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THE GENETIC SYSTEM OF THE L-TYPE PYRUVATE KINASE FORMS IN MAN

SUBUNIT STRUCTURE, INTERRELATION AND KINETIC CHARACTERISTICS OF THE PYRUVATE KINASE ENZYMES FROM ERYTHROCYTES AND LIVER

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Summary

Pyruvate kinase (ATP : pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40) from human liver and red cells has been purified to homogeneity; its subunit structure and some of its kinetic characteristics have been studied. The influence of a partial proteolysis by trypsin on the subunit structure, the isozymic pattern and the kinetic characteristics of red cell and liver enzyme have been investigated.

From the results of this study we may conclude that:

1. Liver (L-type) pyruvate kinase is composed of 4 identical L subunits while the major form of erythrocyte enzyme (PK-R_2) is a heterotetramer designated as $L_2L'_2$, the molecular weight of L′ being slightly higher than that of L subunits (63 000 and 58 000 respectively). Pyruvate kinase PK-R_1, predominant in the erythroblasts and the young red cells, is composed of four identical L′ subunits.

2. A mild tryptic attack is able to transform PK-R_1 into PK-R_2, then PK-R_2 into pyruvate kinase L (PK-L). The same proteolytic treatment transforms the L′ subunits into L ones.

3. Consequently L-type pyruvate kinase seems to be initially synthesized in the erythroid precursors as an L'_4 enzyme secondarily partially proteolysed into $L_2L'_2$. In liver a very active proteolytic system would be responsible for the total transformation into L_4 pyruvate kinase.

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4. L'_4 enzyme exhibits Michaelis-Menten kinetic behaviour with an apparent Michaelis constant of 3.8 mM whereas L_4 enzyme shows both positive and negative homotropic interactions towards phospho*enol*pyruvate and has [S] 0.5 of 1.2 mM. The characteristics of $L_2L'_2$ are roughly intermediate between those of L'_4 and of L_4. Fructose 1,6-biphosphate decreases $[S]_{0.5}$ for these three pyruvate kinase forms without suppressing the differences in the apparent affinity for phospho*enol*pyruvate of these enzymes.

5. L_4 pyruvate kinase is more inhibited by Mg · ATP than L'_4, with $L_2L'_2$ in the intermediate range.

6. Tryptic treatment of each enzyme form studied transforms its kinetic behaviour into that observed for L_4.

We recently found [1] that erythrocytes contain pyruvate kinase (ATP : pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40), which is a tetramer, composed of 2 L and 2 L′ subunits, the latter having the higher molecular weight. Mild tryptic attack apparently transformed the native pyruvate kinase tetramer ($L_2L'_2$) into a L_4 form, which behaved in a similar manner to human liver L-type pyruvate kinase, judged by SDS polyacrylamide gel electrophoresis and electrofocusing.

We report here further studies on erythrocyte pyruvate kinase and its molecular subunits.

Materials and Methods

Materials

The substrates and auxiliary enzymes were from Boehringer Mannheim or Sigma CC. Trypsin came from Worthington Biochemical Corp. Ion-exchangers, CNBr-Sepharose 4 B and Dextran Blue were supplied by Pharmacia. Acrylamide, bisacrylamide and sodium dodecyl sulphate came from Eastman Kodak, Ampholines from LKB, agarose from l'Industrie biologique française. The apparatus and the plates for polyacrylamide gradient gel electrophoresis were from Universal Scientific Limited (London). Complement and goat anti-rabbit-immunoglobulin antibodies conjugated to horseradish peroxidase were from l'Institut Pasteur, Paris.

Methods

Assays. The enzyme activity was measured at 30°C in a Gilford spectrophotomer (model 2400) according to the method of Blume et al. [2]. The protein concentration was measured by Lowry method [3] with bovine serum albumin as standard.

Erythroblast and red cell fractionation. Circulating erythroblasts from patients with various types of blood diseases were fractionated [4] and leukocytes and platelets were removed by filtration through a cellulose column [5]. Large quantities of blood required the use of gelatin sedimentation for removal of these latter two cell types [6].

Enzyme purification. The method used for purifying pyruvate kinase from human liver and erythrocytes has been reported elsewhere [7—9].

The enzyme preparations obtained were homogeneous as judged by electrophoretic, chromatographic and immunologic criteria [7—9]. Their specific activity were 400 I.U./mg of protein for liver L-type enzyme, 300 and 150 I.U./mg of protein for the two erythrocyte forms L_4 and $L_2L'_2$, respectively.

As pyruvate kinase was to be purified from small amounts of blood (in order to fractionate the $L_2L'_2$ and L'_4 forms), the following procedure was used: all procedures were carried out at 4°C. After removal of the leukocytes, the red cells were hemolysed in 10 vols. distilled water/2 mM β-mercaptoethanol/10 mM EDTA/2 mM diisopropylfluorophosphate (or phenylmethylsulfonylfluoride)/10 mM ϵ-aminocaproic acid.

After elimination of stroma by centrifugation, 23% (w/v) solid $(NH_4)_2SO_4$ were added to the hemolysate. The precipitate was dissolved in 11% (w/v) $(NH_4)_2SO_4$ (at pH 8, with solid Tris). A final concentration of 22% $(NH_4)_2SO_4$ precipitated the protein after adjusting to pH 6.6 with 10% (v/v) acetic acid. The precipitate was washed in 21% $(NH_4)_2SO_4$ (at pH 6.6 with solid Tris). The yield ranged from 60 to 80% and specific activity was 2—4 I.U./mg protein.

The second step was the chromatography on Dextran Blue-Sepharose column [7]: the enzymes in 50 mM Tris · HCl (pH 7.5) were applied to the Dextran Blue column equilibrated with the same buffer. The column was washed with 40 mM Tris · HCl (pH 7.5)/80 mM KCl and the most part of the enzyme was eluted by adding 0.1 mM fructose 1,6-biphosphate to this buffer. The concentration of KCl in the buffer was then raised to 200 mM: a second protein peak was then eluted, containing some pyruvate kinase. This second fraction was predominant in erythroblast extracts. The purified enzyme preparations were precipitated by addition of 31 g per 100 ml $(NH_4)_2SO_4$ and stored frozen as a suspension in a 35 g per 100 ml $(NH_4)_2SO_4$ solution. At low protein concentrations, 0.1 mg/ml bovine albumin was added to the eluates and the proteins were coprecipitated with 56 g per 100 ml $(NH_4)_2SO_4$.

Immunologic characterization. The methods of electroimmunodiffusion [10,11], double immunodiffusion [12], immunoneutralization and microcomplement fixation [7,11,13] were used.

The antisera used were raised in rabbits against L-type pyruvate kinase from either erythrocytes or liver [7—9].

Electrophoresis and isoelectrofocusing. Isoelectrofocusing and electrophoresis in polyacrylamide slab gel were performed as described earlier [1]. Pyruvate kinase activity was usually revealed by the positive staining method used by Kahn et al. [10].

Electrophoresis in polyacrylamide gradient gel was performed in 40 mM Tris/glycine (pH 8.7), and using SDS according to the method of Weber and Osborn [14]. In both cases the migration continued for 18 h at 30 mA per plate (3 × 7 × 8 mm). The polyacrylamide gradient of the gels was 2.7—27% (w/v) acrylamide.

The subunit structure of pyruvate kinase R_1 [15,16] from an impure prepation was investigated by direct immunochemical detection of the SDS-dissociated L′ subunits in SDS polyacrylamide gel, according to the method of Olden et al. [17].

Tryptic digestions and in vitro incubations. Tryptic hydrolysis of the pure enzymes was performed at room temperature in 50 mM Tris · HCl (pH 7.3)/

100 mM KCl/4 mM $MgCl_2$/0.1 mM β-mercaptoethanol/500 mM sucrose; protein concentration was 0.2 mg/ml; the ratio trypsin : pyruvate kinase was 1 : 5 (w/w). The enzyme activity was sequentially checked, and proteolysis was stopped with 2 mM diisopropylfluorophosphate. The tryptic treatment of crude extracts or partially purified preparations was performed under the same conditions, with 1 or 100 μg/ml trypsin.

In some experiments crude hemolysates in water were incubated at 37°C overnight with 50 mM $CaCl_2$/1 mM β-mercaptoethanol with and without diisopropylfluorophosphate. The influence, on the electrophoretic changes induced by this incubation, of leukocytes and stroma was checked by using non destromatized hemolysates or blood whose leukocytes were not eliminated. After incubation, pyruvate kinase was purified by $(NH_4)_2SO_4$ fractionation.

Kinetic studies. All the kinetic studies were performed at 37°C. The reaction mixture contained, 100 mM Triethanolamine/HCl (pH 7.5) 100 mM KCl/12 mM $MgCl_2$/1 mM EDTA/0.1 mM NADH and 1 I.U./ml lactate dehydrogenase. Samples were preincubated in the cuvette, containing all ligands except ADP, for 5—10 min. When pure enzyme preparations were used, the concentration of pyruvate kinase in each reaction cuvette was about 10—15 ng/ml. The reaction was started by adding ADP. The reaction rate was obtained from the linear fast phase of the reaction progress curve [18]. $[S]_{0.5}$ for phospho*enol*pyruvate was calculated from a Hill plot using a concentration range from 0.01 to 15 mM, at a constant concentration of 1.5 mM Mg · ADP. Maximal velocity (V) was calculated from the Linewear-Burk plot.

The allosteric inhibition by Mg · ATP^{2-} was determined with 2 mM phospho*enol*pyruvate and approx. 10 mM excess of Mg^{2+} (Mg · ADP = 1.5 mM; Mg · ATP^{2-} from 0.5 to 10 mM).

The activation by fructose 1,6-bisphosphate was determined using 0.25 mM phospho*enol*pyruvate, 1.5 mM Mg · ADP and 0.05—500 μM fructose 1,6-bisphosphate. To avoid breakdown by aldolase (a contaminant of the lactate dehydrogenase preparations), fructose 1,6-bisphosphate was added immediately after the addition of ADP.

Apparent Michaelis constant for Mg · ADP was determined with 5 mM phospho*enol*pyruvate and 0.5 mM fructose 1,6-bisphosphate; Mg · ADP concentration was varied from 0.05 to 1.5 mM.

Results

Electrophoretic pattern of crude or partially purified extracts

The erythroblasts exhibit a single pyruvate kinase form on thin layer polyacrylamide gel electrophoresis (Fig. 1) identified as the R_1 form [15,16]. Both R_1 and R_2 forms are found in hemolysates, and L pyruvate kinase is the predominant form in liver, according to the results previously published [15,16]. Partial purification did not modify the electrophoretic pattern, provided that leukocytes and stroma were carefully eliminated from the hemolysates and that anti-proteolytic substances were added to all the buffers.

Influence of trypsin on the pyruvate kinase forms from liver and red cells

Trypsin treatment of liver, red cell or erythroblast extracts did not change

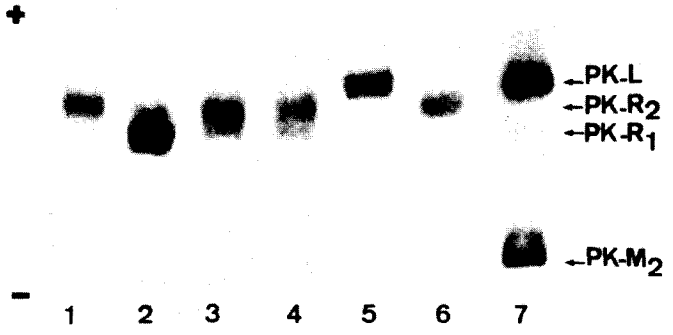

Fig. 1. Electrophoretic pattern in polyacrylamide slab gel of the various enzyme forms of pyruvate kinase. 1: Old red cells. 2: Circulating erythroblasts from a patient in phase of recovery of a bone marrow aplasia. 3: Normal red cells. 4: Normal red cells (from a different control). 5: Purified L-type liver pyruvate kinase. 6: Purified $L_2L'_2$ pyruvate kinase from red cells. 7: Liver homogenate. All the red cell preparations were obtained by $(NH_4)_2SO_4$ fractionation.

either the enzyme activity or the immunologic reactivity of pyruvate kinase. When erythroblasts or red cell extracts were incubated with 0.1 mg/ml trypsin (in the absence of phenylmethylsulphonylfluoride or diisopropylfluorophosphate), both forms R_1 and R_2 were readily transformed into an active band, electrophoretically indistinguishable from the pyruvate kinase L from liver.

When the same extracts were incubated with 1 μg/ml trypsin, a sequential transformation of $R_1 \rightarrow R_2 \rightarrow L$ was observed (Fig. 2). Trypsin had no apparent effect on the liver L-type enzyme.

Modifications of the electrophoretic pattern

The incubation of destromatized hemolysates from red cells (free of leukocytes) led to a small relative decrease in the intensity of the R_1 band, while this latter form disappeared when stroma were not removed from the hemolysate, the R_2 form being the only band found on electrophoresis. The antiproteolytic agents totally inhibited this disappearance of the R_1 band. The disappearance

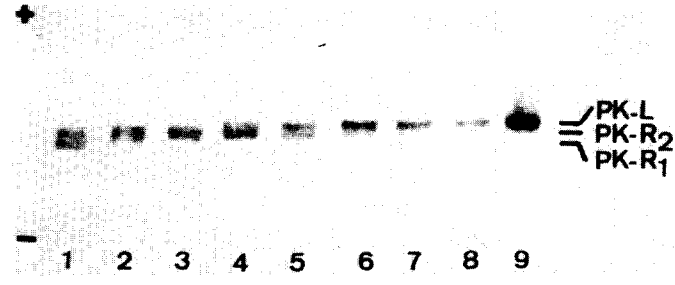

Fig. 2. Influence of a mild tryptic attack on the electrophoretic pattern of red cell pyruvate kinase. The concentration of trypsin was 1 μg/ml (i.e. 0.5 μg/mg of red cell proteins). 1: Preparation incubated for 1 h without trypsin and with antiproteolytic agents; 2: 5 min + trypsin; 3: 15 min + trypsin; 4: 30 min + trypsin; 5: 45 min + trypsin; 6: 1 h + trypsin; 7: 2 h + trypsin; 8: 4 h + trypsin; 9: purified liver pyruvate kinase L.

of the cathodic band R_1 form seemed to be due to transformation into an R_2 form rather than to degradation.

When the blood samples were not cleared of white cells before hemolysis, incubation resulted in the disappearance of the cathodic band R_1 and the appearance of an anodic band migrating as the L form from liver. This modification was not prevented by antiproteolytic agents, which may signify that some leukocyte proteolytic enzymes were not blocked either by serine reagents or ϵ-aminocaproic acid.

Purification of the different enzyme forms

Most techniques used for purifying liver and erythrocyte pyruvate kinase have been already reported in previous papers [1,7—9], and the enzymes obtained by these procedures have been characterized. When the different pyruvate kinase forms were to be fractionated from minute amounts of reticulocyte-rich blood, a single broad active peak was eluted by adding fructose 1,6-bisphosphate to the buffer containing 80 mM KCl; then the buffer containing 200 mM KCl and 0.1 mM fructose 1,6-bisphosphate was directly applied to the column, eluting a second pyruvate kinase peak and other proteins. This latter fraction, obviously, was not homogeneous; its pyruvate kinase specific activity was about 60 I.U./mg of proteins.

These procedures yielded the highest recovery with the L form from liver, followed by the R_2 form; the R_1 form was the most unstable.

Electrophoretic characterization of the purified enzyme forms

Upon polyacrylamide slab gel electrophoresis as well as upon isoelectrofocusing in various media pure pyruvate kinase L from liver was identical with that from crude of partially purified liver extracts (Fig. 1).

The first peak of activity eluted from Dextran Blue Sepharose 4B column from purification of "outdated-blood enzyme" [1] was identified as an L form. Gradient gel electrophoresis (Fig. 3) confirmed the identity of "erythrocyte pyruvate kinase I" [1] with pyruvate kinase L from liver.

The second peak of the erythrocyte enzyme migrated as the R_2 form in polyacrylamide slab gel (Fig. 4). The R_1 and R_2 forms were observed in a reticulocyte-rich blood sample. The R_2 form, from outdated blood or reticulocyte-rich blood, migrated in the same position. The R_1 form migrated as pyruvate kinase from erythroblast crude extracts.

Molecular weight and subunit structure

Gradient gel electrophoresis (Fig. 3) showed that molecular weight of the R_2 form was slightly higher than that of the L form from liver. Fig. 5 shows that both L-type from liver and "erythrocyte pyruvate kinase I" exhibited a single, and probably identical, protein band, designated as L subunit [1]. By contrast, erythrocyte R_2 form enzyme could be dissociated by SDS into two types of subunits, one migrating as the subunit L, the other migrating more slowly and called L′ subunit. The molecular weight of the L and L′ subunits was estimated (from electrophoresis of the dissociated enzyme in SDS polyacrylamide gradient gel [19], using RNA polymerase, bovine serum albumin and trypsin inhibitor as standards) at 57 000—58 000 for the L and 62 000—63 000 for the L′

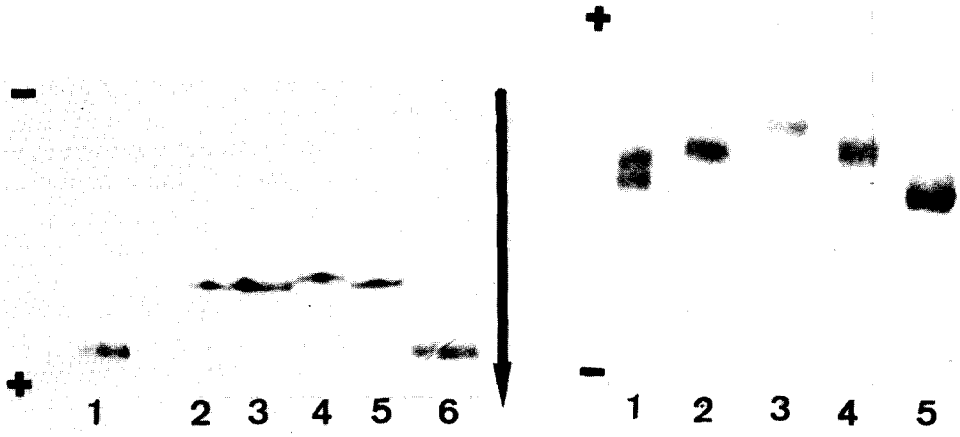

Fig. 3. Electrophoresis in polyacrylamide gradient gels (2.7—27% acrylamide, w/v) of pure pyruvate kinase preparation. Staining with Coomassie Blue. 1 and 6: marker aldolase; 2: liver L form; 3: erythrocyte pyruvate kinase from stored blood, first peak; 4: erythrocyte pyruvate kinase from stored blood, second peak (i.e. form $L_2L_2' = R_2$); 5: R_2 form treated by trypsin for 2 h.

Fig. 4. Control of the fractionation of both erythrocyte pyruvate kinases (PK-R_1 and PK-R_2) from a reticulocyte-rich blood sample by chromatography on Dextran Blue Sepharose 4 B column. 1: unfractionated extract; 2: pure L_2L_2' pyruvate kinase; 3: pure L_4 pyruvate kinase; 4: pyruvate kinase fraction eluted by 0.1 mM fructose 1,6-bisphosphate at 80 mM KCl; 5: pyruvate kinase fraction eluted by 200 mM KCl + 0.1 mM fructose 1,6-bisphosphate.

subunits. It was not possible to directly investigate the subunit structure of the R_1 enzyme by standard SDS polyacrylamide gel electrophoresis due to the small yield. Thus, immunochemical detection of the dissociated subunits in SDS polyacrylamide slab gel was used. This experiment confirmed that the L form enzyme exhibited only one subunit (L), while both L and L′ subunits were found with the R_2 form.

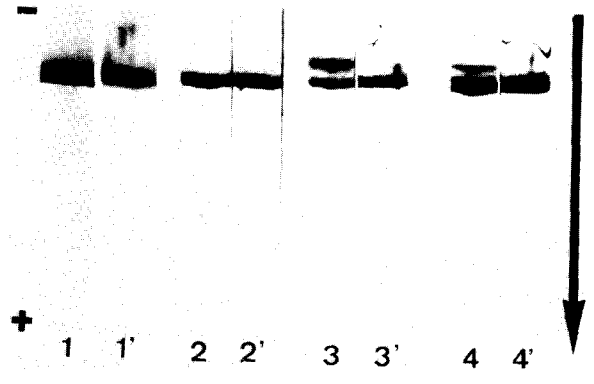

Fig. 5. SDS polyacrylamide gel electrophoresis of untreated (1, 2, 3, 4) and trypsin-treated (1′, 2′, 3′, 4′) pure pyruvate kinase preparations (treatment for 2 h by trypsin). 1 and 1′: liver pyruvate kinase L; 2 and 2′: erythrocyte pyruvate kinase from stored blood, first peak; 3 and 3′: erythrocyte pyruvate kinase from stored blood, second peak (i.e. R_2); 4 and 4′: mixture of liver L form and of erythrocyte R_2.

The R_1 form was composed of a single type of subunit migrating as the L′ subunits of the R_2 form. The R_1 preparation was slightly contaminated with PK-R_2 and a very faint band migrating as the L subunits could also be detected, its intensity was less than 10% of the L′ subunits.

From these results we might attribute the following formulae to the enzyme preparations: L_4 for liver L-type enzyme and erythrocyte enzyme I, $L_2L'_2$ for erythrocyte R_2 enzyme and L'_4 for erythrocyte R_1 enzyme.

Influence of trypsin treatment

Mild tryptic attack converted R_1 and R_2 forms of pyruvate kinase into a form with similar electrophoretic mobility to liver pyruvate kinase L (Fig. 6). The transformation of the pure R_2 form into L enzyme was associated with an increase of enzyme activity 1.6—2 fold. Trypsin-treated erythrocyte enzyme is probably also a tetrameric molecule (as is pyruvate kinase L) according to gradient gel electrophoresis (Fig. 3). Migration in SDS polyacrylamide gels of the dissociated trypsin-treated $L_2L'_2$ enzyme showed that this form was made from a single type of subunit, indistinguishable from the L subunit (Fig. 5). We thus conclude that the L′ subunits are converted into L subunits by partial proteolysis.

The properties of L_4 pyruvate kinase were not altered by trypsin treatment.

Immunologic characterization

The ratio of enzyme activity to pyruvate kinase-related antigen concentration measured by immunoneutralization was similar or slightly higher in erythroblast preparations than in whole hemolysates: it was also similar to that found for pure L_4 pyruvate kinase. Therefore, an inactive form was not detected in the erythroid precursors; i.e. the R_1 form is as active as the R_2 and L-type enzyme from liver.

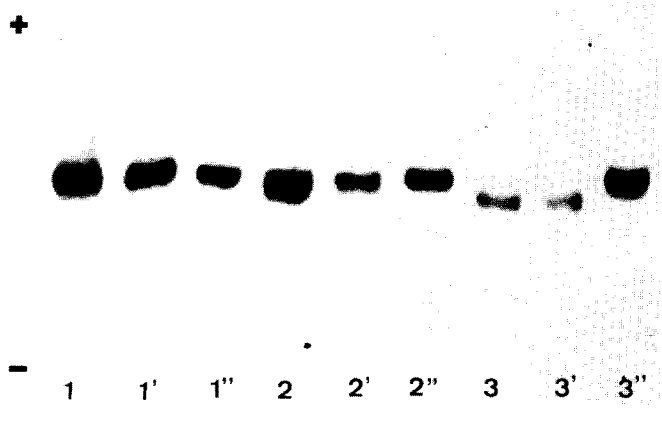

Fig. 6. Electrophoretic pattern in polyacrylamide slab gel of pure pyruvate kinase preparation from liver and red cells, and influence of the treatment with trypsin; stained for enzyme activity. 1, 2, 3: untreated enzymes; 1′, 2′, 3′: enzymes incubated without trypsin; 1″, 2″, 3″: enzymes incubated with trypsin; 1, 1′ and 1″: liver L form; 2, 2′, 2″: erythrocyte pyruvate kinase, peak 1; 3, 3′, 3″ erythrocyte R_2 form. The treated enzymes were incubated with trypsin for 2 h. The "untreated enzymes" refer to the absence of either incubation or tryptic treatment of the preparations.

Pure L_4 and $L_2L'_2$ pyruvate kinases were immunologically identical as judged by double immunodiffusion and micro-complement fixation. An electroimmunodiffusion experiment using pure L_4 enzyme (from liver or from stored blood) and $L_2L'_2$ form showed that all these enzymes contained an identical amount of L-type-related antigen per mg of protein (Fig. 7). Although all accurate antigenic quantification is to be interpreted with caution when hybrid molecules are studied, these findings seemed to indicate that the antigenicity of the L′ subunits with respect to anti-L-type antiserum was identical with that of the L subunits. This was confirmed after SDS polyacrylamide electrophoresis of the dissociated L and L′ subunits, which showed equal levels of reactivity against anti-L-type antiserum.

Kinetic behaviour towards phosphoenolpyruvate, and the influence of fructose 1,6-bisphosphate

In the absence of fructose 1,6-bisphosphate, the L'_4 form showed Michaelis-Menten kinetic behaviour as can be seen from the Lineweaver-Burk and Hill plots (Fig. 8A and B). The mean of three independent experiments yielded an apparent K_m = 3.8 mM.

The L_4 form from liver and erythrocytes, however, showed very complex kinetic patterns, with characteristics of both positive and negative homotropic interactions (Fig. 9A and B, Table II): Hill coefficients (h) were 1.6—1.8 at low phospho*enol*pyruvate concentration and 0.6 at intermediate phospho*enol*pyruvate concentration; $[S]_{0.5}$ for phospho*enol*pyruvate was about 1.6 mM.

The characteristics of $L_2L'_2$ were comparable to those of L_4, but with weaker homotropic interactions and a higher $[S]_{0.5}$ (Fig. 8A and B, Table I and II).

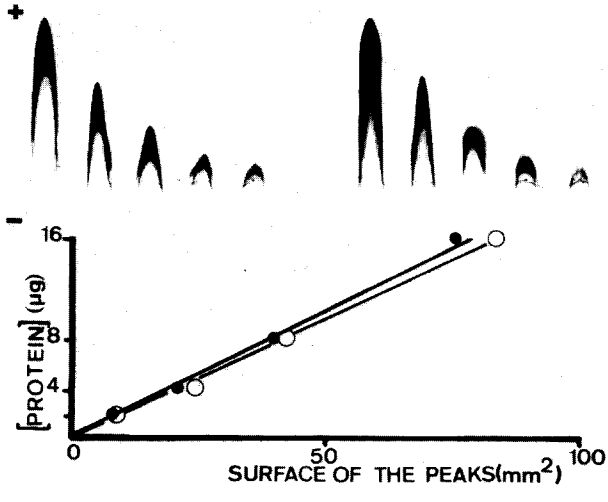

Fig. 7. Electroimmunodiffusion analysis of pure pyruvate kinase from liver (L) and erythrocytes (R_2). Staining with Amido Black. left: pyruvate kinase L; right: R_2 form (from stored blood). The dilutions applied to the gel contained about 16, 8, 4 and 2 µg of proteins. The gel contained 0.05 ml/ml of anti-L-type serum in 30 mM sodium barbital buffer (pH 8.6). The migration ran for 3 h at 2°C at 25—30 V/cm. Below, the straight line of protein concentration versus surface of the immunoprecipitate peaks.

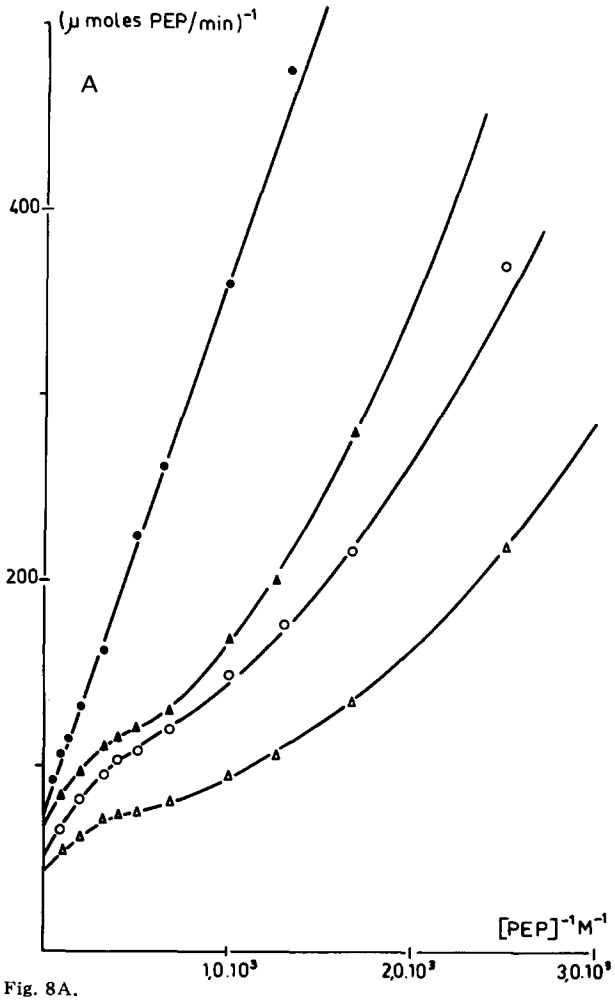

Fig. 8A.

In the presence of 0.5 mM fructose 1,6-bisphosphate no positive homotropic interaction was observed in any of the L-type enzymes studied, while the negative homotropic interactions persisted (Fig. 9A and B, Table II). $[S]_{0.5}$ for phospho*enol*pyruvate in the presence of fructose 1,6-bisphosphate was about 0.12—0.16 mM for L_4, 0.32 mM for $L_2L'_2$ and 1.3 mM for L'_4 (Table I).

Trypsin treatment of either L'_4 or $L_2L'_2$ converted the kinetic behaviour of these enzymes into a type very similar to that of L_4 pyruvate kinase (Tables I and II, and unpublished data).

Allosteric inhibition by Mg · ATP^{2-}

The allosteric inhibition by Mg · ATP^{2-} was stronger for the L_4 form than for L'_4, with L'_2L_2 at an intermediate value: 50% inhibition was obtained at 7.0 mM Mg · ATP^{2-} for L'_4, at 3.4 mM for L'_2L_2 and at 2.5 mM for L_4. After treatment with trypsin, a 50% inhibition was obtained at 2.5—3.5 mM Mg · ATP^{2-} for all three pyruvate kinase forms.

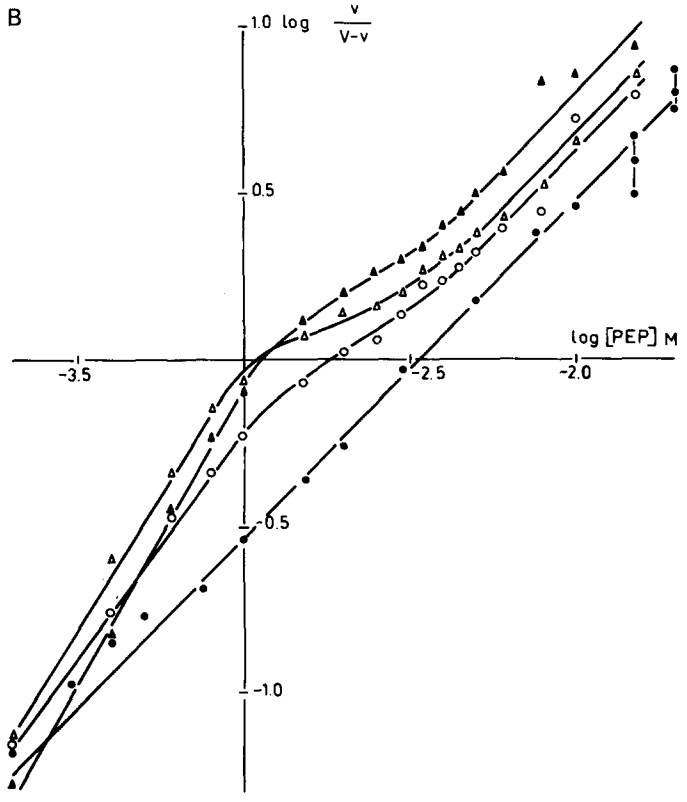

Fig. 8. Lineweaver-Burk (A) and Hill (B) plots of the different forms of L-type pyruvate kinase from human erythrocytes and liver. ●——●: L_4' from erythrocytes; ○——○: $L_2'L_2$ from erythrocytes; ▲——▲: L_4 from erythrocytes; △——△: L_4 from liver. Many points (shown in the Hill plot) are omitted from the Lineweaver-Burk plot for clarity.

TABLE I

$[S]_{0.5}$ FOR PHOSPHO*ENOL*PYRUVATE

The $[S]_{0.5}$ for phospho*enol*pyruvate of the various enzyme forms studied, before and after tryptic treatment. Values are given in the presence and in the absence of 0.5 mM fructose 1,6-bisphosphate (Fru-1,6-P_2). L_4 pyruvate kinase "from erythrocytes" refers to enzyme isolated from old outdated blood and eluted from the Dextran Blue-Sepharose column in the first peak; it resulted from the in vitro partial hydrolysis of L_2L_2' and L_4' during the purification procedure. All values were obtained from three independent experiments of which the range is given.

		Erythrocytes		Liver	
		Before tryptic treatment	After tryptic treatment	Before tryptic treatment	After tryptic treatment
L_4'	— Fru-1,6-P_2	3.5 —4.1 mM	1.0 —1.4 mM		
	+ Fru-1,6-P_2	1.1 —1.5 mM	0.08—0.15 mM		
L_2L_2'	— Fru-1,6-P_2	2.0 —2.9 mM	0.98—1.2 mM		
	+ Fru-1,6-P_2	0.24—0.40 mM	0.10—0.12 mM		
L_4	— Fru-1,6-P_2	1.1 —1.3 mM	1.0 —1.2 mM	1.1 —1.3 mM	0.77—0.12 mM
	+ Fru-1,6-P_2	0.09—0.15 mM	0.08—0.10 mM	0.15—0.17 mM	0.10—0.12 mM

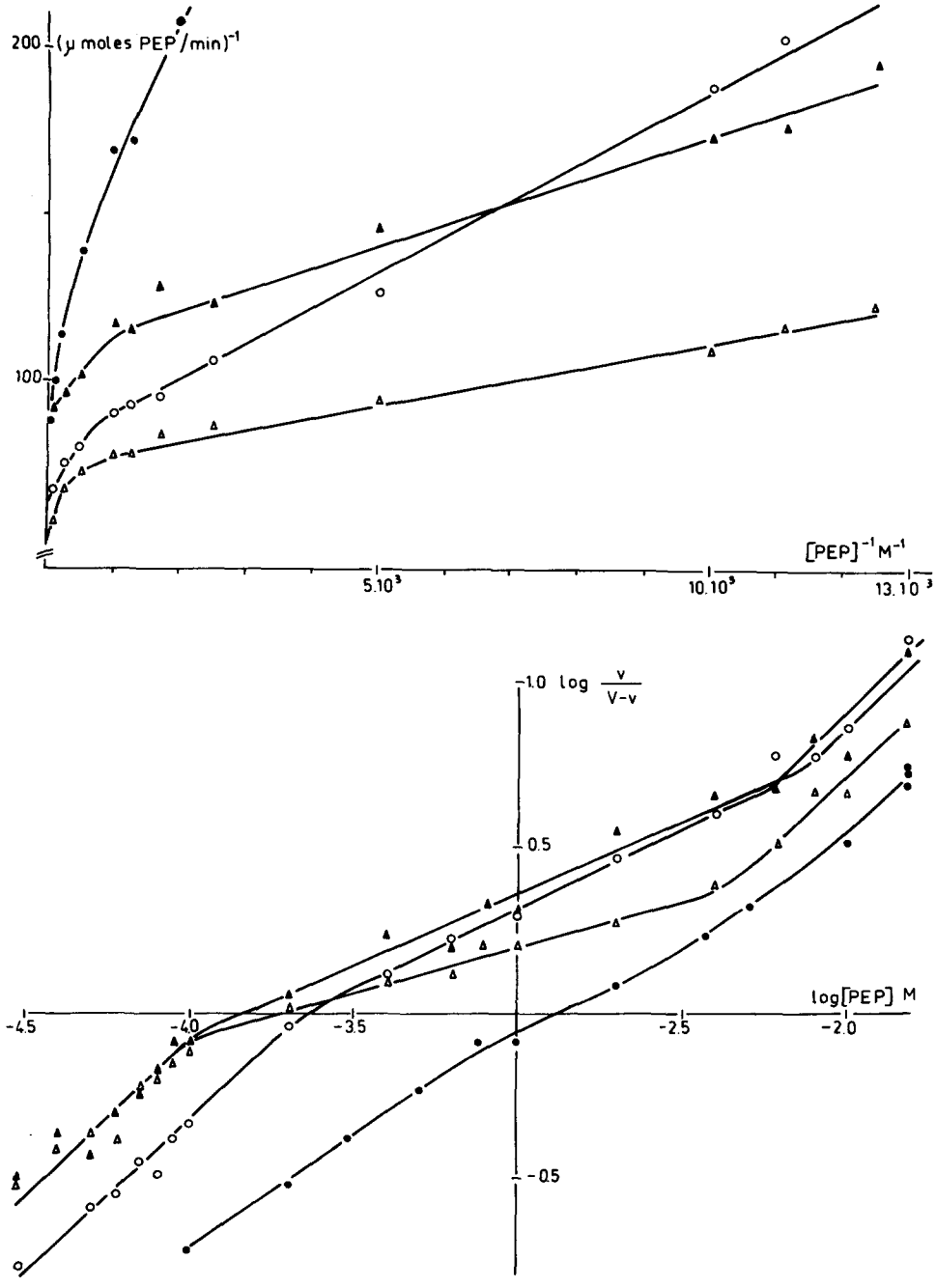

Fig. 9. Lineweaver-Burk (A) and Hill (B) plots of the same samples as in Fig. 1, in the presence of 0.5 mM fructose 1,6-bisphosphate. Legend as in Fig. 8.

Activation by fructose 1,6-bisphosphate

At the low phospho*enol*pyruvate concentrations used (0.25 mM), all pyruvate kinase forms were strongly activated by fructose 1,6-bisphosphate. No differences were found in the half-maximal activation of the different forms and

TABLE II

HILL COEFFICIENTS (h)

The Hill coefficients (h) of the various enzyme forms studied, before and after tryptic treatment. Values of experiments performed in the presence and absence of 0.5 mM fructose 1,6-bisphosphate are given. Because Hill-plots are "non linear" different Hill coefficients are calculated at low and intermediate phosphoenolpyruvate (PEP) concentration. The low phosphoenolpyruvate concentrations are those concentrations yielding $V_i < 0.5\ V$. The intermediate concentrations are those yielding V_i ranging from 0.5 to 0.7 V. The values given in this table represent the mean of three independent determinations.

		Erythrocytes				Liver			
		Before tryptic treatment		After tryptic treatment		Before tryptic treatment		After tryptic treatment	
		low PEP	intermediate PEP	low PEP	intermediate PEP	low PEP	intermediate PEP	low PEP	intermediate PEP
L_4'	— Fru-1,6-P_2	1.0	1.0	N.D.*	N.D.*				
	+ Fru-1,6-P_2	0.9	0.7	N.D.*	N.D.*				
L_2L_2'	— Fru-1,6-P_2	1.4	0.8	1.9	0.6				
	+ Fru-1,6-P_2	1.0	0.5	1.0	0.4				
L_4	— Fru-1,6-P_2	1.8	0.6	2.1	0.6	1.6	0.6	1.9	0.5
	+ Fru-1,6-P_2	1.0	0.5	1.0	0.4	1.0	0.6	1.0	0.4

* N.D.: not determined, small number of experiments.

trypsin treatment did not induce any change in affinity towards this effector. Half-maximal activation was obtained in the range 0.1—0.3 μM fructose 1,6-bisphosphate.

Apparent Michaelis constant for Mg · ADP

Apparent K_m for Mg · ADP was identical for all three pyruvate kinase forms, in the range 0.16—0.21 mM.

Discussion

From the results reported above, it appears that the precursor L-type pyruvate kinase in the erythroblasts is L_4', a homotetramer composed of four identical subunits with a mol. wt. of about 63 000. Red cell aging is associated with a partial proteolysis of 2 L' subunits into L ones, resulting in the appearance of the heterotetramer L_2L_2'; mol. wt. of the L subunit is about 58 000. In the liver, the homotetramer L_4 is the only L-type pyruvate kinase to be detected.

A very mild proteolytic attack by trypsin is able to transform L_4' into L_2L_2', the transformation of L_2L_2' into L_4 requiring a more protracted or a more drastic proteolytic treatment. In vivo, the absence of L_4 in the old red cells could be due to the absence in these cells of any proteolytic system active enough to convert L_2L_2' into L_4, this conversion requiring a proteolytic system more active than that required for the conversion $L_4' \rightarrow L_2L_2'$. By contrast such active proteolytic substances may be liberated from stroma and leukocytes, and are most likely responsible for the appearance of L_4 when pyruvate kinase is purified from a large amount of stored blood, the leukocyte contamination of which cannot be totally eliminated. As for the liver, the mechanism of the

TABLE III

REPARTITION OF THE VARIOUS L-TYPE PYRUVATE KINASE FORMS IN ERYTHROID AND HEPATIC CELLS

L_4 enzyme found in the pure erythrocyte pyruvate kinase preparation results from the in vitro transformation of L'_4 and $L_2L'_2$ during the purification procedure.

Cell extracts	Pyruvate kinase forms		Purified preparation
	Isozyme designation	Subunit structure	
Erythroblasts	PK-R_1	L'_4	
Erythrocytes	PK-R_2	$L_2L'_2$	Erythrocytes
Liver	PK-L	L_4	Liver

appearance in vivo of L_4 pyruvate kinase can only be hypothesized: taking into account the richness of this tissue in proteolytic enzymes, we assume that the L′ subunits synthesized in the hepatocytes could be readily hydrolysed into L subunits, by a liver-specific proteolytic system or by wide-spread proteolytic enzymes.

Table III summarizes these data and recalls the correspondence between the classical forms PK R_1, PK R_2 and PK L, and their tetrameric composition.

We have shown that all the L_4 enzymes obtained from liver, red cells, or in vitro treatment of L'_4 and $L_2L'_2$ by trypsin had similar mol. wt., electrophoretic migration in various media, isoelectric point, specific activity, immunological properties and kinetics. The exact cleavage point of the L′ subunits, however, is not necessarily identical in these different conditions and these different L_4 forms could differ by a small number of residues which do not significantly modify the properties of the L_4 tetramers.

In our recent preliminary studies [1,20] we hypothesized that the L′ subunits could be inactive: indeed, specific activity (and fructose 1,6-bisphosphate-binding sites) of $L_2L'_2$ was half that of L_4 and increased as L′ was transformed into L subunit by tryptic treatment. As a matter of fact the data reported in this paper (namely, the identical ratio of enzyme activity to antigen concentration in erythroblasts and liver, and the absence of any change in enzyme activity when L'_4 from crude extracts is transformed into L_4 by tryptic treatment) enable us to rule out this hypothesis. The instability of L'_4 and $L_2L'_2$ during the purification procedure allows to put forward the hypothesis summarized in Fig. 10: during the purification both L′ subunits of $L_2L'_2$ could be converted into inactive L′ forms, which could be reactivated as they are transformed into L subunits by tryptic digestion. By contrast, L'_4 could undergo an irreversible degradation.

If the transformation of L′ into L subunits is not associated in vitro with enzyme activation, it results, nevertheless, in dramatic kinetic changes, the importance of which could be crucial in the regulation of glycolysis in red cells and liver.

Positive homotropic interactions for the substrate phospho*enol*pyruvate at low phospho*enol*pyruvate concentration do not exist for L'_4, appear for $L_2L'_2$ and are reinforced for L_4 pyruvate kinase. In the same way, negative homo-

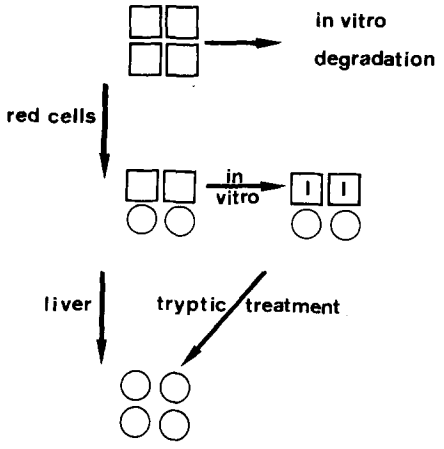

Fig. 10. Hypothetical representation of the postsynthetic evolution of L'_4 enzyme, in vivo and in vitro. □ = subunit L'; [i] = inactive subunit L'; ○ = subunit L.

tropic interactions for the phospho*enol*pyruvate at intermediate phospho*enol*-pyruvate concentrations appear for $L_2L'_2$ and are reinforced for L_4 enzyme. All three pyruvate kinase forms seem to be identically activated by fructose 1,6-bisphosphate; in the presence of this allosteric activator, however, the differences between kinetic behaviour of these enzymes for phospho*enol*pyruvate persist. Finally, the ATP inhibition increases with the transition $L'_4 \rightarrow L_2L'_2 \rightarrow L_4$.

The complex kinetic properties of pyruvate kinase, associating positive and negative homotropic interactions, have been already described by other authors [21—25]. These findings are currently under further study in our laboratory and will be detailed in another paper (Sprengers, E.D. et al., in preparation).

Although it is tentative to draw conclusions about the in vivo function of pyruvate kinase from our incomplete in vitro kinetic studies, we would like to point out several possibilities:

(1) the improvement of the regulatory properties of L_4 with respect to L'_4 would be indispensable to 'turn off' liver pyruvate kinase during gluconeogenesis and to prevent futile cycling of phospho*enol*pyruvate

(2) Instability and low phospho*enol*pyruvate affinity of L'_4 constitute defavourable kinetic characteristics; in the patients with hereditary deficiency in pyruvate kinase activity reticulocytosis is high and consequently, the mutated L'_4 form could be the predominant form, and could be responsible for the special sensitivity to hemolysis of the pyruvate kinase-deficient reticulocytes [26].

In conclusion, we describe in this work a phenomenon of postsynthetic 'maturation of enzyme' which, to our knowledge, is unique up to now: an enzyme composed of a single gene product is sequentially transformed into three enzyme forms, found in different cells and having different properties. Such a phenomenon may not be restricted to pyruvate kinase, and may play a major role in the regulation of several enzymatic systems.

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