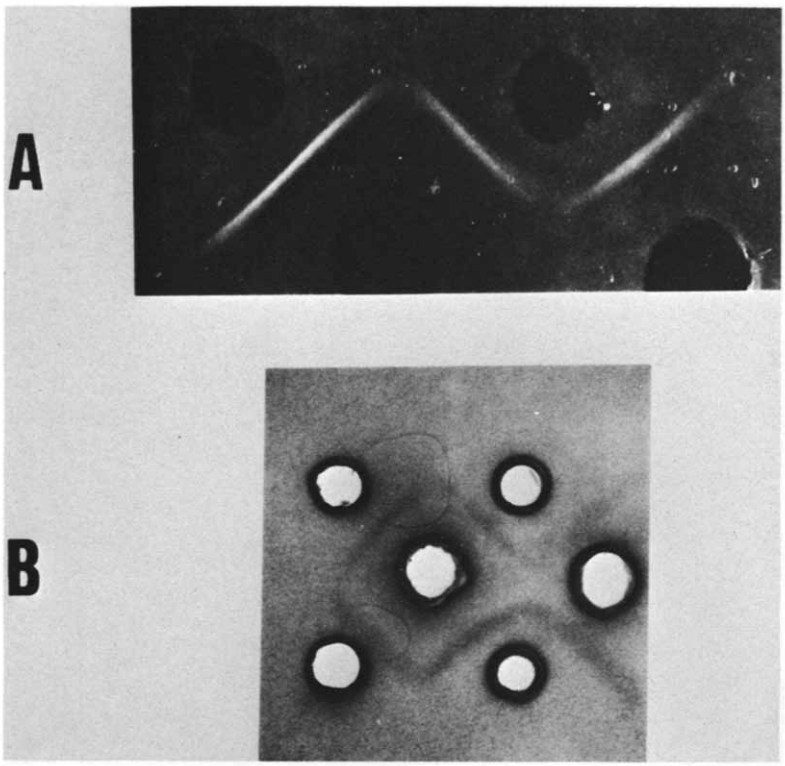

Fig. 1. Immunodiffusion of highly purified L-type pyruvate kinase against the anti L-type serum A: the upper wells were filled with the purified L-type pyruvate kinase (20 μg right and 10 μg left). The lower wells contained rabbit antiserum pure and 1 : 2 diluted. The gel was 1% agarose in 100 mM phosphate buffer, pH 7.2, containing sodium azide, 0.02% (w/v). B: the precipitation lines were stained for enzymatic activity.

tween the polyvalent antiserum and the purified preparation (Fig. 1) as well as between the anti highly purified pyruvate kinase serum and liver crude extracts.

Acrylamide gel electrophoresis in Tris/glycine buffer at pH 8.7 showed one markedly predominant protein band and another very slight band migrating more quickly towards the anode than the major band.

Sodium dodecyl sulphate acrylamide gel electrophoresis showed only one protein band (Fig. 2).

Immunological studies

L-type pyruvate kinase was 90—95% neutralized by the antisera. This neutralization was an inactivation and not simply an immunoprecipitation since the elimination of the antigen-antibody complex by centrifugation does not change the "titre" of the antisera.

The titre of the polyvalent antiserum used was approximately 45—70 I.U. of L-type pyruvate kinase inactivated by 1 ml of antiserum. The titre of the anti highly purified pyruvate kinase serum was approximately 30—35 I.U./ml. The slight enzymatic residual activity after inactivation by an excess of antiserum

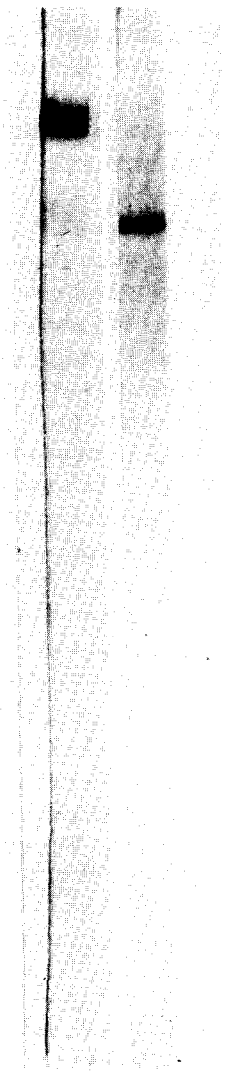

Fig. 2. Polyacrylamide gel electrophoresis and sodium dodecyl sulphate polyacrylamide gel electrophoresis of purified preparation Left: acrylamide gel electrophoresis. 6% acrylamide in 4 mM Tris glycine buffer pH 8.7 10 μg of proteins were applied to the gel. Proteins were stained with Coomassie blue. Right: sodium dodecyl sulphate polyacrylamide gel electrophoresis, according to the method of Weber and Osborn [21]. 10 μg of proteins were applied to the gel. Proteins were stained with Coomassie blue.

probably explains that the immunoprecipitate line could be specifically stained for pyruvate kinase activity.

Molecular weight

Electrophoretic mobility in sodium dodecyl sulphate acrylamide gel corresponded to a subunit of 59 500—61 000 daltons (Fig. 3). L-type pyruvate kinase was eluted from a Sephadex G-200 column in a single peak whose molecular weight was 220 000—240 000 in the course of 3 different experiments.

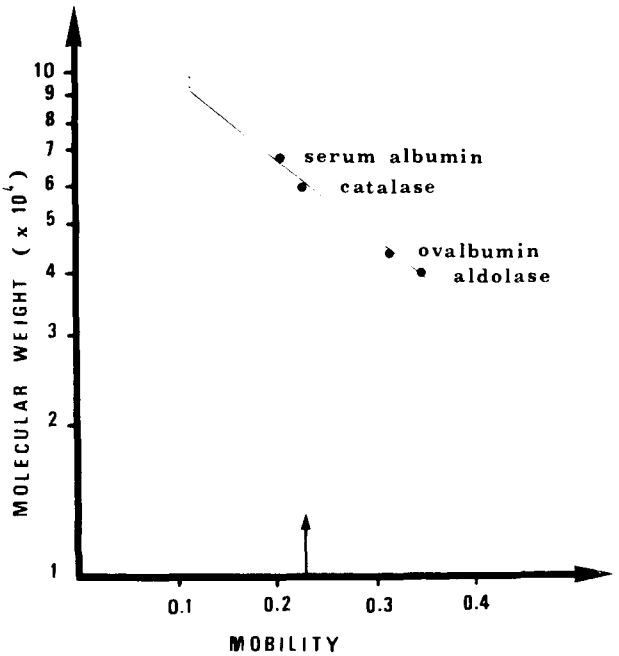

Fig. 3. Determination of the molecular weight of L-type pyruvate kinase subunits. Molecular weight was estimated by electrophoresis in sodium dodecyl sulphate polyacrylamide gel [21]. Standard molecular weight used were bovine serum albumin, hen egg albumin, aldolase from rabbit muscle and catalase from beef liver. The arrow indicates the mobility of L-type pyruvate kinase. The molecular weights of the markers proteins are those found in literature [21].

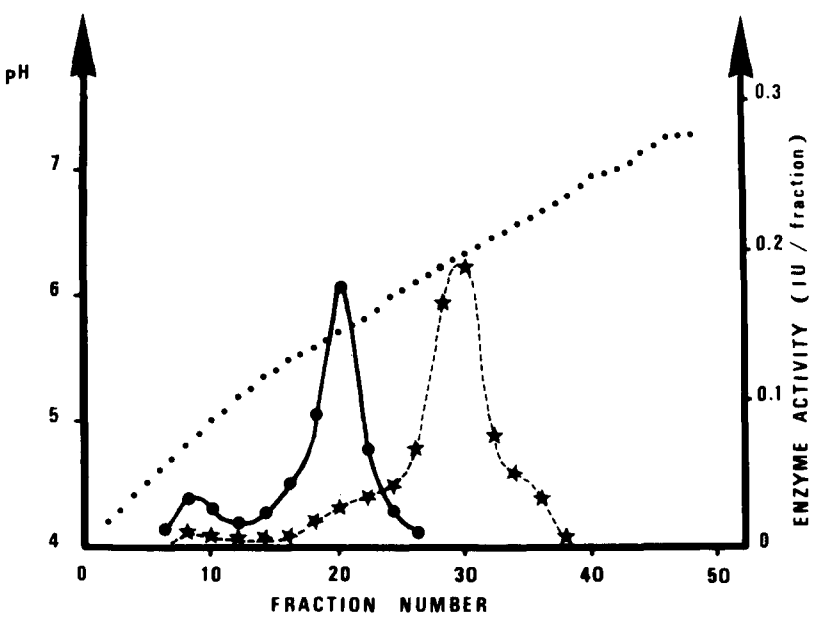

Fig. 4. Electrofocusing pattern of highly purified human liver L-type pyruvate kinase and liver crude extract in sucrose gradient. Electrofocusing was carried out in continous sucrose gradient, in the pH range 5—8. ●———●: purified human liver L-type pyruvate kinase. ★———★: liver crude extract. . . . . .: pH. This schema represents the results of two different experiments.

Isoelectrofocusing in sucrose gradient (Fig. 4)

Purified pyruvate kinase from human liver was eluted from the focusing column in a major peak, the isoelectric point of which was 5.85 ± 0.09 (n = 5). By contrast the major isozymic form from liver crude extract had an isoelectric point of 6.28 ± 0.03 (n = 5). Another acidic form with an isoelectric point of about 5 seemed to exist both in liver crude extract and purified preparation. M_2-type pyruvate kinase has an isoelectric point of about 7.7 and was not resolved in the pH gradient chosen (Marie et al., unpublished data). The purified L-type pyruvate kinase previously incubated with the fraction P2 from liver extract had a focusing pattern identical to that of the enzyme from crude extract.

Isoelectrofocusing in acrylamide ampholine slab gel

Fig. 5 shows the electrofocusing pattern of highly purifed preparation and pyruvate kinase from liver crude extract. Highly purified enzyme showed at

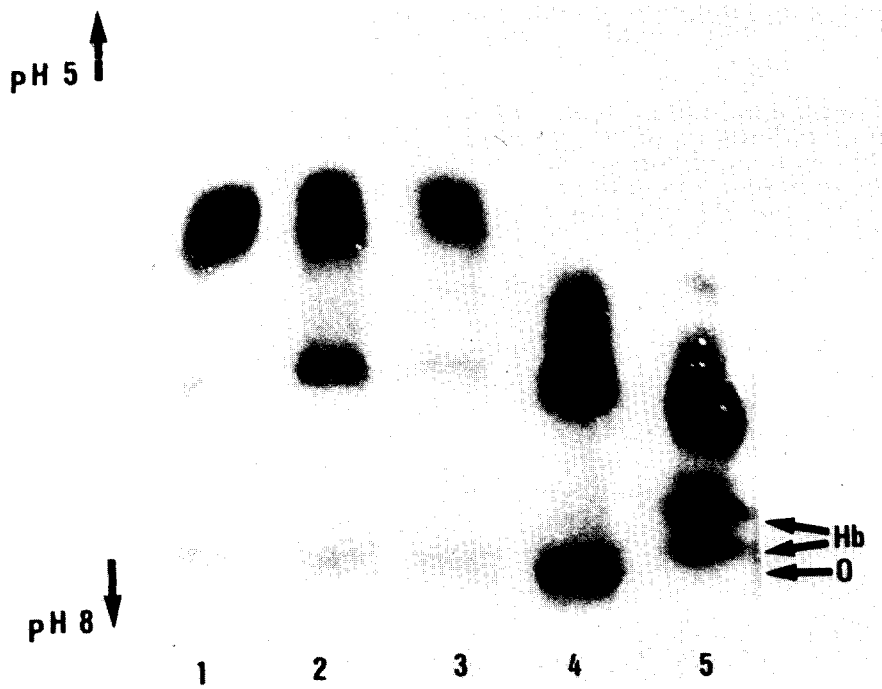

Fig. 5. Polyacrylamide slab gel electrofocusing of highly purified and crude extract L-type pyruvate kinase The extracts were focused on a polyacrylamide ampholine slab gel according to methods reported in Materials and Methods. Before focusing the preparations were incubated overnight at room temperature either with 2 mM fructose 1,6-diphosphate or 50 mM dithiothreitol in 50 mM Tris/chloride buffer, pH 7.3, containing 4 mM $MgCl_2$, 100 mM KCl, 1 mM EDTA, 1 mM ε-aminocaproïc acid and 500 mM sucrose. 1, Purified enzyme incubated with 2 mM fructose 1-6 diphosphate. 2, Purified enzyme incubated with only the buffer. 3, Purified enzyme incubated with 50 mM dithiothreitol. 4, Liver crude extract previously dialysed and incubated with the buffer. The addition of fructose 1,6-diphosphate to the liver crude extract does not change the electrofocusing pattern of L-type pyruvate kinase. With the ampholines chosen, the M type pyruvate kinase of liver focused at the cathodic extremity of the gel. 5, Hemolysate stored frozen at −80°C for 48 h. Hb: hemoglobin; 0 : origin.

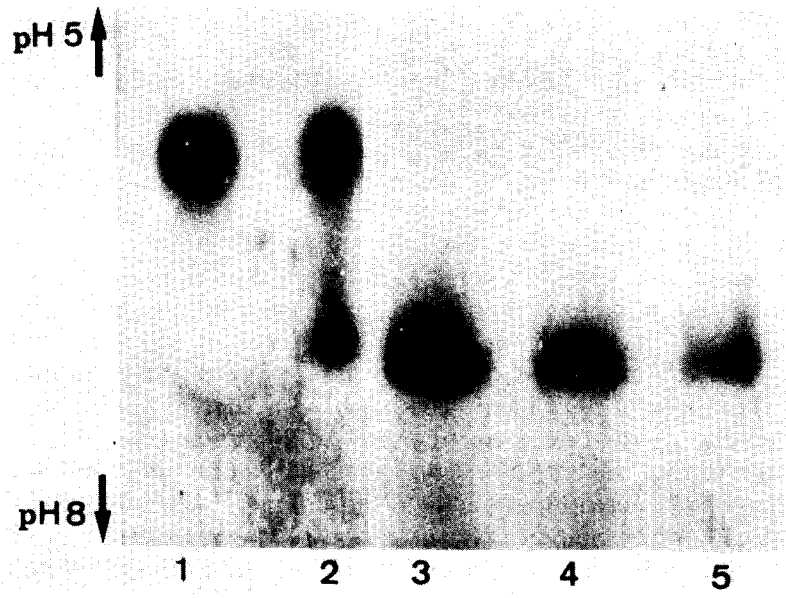

Fig. 6. Influence of fructose 1,6-diphosphate and of the ammonium sulphate fraction of liver extract P2 upon the electrofocusing pattern of highly purified L-type pyruvate kinase. Before the electrofocusing the L-type enzyme was incubated with: 1, 2 mM 1,6-diphosphate fructose. 2, buffer alone. 3, the fraction P2. 4, both 2 mM 1-6 fructose diphosphate and the fraction P2. In "5", liver crude extract.

least two major isozymic forms. One with cathodic migration was the major isozymic form in liver crude extract; the other with anodic migration was the major component after incubation with fructose 1,6-diphosphate and a high concentration of SH reagents. This component seems in fact to correspond to two bands poorly separated by the focusing method. The previous incubation in the presence of L(+)-Alanine, pyruvate or phospho*enol*pyruvate had no effect on the electrofocusing pattern, while ATP seemed to slightly shift the equilibrium towards the cathodic form. The previous incubation of highly purified L-type pyruvate kinase with the ammonium sulphate fractions of liver extract (P1 and P2) stabilized these enzymes into their cathodic form. The final supernatant obtained after precipitation of the fraction P2 had no effects. Fig. 6 shows that the fraction P2 was able to change the electrofocusing pattern of the L-type enzyme into its cathodic form, even in the presence of a high concentration of fructose 1,6-diphosphate (1 mM), and therefore suppressed this ligand's own influence.

The liver factor responsible for such an isozymic change seemed to be of proteic nature since it was totally destroyed by treatment with trypsin. Further characterization of these proteic factors able to stabilize the cathodic conformation of L-type pyruvate kinase are now in progress in this laboratory.

Discussion

The main problem posed by the purification of liver L-type pyruvate kinase of various mammals is the instability of the enzyme resulting in very poor

yields: 2.7% in beef [2], 1.6% [1] and 17% [4] in rat and, (starting with already fractionated preparation) 16% in pig [3]. Several methods have been used in order to avoid such an inactivation during purification: stabilization by various ligands [1,4], sucrose or glycerol [2,4,7].

In our opinion sucrose is an effective stabiliser for L-type pyruvate kinase during the dialysis steps and the various chromatographies. The inactivation is especially marked in the last steps of purification. Thus, molar sucrose solution was used in all the steps after that of selective elution with ATP.

The final preparation was homogeneous according to the criteria of purity chosen: immunodiffusion, acrylamide and sodium dodecyl sulphate acrylamide gel electrophoresis.

Acrylamide gel electrophoresis, however, showed a very slight additional band, faster than the major protein band. The hypothesis that this extra band represents a monomeric or dimeric form of pyruvate kinase (the major band being the tetrameric form of the enzyme) can be made since only one band was seen after dissociation by β-mercaptoethanol and sodium dodecyl sulphate.

Specific activity of purified human L-type pyruvate kinase (190 I.U./mg of proteins) was intermediate between the values found for bovine enzyme (63.4 I.U./mg) [2] and for rat enzyme (450 I.U./mg of proteins) [4]. It is difficult to compare the specific activities of human liver L-type and erythrocyte pyruvate kinase because several different values have been reported for erythrocyte pyruvate kinase, from 77 [22] to 150 I.U./mg [9]. Liver L-type enzyme, however, seems to have a higher specific activity than erythrocyte enzyme.

The specific elution of an enzyme from non-specific exchanger by a ligand has been previously demonstrated to be a powerful purification methods [23–27]. Carminattie et al. [28] used such a method for eluting rat liver pyruvate kinase from a CM-cellulose column by frucrose 1,6-diphosphate.

As previously reported [3] fructose 1,6-diphosphate alone is able to elute pyruvate kinase from CM-Sephadex, though with difficulty. However, in contrast to the results found by these authors [3], our previous experiments seemed to indicate that CM-Sephadex chromatography gave markedly higher yield than CM-cellulose chromatography. This is one of the reasons for the choice of CM-Sephadex and other ligands besides simply fructose 1,6-diphosphate. ATP eluted pyruvate kinase from a CM-Sephadex column in a sharp peak with or without fructose 1,6-diphosphate. However the yield of this step seemed to be slightly better (65%) with than without 0.1 mM fructose 1,6-diphosphate.

Of course ATP was able to elute other proteins than pyruvate kinase. It also eluted other kinases [27] and even hemoglobin which we have always found to be a contaminant of the ATP-eluates.

At step 5 both phospho*enol*pyruvate and fructose 1,6-diphosphate (probably bound to two different sites of the enzyme [29,30]) were required to promote a sharp elution peak. Each of these ligands separately resulted in an incomplete and tailing elution.

Molecular weight of the subunit (about 60 000) indicated that the 220 000–240 000-dalton L-type pyruvate kinase is a tetrameric enzyme as are L-type enzyme from other mammals [2] and human erythrocyte pyruvate kinase [15, 22].

Molecular weight of human erythrocyte pyruvate kinase subunit (60 000) [15] is similar to that herein reported for L-type enzyme. The enzymes however differ in their electrophoretic pattern after dissociation by sodium dodecyl sulphate and β-mercaptoethanol: L-type pyruvate kinase exhibited only one protein band, while erythrocyte pyruvate kinase was reported by Peterson et al. [15] to exhibit two distinct bands with similar mobilities.

Whether these two bands are the product of the same gene (one of the bands arising from the initial gene product through postranslational modifications), or the products of two different genes remains unresolved. The study of the immunological reactivity of entire and dissociated erythrocyte pyruvate kinase with both anti L-type and anti M2-type antisera constitutes a means to the clarification of this problem. Such a work is now in progress in our laboratory.

In the same way, the existence of several conformers of L-type pyruvate kinase and the mechanism of the equilibrium between these forms cannot yet be clearly explained. The results herein reported resemble those previously reported by several authors [8,10,31—33], as well as ourselves [13,18] concerning the multiple interconvertible forms of erythrocyte pyruvate kinase.

Hess et al. [34] also reported multiple interconvertible forms of pyruvate kinase from pig liver. Two main forms were found by this author: the acidic form (pI = 5.3), fructose 1,6-diphosphate-loaded, which is the main enzyme form in liver crude extract, and the alkaline form (pI = 6.1), fructose 1,6-diphosphate-free, which is mainly found in purified preparation. Our results were at variance with those of Hess et al. [34] since in our experiments the major acidic form (pI = 5.85) was predominant in the purified preparation as judged by acrylamide ampholine slab gel and was the only form found by focusing in sucrose gradient (Fig. 5). Fructose 1,6-diphosphate was eliminated before all these experiments by extensive dialysis for 36 h with multiple changes of the dialysis buffer; furthermore, high concentration of dithiothreitol as well as fructose 1,6-diphosphate was able to shift the equilibrium towards the acidic form in acrylamide ampholine gel electrofocusing. The predominance of the L-type cathodic isoenzyme in the liver crude extracts seems to be due to presence in these tissues of protein factors stabilizing the cathodic conformation of pyruvate kinase.

The mechanism of the interactions between these proteic factors and pyruvate kinase remains unclear. Before the incubations with pyruvate kinase, all the extracts were extensively dialysed against buffers devoid of phosphorylated compounds, so that a phosphorylation-dephosphorylation mechanism (as reported for pyruvate kinase from pig liver and rat liver [4—35]) is unlikely if, as hypothesized by Hess et al. [34], the acidic forms are enzyme forms tightly binding fructose 1,6-diphosphate (i.e. if the previous dialysis was unable to remove this tightly bound fructose 1,6-diphosphate), then the "proteic factors" could be enzymes destroying the fructose 1,6-diphosphate bound to pyruvate kinase (for instance, phosphatases). These various hypotheses are currently being tested in our laboratory.

In conclusion, L-type pyruvate kinase from human liver seems to be a molecule closely related to erythrocyte enzyme in the following ways: similar molecular weights of the entire molecule as well as of its subunits; similar immunological reactivities with anti erythrocyte pyruvate kinase serum [8] and

with anti L-type serum [5,18]; common absence of immunological cross reactivity with anti M2-type serum [5,8,18]; existence in both of interconvertible forms in function of the degree of purification and of the presence of various effectors (especially fructose 1,6-diphosphate); and finally, similar kinetic properties [9,10].

The immunological and structural studies of purified pyruvate kinases from erythrocyte and from liver should allow the elucidation of the nature of the differences between these two enzymes.

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