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HUMAN ERYTHROCYTE PYRUVATE KINASE

TOTAL PURIFICATION AND EVIDENCE FOR ITS ANTIGENIC IDENTITY WITH L-TYPE ENZYME

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Summary

Erythrocyte pyruvate kinase (ATP:pyruvate 2-0-phosphotransferase, EC 2.7.1.40) has been purified 40 000 times from human erythrocytes, according to an original method.

The whole purification procedure included toluene extraction, ammonium sulphate fractionation, DEAE-Sephadex batchwise chromatography and affinity chromatography on a Dextran Blue-Sepharose column with specific elution by fructose 1,6-diphosphate.

The final preparation had specific activity of 290 I.U./mg of proteins and the overall yield was about 30%. Pyruvate kinase showed only one protein band as judged by sodium dodecyl sulphate acrylamide gel electrophoresis. Pure enzyme was injected into rabbits and monospecific antiserum was obtained able to neutralize, per ml, 150 I.U. of erythocyte-type pyruvate kinase as well as of L-type enzyme.

L-type and erythrocyte-type pyruvate kinases showed reactions of complete identity when tested in immunodiffusion against anti-erythrocyte type pyruvate kinase sera; in all cases a single precipitation line could be detected. L-type pyruvate kinase when mixed with anti-erythocyte pyruvate kinase serum suppressed all ability of that antiserum to react immunological with erythocyte enzyme.

Finally the microcomplement fixation curves using anti-erythrocyte pyruvate kinase serum were identical for erythrocyte and L-type enzymes.

From these results it appeared that no antigenic difference between L-type and erythocyte enzyme could be detected. Consequently the most likely hypothesis is that both these enzymes are coded by the same single gene, the slight

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electrophoretic differences between them being due to post-synthetic tissue-specific changes.

Total purification of human erythrocyte pyruvate kinase (ATP:pyruvate 2-0-phosphotransferase, EC 2.7.1.40) is required to advance in the understanding of at least three problems of current interest: the genetic determinism of this enzyme (and its relationship with L-type pyruvate kinase), the nature of its interconvertible forms and the nature of the structural modifications responsible for the congenital defects in pyruvate kinase activity. In addition, an indispensable condition for such a structural study of the mutant pyruvate kinase molecules will be the availability of a rapid and easy purification procedure for obtaining pure enzyme in high yield.

Up to now, the most elaborate purification procedures published by various authors resulted in preparations whose specific activities were 77.4 I.U./mg [1], 120 I.U./mg [2] and 150 I.U./mg [3]. Criteria of purity of the final pyruvate kinase preparations were usually poorly examined, except by Chern et al.: these authors reported that purified preparation showed single protein band as judged acrylamide gel electrophoresis at pH 8.7. After dissociation by sodium dodecyl sulphate and β-mercaptoethanol, however, sodium dodecyl sulphate acrylamide gel electrophoresis showed two discrete protein bands [1].

Using the same type of enzyme preparation and of antiserum, Lincoln et al. reported that, upon immunodiffusion against anti-erythocyte pyruvate kinase serum, the purified erythrocyte pyruvate kinase preparation exhibited two precipitation lines, one of them having a partial identity relation with the line of L-type enzyme [4].

The yields of the purification procedures described by the authors cited above were rather low, around 8%.

We describe herein a new purification method allowing us to obtain pure pyruvate kinase of high specific activity (about 300 I.U./mg of proteins at 30°C) with yield as high as 30%. Pure enzyme showed a single type of subunit detected by sodium dodecyl sulphate acrylamide gel electrophoresis *. Finally erythrocyte pyruvate kinase appeared to be antigenically identical to L-type enzyme.

Materials and Methods

Materials

DEAE Sephadex A50, cyanogen bromide-activated Sepharose 4B and Sephadex G25 were supplied by Pharmacia.

The substrates for the enzymatic reactions and the intermediate enzymes were furnished by Boehringer Mannheim. Acrylamide, bisacrylamide and sodium dodecyl sulphate were purchased from Eastmann Kodak. Coomassie Blue R250 came from Sigma C.C. ; Dextran Blue was obtained from Pharmacia.

Enzymatic activities were measured in a Zeiss PMQ II spectrophotometer

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* See Note added in proof, p. 104.

connected to a Servogor recorder. The column eluates were monitored at 280 nm with a Beckman DBG spectrophotometer coupled to a recorder. The conductivity of the buffers was measured with a Biolyon conductivity meter.

Methods

Assays. The enzymatic activity was measured at 30° C according to the method of Blume et al. [5] with 1.5 mM ADP and 5 mM phospho*enol*pyruvate. The protein concentration was measured according to the method of Lowry et al. [6] against a standard of crystallized serum albumin.

Preparation of the Dextran Blue-Sepharose column. The activated Sepharose 4B was covalently coupled with Dextran Blue according to the method described by Ryan and Vestling [7].

Electrophoreses. Polyacrylamide gel electrophoresis was performed in 40 mM Tris/glycine buffer, pH 8.7, containing 7.5% (W/V) acrylamide. The migration continued for 1.5 h at 2.5 mA per gel.

Acrylamide sodium dodecyl sulphate electrophoresis was performed according to the method of Weber and Osborn [8]. The proteins were stained with Coomassie Blue [9].

Molecular weight of the subunits was estimated by sodium dodecyl sulphate gel electrophoresis by using as standards: dissociated bovine serum albumin, albumin from hen egg, aldolase from rabbit muscle and catalase from beef liver [8].

Immunisation of rabbits. Rabbits were immunized against pure erythocyte pyruvate kinase according to the following methods: 250 μg of enzyme in 500 μl of 10 mM sodium phosphate buffer pH 7 containing 500 mM sucrose, 150 mM NaCl, 1 mM EDTA and 1 mM β-mercaptoethanol were emulsified with 500 μl of Freund's complete adjuvant, then injected three times every 10 days in multiple intradermal injections on the back (0.1 ml per location). The same amount of enzyme, similarly emulsified with Freund's complete adjuvant, was intramuscularly injected on the 45th day, then enzyme without Freund's adjuvant was intravenously injected on the 52nd and 58th days. Animals were killed on the 63rd day, their serum was heated for 30 min at 60° C, mixed with 0.02% (W/V) sodium azide, then stored frozen at −80° C in 1-ml aliquots.

Double immunodiffusion tests. Erythrocyte and L-type pyruvate kinase from purified preparations [10] as well as from crude extracts were tested against both anti-erythrocyte and anti L-type pyruvate kinase sera [10]. In one experiment 10 μl of anti-erythrocyte pyruvate kinase serum were mixed with 10 μg of L-type enzyme before being tested against erythrocyte pyruvate kinase.

Microcomplement fixation. Microcomplement fixation tests compared erythrocyte and L-type pyruvate kinase with respect to anti-erythrocyte pyruvate kinase serum. The method of Levine and Vunakis [11] was slightly modified in that the reaction mixture had a final volume of 1.4 ml instead of 7 ml as described by these authors.

Anti-erythrocyte pyruvate kinase was diluted 26 400 times (final dilution). Dilution of lyophilized guinea pig serum (C′) varied from 1 : 1080 to 1 : 1320. The amounts of pure antigens used (erythrocyte and L-type enzymes) ranged from

1 to 30 ng. As partially purified extracts were to be used, the total pyruvate kinase activity mixed with the reaction mixture varied from $3 \cdot 10^{-4}$ I.U. to $10 \cdot 10^{-3}$ I.U.

Results

Purification procedure

All the steps of the purification were carried out at +4°C. Unless otherwise indicated the buffers contained 1 mM EDTA, 1 mM ϵ-aminocaproic acid and 2 mM β-mercaptoethanol.

Step 1: toluene extraction. Red cells were lysed in one volume of water containing 1 mM EDTA, 1mM ϵ-aminocaproic acid and 2 mM β-mercaptoethanol. Cold toluene was added up to a concentration of about 10% (W/V) and vigorously emulsified with the hemolysate. After centrifugation for 40 min at 10 000 × *g* the upper fat layer was sucked of and the hemolysate was decanted.

Step 2: Ammonium sulphate fractionation. The hemolysate was precipitated by adding 23 g of solid ammonium sulphate per 100 ml. The precipitate was collected by centrifugation was dissolved in 50 mM Tris/chloride buffer pH 8 fer pH 7 containing 23 g of solid ammonium sulphate per 100 ml. After centrifugation, the precipitate was washed again with 2 l of 10 mM phosphate buffer pH 6 containing 21 g of solid ammonium sulphate per 100 ml. The precipitate collected by centrifugation was dissolved in 50 mM Tris/chloride buffer pH 8 containing 11 g of ammonium sulphate per 100 ml.

The supernatant was collected and precipitated by adding 25 g of solid ammonium sulphate per 100 ml. The precipitate was collected again by centrifugation and applied to a G-25 column (50 × 4 cm) equilibrated with 10 mM buffer pH 7.2 containing 0.1 mM fructose 1,6-diphosphate.

Step 3: DEAE-Sephadex, batchwise. DEAE-Sephadex A-50 previously equilibrated with the buffer described above was added to the erythrocyte pyruvate kinase preparation and gently stirred for 30 min at 4°C.

The ion exchanger was gathered in a buchner funnel and washed at room temperature with 10 mM phosphate buffer, pH 7, containing 0.01 mM fructose 1,6-diphosphate. The elution was promoted by 10 mM phosphate buffer, pH 7, containing 500 mM sucrose and 40 mM $MgSO_4$.

The eluate was precipitated by adding solid ammonium sulphate (36 g per 100 ml).

The precipitate was collected by centrifugation and was deposited at the top of a G-25 column (50 × 2 cm) equilibrated with 50 mM Tris/chloride buffer, pH 7.5, containing 40 mM NaCl (conductivity: 5700 $\mu\Omega^{-1}$).

Step 4: Affinity chromatography on Dextran Blue-Sepharose 4B column and specific elution by fructose 1,6-diphosphate. All of this step was performed at 15°C. The preparation was applied to the affinity chromatography column (2 × 20 cm) equilibrated with the 50 mM Tris/chloride buffer pH 7.5 described above. The column was washed with 100 ml of this buffer, then with 40 mM Tris/chloride buffer pH 7.5 containing 80 mM NaCl (conductivity: 7000 $\mu\Omega^{-1}$ until all absorbance at 280 nm disappeared. The elution was effected by adding to the washing buffer 0.1 mM fructose 1,6-diphosphate. The active fractions were pooled and precipitated by solid ammonium sulphate (31 g per 100 ml). The

TABLE I

STEPS OF THE PURIFICATION PROCEDURE

	Activity (I.U.)	Proteins (mg)	Specific activity (I.U./mg)	Accumulation purification (-fold)	Yield (%)
Hemolysate	6900	932 000	0.0074	1	100
Ammonium sulphate precipitation and G-25 Sephadex chromatography	4899	1 620	3	405	71
Batch of DEAE-Sephadex and G-25 Sephadex chromatography	3200	198	16.2	2 189	46
Dextran Blue-Sepharose 4B column with elective elution by fructose 1,6-diphosphate	1984	6.8	290	39 000	29

precipitate was collected after centrifugation and the highly purified erythrocyte pyruvate kinase preparation was stored frozen (−80° C) in a 50 mM Tris/chloride buffer, pH 8, containing 10 mM dithiothreitol, 500 mM sucrose and solid ammonium sulphate (36 g per 100 ml). This purification procedure allowed us to obtain a preparation with a specific activity of 290 I.U. per mg of protein, i.e. a 39 000-fold purification with an overall yield of 29%.

Table I summarizes the different steps of the purification procedure.

Criteria of purity. The double immunodiffusion test showed a single precipitation line between anti-erythrocyte pyruvate kinase sera and the highly purified preparation (Fig. 1). Acrylamide electrophoresis in Tris/glycine buffer, pH 8.7, and sodium dodecyl sulphate acrylamide gel electrophoresis showed a

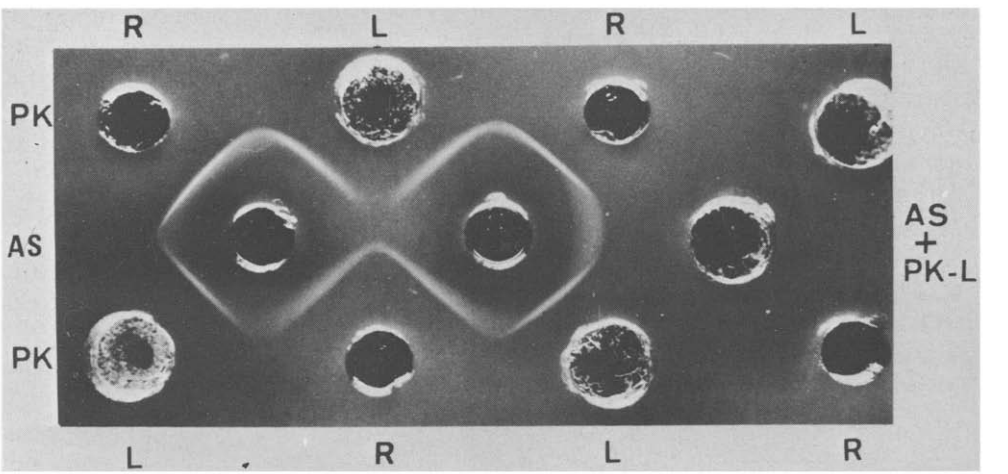
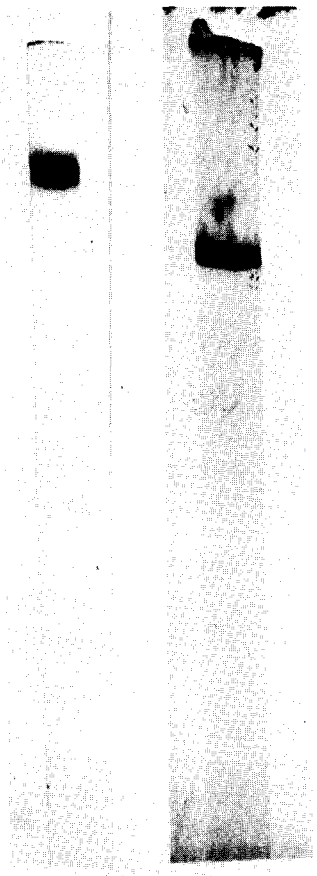


Fig. 1. Double immunodiffusion analysis of L-type and erythrocyte pyruvate kinases. R = erythrocyte enzyme; L = L-type enzyme; AS = anti-erythrocyte pyruvate kinase serum. Both the centre wells located at the left side of the gel contained 25 µl of 1 : 2 antiserum. The centre well located at the right side of the gel contained a mixture of 10 µl of antiserum with 10 µg of pure L-type pyruvate kinase. Outer wells contained either from 5 to 20 µg of pure erythrocyte enzyme, or from 1.5 to 3.5 I.U. of partially purified L-type enzyme.



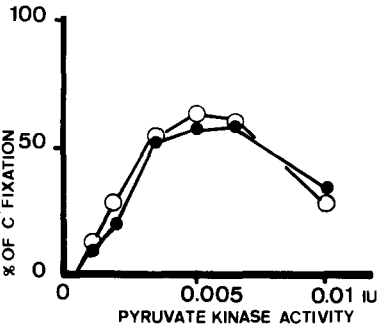


Fig. 2. Polyacrylamide gel electrophoresis and sodium dodecyl sulphate polyacrylamide gel electrophoresis of pure erythrocyte pyruvate kinase. A : electrophoresis in Tris/glycine buffer pH 8.7. B : sodium dodecyl sulphate polyacrylamide gel electrophoresis. 10 μg of protein were deposited on the gels.

Fig. 3. Microcomplement fixation analysis of erythrocyte and L-type pyruvate kinases with anti-erythrocyte pyruvate kinase serum. Ammonium sulphate fractionated preparations from hemolysate and liver extract were used as source of antigen. Lyophylised guinea pig serum was 1080 times diluted and antiserum was diluted. 26 400 times.

single protein band, even when the amount of protein deposited on the gel was as high as 50 μg (Fig. 2).

Electrophoretic mobility of pyruvate kinase subunits in sodium dodecyl sulphate acrylamide corresponds to a molecular weight of about 59 000—60 000.

Immunological studies

Double immunodiffusion analysis. Fig. 1 shows that pure erythrocyte enzyme as well as partially purified extracts gave a single precipitation line as tested against specific antiserum. L-type enzyme from purified preparation or liver crude extracts showed a similar pattern.

These precipitation lines, corresponding to L-type and erythrocyte enzymes, showed a reaction of complete identity (i.e. no 'spur' could be detected

between these lines). An identical result has been previously reported with anti-L-type serum [10]. Moreover the addition of L-type pyruvate kinase in excess to anti-erythrocyte pyruvate kinase serum suppressed all ability of that antiserum to precipitate erythrocyte as well as L-type enzyme.

Micro-complement fixation (Fig. 3). The pattern of the complement fixation curves in the presence of anti-erythrocyte pyruvate kinase serum was identical for either erythrocyte or L-type enzymes, whether the experiment was performed with pure enzymes or with ammonium sulphate fractionated preparations.

Discussion

Although an accurate comparison between results obtained with various titration techniques is impossible, specific activity of purified erythrocyte pyruvate kinase and overall yield of the purification seem to be, with the method described above, far higher than those obtained with all the other methods so far described [1–3].

The main advantages of such a procedure are its rapidity (about 1 week), its simplicity and the high yield obtained. These characteristics would be fundamental for purifying an unstable mutant enzyme with deficiency in catalytic activity.

The determinant step of the method we described is the affinity chromatography on Dextran Blue-Sepharose 4B column. The property of the enzyme of combining with Dextran Blue and to Cibacronblau has been known since 1968 [12] and has been used by several authors to purify yeast [13] and erythrocyte pyruvate kinase [3,14]. We have introduced two main changes into these methods. Firstly, Dextran Blue covalently attached to Sepharose 4B [7] was used instead of free Dextran Blue [3,14], or instead of a Cibacronblau-Sephadex G-200 column [13]. Secondly, and above all, pyruvate kinase was not eluted by ionic strength but, specifically, by a low concentration of fructose 1,6-diphosphate. When the enzyme is eluted from the Dextran Blue-Sepharose column by ionic strength, specific activity of pyruvate kinase in the eluate is only 80 I.U./mg of protein (instead of 290). Thus, the method we propose is a combination of polyspecific affinity chromatography with specific affinity elution. It has been well established that Dextran Blue-Sepharose constituted an affinity column for the dinucleotide fold in proteins [15]: consequently it can be speculated that fructose 1,6-diphosphate acts by changing the conformation of pyruvate kinase (and thus by modifying its dinucleotide fold) rather than by directly interfering at the site of binding between the protein and the blue Dextran-Sepharose resin.

The final enzymatic product is homogeneous as tested by immunodiffusion and sodium dodecyl sulphate polyacrylamide gel electrophoresis.

These results are at variance with those of Peterson et al. [16] and of Lincoln et al. [4]: these authors, using the same type of preparation [1], reported that erythrocyte pyruvate kinase showed two discrete bands by sodium dodecyl sulphate polyacrylamide gel electrophoresis, and sometimes [4], two precipitation lines as tested by immunodiffusion against anti-erythrocyte type enzyme.

If, indeed, as assured by the authors [1,16], the pyruvate kinase preparation subjected to sodium dodecyl-sulphate acrylamide gel electrophoresis was homogeneous, the following hypothesis might be put forward for explaining the discrepancies between the data of Peterson et al. [16] and ours: either in the course of the red blood cell life, or, mainly during the storage of blood or the first steps of the purification procedure some of the enzyme molecules (or some of the subunits of the same molecule) could be modified by partial proteolysis. Such a proteolysis could be responsible for the low specific activity found by Chern et al. [1], for the presence of a protein band having a slightly reduced molecular weight found by sodium dodecyl sulphate acrylamide gel electrophoresis and for the heterogeneity inconstantly found by immunodiffusion [1,4]. The extent of the proteolysis might be variable in function of blood storage time and (or) of the type (and of the brevity) of the purification procedure. This hypothesis should be tested by studying the C and N termini of homogeneous pyruvate kinase preparations and by comparing the sodium dodecyl sulphate polyacrylamide gel electrophoresis pattern of various preparations purified from blood samples stored for a variable time in different conditions.

Preliminary results obtained in our laboratory seem to indicate that, indeed, certain preparations purified by the method described above show by sodium dodecyl sulphate polyacrylamide gel electrophoresis an additional protein band with similar mobility. When two bands were detected, however, one of them was always markedly predominant and, therefore, this pattern could not be explained by the existence of two types of subunits in each of the erythrocyte pyruvate kinase molecules. The post-synthetic changes due to partial proteolysis are now well documented, especially in the case of α-crystallin of lens [17]. In addition, we have recently showed that erythrocyte glucose-6-phosphate dehydrogenase seemed to differ from the leukocyte enzyme by its C-terminal end, and that this difference was the most likely due to a partial hydrolysis of at least a C-terminal lysine found in the native form of the enzyme existing in granulocytes [18].

The question can also be raised of whether such a hypothetical use by Lincoln et al. [4] of modified forms of erythrocyte pyruvate kinase could account for another discrepancy with our results: one of the precipitation lines obtained by these authors between erythrocyte pyruvate kinase and its specific antiserum showed a "partial identity" (i.e., a 'spur' phenomenon) with the precipitation line of L-type enzyme. By contrast, in our immunodiffusion experiments erythrocyte and L-type enzymes from crude extracts as well as from partially or totally purified preparations showed a complete identity reaction as tested against both anti-erythrocyte and L-type pyruvate kinase sera. Moreover the total consumption of the anti-erythrocyte enzyme antibodies by L-type pyruvate kinase seemed to indicate that this antiserum did not contain two types of antibody, one of them recognizing L-type subunit and the other a second antigenically different subunit. Antigenic identity by immunodiffusion is thought to signify that the two antigens being compared bear the same antigenic determinant, recognized by the antiserum [19,20]. Since such an identity was found with both the specific antisera, it appeared that the nature of the antigenic determinants was identical for either of the pyruvate kinases studied. The significance of the identity of two antigens compared by micro-

complement fixation is different: it is that both the antigens bear an identical number of antigenic determinants and that the affinities of these determinants for antibody are identical [11,20]. Consequently combination of the method of double immunodiffusion against both the specific antisera with that of micro complement fixation could demonstrate that two antigens are immunologically identical. These data, together with those of Lincoln et al. [4] and of Kahn et al. [21] showing that no immunological cross-reactivity between M_1 or M_2 type and erythrocyte pyruvate kinases could be detected, seem to indicate that both L-type and erythrocyte pyruvate kinases are composed from a single type of subunit, coded by a single gene. Post-synthetic tissue-specific changes could account for the slight differences found between the electrophoretic properties of these enzymes. The nature of these changes remains entirely unknown; partial proteolysis, as discussed above, should be considered.

Note added in proof (Received December 6th, 1976)

In fact, as reported and discussed hereafter, purified erythocyte pyruvate kinase shows in some preparations several bands having slightly different mobilities in sodium dodecyl sulfate acrylamide gel. In all cases, however, enzyme from liver (L-type) and from red cells are antigenically identical.

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