EVIDENCE FOR A POSTSYNTHETIC PROTEOLYTIC TRANSFORMATION OF HUMAN ERYTHROCYTE PYRUVATE KINASE INTO L-TYPE ENZYME

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1. Introduction

The relationship between liver (L-type) and erythrocyte pyruvate kinase remains a subject of discussion. It has been previously demonstrated that these enzymes are kinetically [1,2] and immunologically [3–5] related. In some cases liver (L-type) pyruvate kinase from patients with hereditary erythrocyte pyruvate kinase deficiency was shown to be defective [2,6–8]. Erythrocyte and L-type enzymes, however, can be distinguished by their electrophoretic mobilities [2,9]. Some authors [9] have suggested that erythrocyte enzyme might be a hybrid between L-type and non-L-type subunits: the demonstration by Peterson et al. [10] that, by sodium dodecylsulphate–acrylamide gel electrophoresis, erythrocyte pyruvate kinase was resolved into two distinct bands with similar mobility, supported this assumption.

We have purified erythrocyte enzyme by a new method, previously reported [11]. The crucial step of this purification procedure was an affinity chromatography on a blue-dextran Sepharose 4 B column, with selective elution by fructose-1,6-diphosphate. Under some conditions, red cell pyruvate kinase was eluted from the blue-dextran Sepharose column in two peaks.

The purpose of this work was to study the nature of those two molecular forms of the erythrocyte enzyme in relation to the L-type enzyme from liver.

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We have been able to prove that one of the erythrocyte forms was a homotetramer composed of 4 identical L-type subunits, while the other one was a hybrid composed of 2 L-type subunits and 2 subunits with a slightly higher molecular weight. The specific activity of this latter form was half that of the former. This heterotetramer could be activated and transformed into a homotetramer similar to L-type enzyme under the influence of a limited proteolysis by trypsin.

From these results it might be speculated that erythrocyte pyruvate kinase might be synthesized as a precursor enzyme whose postsynthetic partial proteolysis could lead to a progressive transformation into L-type enzyme; such a modification seems to be associated with an increase of the catalytic efficiency of pyruvate kinase.

2. Materials and methods

Ion-exchangers, cyanogen bromide-activated Sepharose 4 B and blue-dextran were supplied by Pharmacia. The substrates for the enzymatic reaction and the intermediate enzymes were furnished by Boehringer-Mannheim. Trypsin (TRTPCK) came from Worthington Biochemical Corporation. Acrylamide and sodium dodecylsulphate–acrylamide gel electrophoresis was performed as reported elsewhere [12]. Slab-gel electrophoresis was performed according to the methods described by Imamura et al. [9], slightly modified in that the gel was 4% acrylamide

instead of 3.34% and the pH 8 instead of pH 8.2. The enzymes were focused in acrylamide–ampholine gels as described in ref. [12]. The gels were stained either for proteins, by Coomassie Blue, or for pyruvate kinase activity; in the latter case the enzymatic activity was revealed either by defluorescence under ultraviolet, or by positive staining, via the hexokinase and glucose-6-phosphate dehydrogenase reactions [13].

The molecular weight of the whole molecules and dissociated subunits of the erythrocyte enzymes have been determined by gel filtration and by sodium dodecylsulphate–acrylamide gel electrophoresis, respectively as previously described for L-type pyruvate kinase [12].

The tryptic hydrolysis was performed at room temperature: 100 μg pure pyruvate kinase were diluted in 500 μl 50 mM Tris–chloride buffer, pH 7.3, containing 100 mM KCl, 4 mM $MgCl_2$, 0.1 mM β-mercaptoethanol and 500 mM sucrose, then incubated either alone or with 20 μg trypsin. Tryptic digestion was stopped after various incubation times by adding 2 mM di-isopropylfluorophosphate to the reaction mixtures. L-Type pyruvate kinase from human liver was purified to homogeneity by a procedure whose last step was identical with that described for the red cell enzyme (i.e., an affinity chromatography on blue-dextran Sepharose 4 B column with elective elution by fructose-1,6-diphosphate [14].

Enzyme activity was measured at 30°C, in Tris–chloride buffer pH 8, according to Beutler [15] and Blume et al. [16].

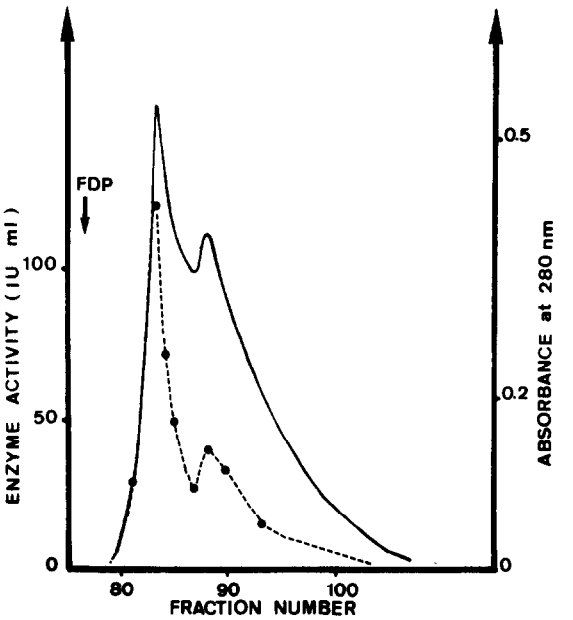

Fig.1. Elution of erythrocyte pyruvate kinase from the blue-dextran Sepharose 4 B column. The preparation obtained after the second step of the purification procedure described in ref. [11] was desalted by chromatography on a Sephadex G-25 column equilibrated with a 50 mM Tris–chloride buffer, pH 7.5, containing 1 mM EDTA, 2 mM β-mercaptoethanol, 1 mM ε-aminocaproic acid and 40 mM NaCl, then applied to the dextran-blue Sepharose 4 B column equilibrated with the same buffer. The column was then washed with a 40 mM Tris–chloride buffer, pH 7.5, containing 60 mM KCl and the same protectors as above; elution was initiated by adding 0.1 mM fructose-1,5-diphosphate to this buffer. (-•-•-) Pyruvate kinase activity, (———) absorbance at 280 nm.

3. Results

The elution pattern of erythrocyte pyruvate kinase from the last step of purification is shown in fig.1.

The active fractions from each peak were collected separately. Specific activity of the erythrocyte enzyme from the first peak (designated as red cell pyruvate kinase I) was 300 IU/mg protein, whereas specific activity of the enzyme from the second peak (pyruvate kinase II) was only 150 IU/mg protein. Acrylamide gel electrophoresis, in slab-gels or in disc-gels (fig.2) showed that each preparation contained a single molecular form, enzyme I exhibiting a more anodic migration than enzyme II.

Isoelectrofocusing in acrylamide–ampholine gel, in tubes or in thin layer, confirmed that isoelectric point of enzyme I was slightly higher (i.e., more alkaline) than that of enzyme II. It should be noted that L-type pyruvate kinase from liver and erythrocyte pyruvate kinase I were similar, according to their electrophoretic mobility in various conditions and their isoelectrofocusing patterns.

As previously described for L-type and erythrocyte pyruvate kinase [11,12] the molecular weight of enzyme I and enzyme II estimated by gel filtration was about 220 000–240 000. Sodium dodecylsulphate–acrylamide gel electrophoresis (fig.3) revealed a single protein band for enzyme I. This band corresponds to a subunit having exactly the same migration (i.e., the same mol. wt ≃ 60 000) as

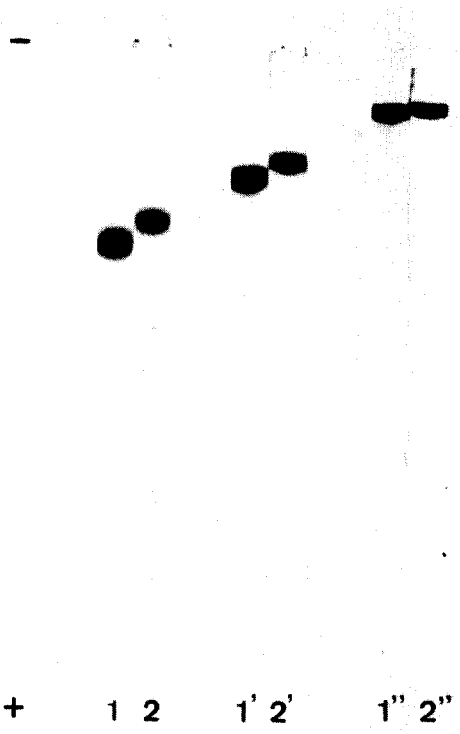

Fig.2. Acrylamide gel electrophoresis of red cell pyruvate kinase I and II. Gels were 4% (1 and 2), 6% (1′ and 2′) and 8% (1″ and 2″) acrylamide (w/v), in 40 mM Tris–lycine buffer, pH 8.7, containing 1 mM EDTA. 20 μg of protein were deposited at the top of the gels. Protein were stained with Coomassie Blue.

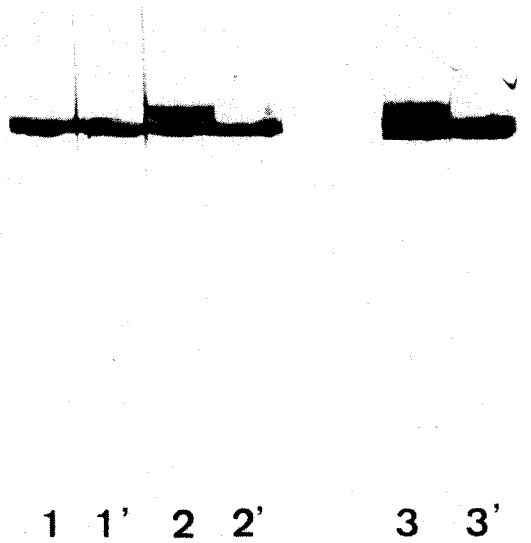

Fig.3. Sodium dodecylsulphate–acrylamide gel electrophoresis of the trypsin-treated and non-treated forms of erythrocyte pyruvate kinases. (1, 1′) Red cell pyruvate kinase I, non-treated (1) and treated (1′). (2, 2′) Red cell pyruvate kinase II, non-treated (2) and treated (2′). (3, 3′) Mixture of L-type pyruvate kinase from liver and of erythrocyte pyruvate kinase II, non-treated (3) and treated (3′). This latter experiment shows that the 'light' subunit of the red cell enzyme II has a migration (i.e., a molecular weight) identical with that of the L-type subunits, and that the treatment by trypsin converts the 'heavy' subunit 'L″ into a subunit having a molecular weight identical with that of the L-type subunits.

the L-type subunit. By contrast enzyme II showed, by sodium dodecylsulphate–acrylamide gel electrophoresis, two distinct protein bands: one being identical with that found with L-type pyruvate kinase or with red cells pyruvate kinase I, the other showing a slower migration (i.e., with a molecular weight slightly higher than for L-type subunit).

The mild treatment by trypsin, as described in Materials and methods, resulted in enzyme activity and electrophoretic mobility changes of red cell pyruvate kinase II, whereas the characteristics of L-type enzyme or enzyme I were not significantly affected. Enzyme activity of red cell enzyme II increased progressively up to 160% of the initial activity; its electrophoretic mobility in acrylamide slab-gel became identical with that of enzyme I. Finally, the additional protein band shown by sodium dodecylsulphate–acrylamide gel electrophoresis (fig.3) (i.e., the 'heavy' subunit) disappeared.

Molecular weight of the undissociated trypsinized enzyme II was similar to that of the untreated enzyme.

In other words, after treatment by trypsin, enzyme II was composed from four identical subunits having a molecular weight identical with that of the L-type subunits.

4. Discussion

The results reported above establish clearly that at least two forms of pyruvate kinase can be purified from human erythrocytes. The first one is a homotetramer composed of 4 identical L-type subunits,

and may be designated as 'L4'; the second form is a heterotetramer tentatively designated as '$L_2L'_2$'.

In a previous paper Peterson et al. [10] have also demonstrated that, in some cases, erythrocyte pyruvate kinase could be dissociated by sodium dodecylsulphate–acrylamide gel electrophoresis into two protein bands with similar mobilities. These authors, however, hypothesized that one of these bands might correspond to an M-type subunit. We have recently shown [17] that there was no interaction between anti-leukocyte (M2) serum and erythrocyte enzyme, while Lincoln et al. [18] proved that anti-muscle (M1) pyruvate kinase serum did not cross-react with red cell pyruvate kinase.

This paper demonstrates that, under the influence of a partial proteolysis by trypsin, the 'L′' subunit can be changed into a form which cannot be distinguished from L-type subunit, by electrophoresis of the whole or dissociated molecules. We have demonstrated in another work (to be published) that, when studied with an anti L-type pyruvate kinase serum, the L′ and L subunits seemed to be antigenetically identical. Consequently it can be speculated that the L′ subunit is the precursor of L-type subunit, the conversion of L′ into L subunit involving the cleavage of a peptide located to an extremity of the molecule.

The fact that erythrocyte pyruvate kinase II (i.e., $L_2L'_2$) had a specific activity half that of enzyme I (L_4), and that its activity increased markedly when this enzyme was treated by trypsin, could indicate that the L′ subunits are totally or partially inactive, their activation requiring the proteolytic cleavage described above.

Up to now we do not know whether or not a precursor molecule composed of 4 L′ subunits exists in the erythroid precursors, or in the young red cells. It is possible that such a hypothetical L'_4 form might be lost during the purification procedure, because it could be unable to be fixed on the blue-dextran Sepharose column or to be eluted by fructose-1,6-diphosphate [19]. The search for such an inactive precursor 'L'_4' requires electrophoretic and immunologic methods.

Another main question is raised by the above results: why were no '$L_2L'_2$' molecules found in the liver? It could be expected that these nucleated cells, synthesizing proteins actively, would contain more precursor molecules than red cells, unless the proteolytic activation of the precursor pyruvate kinase molecules is especially active in liver.

In conclusion, it seems that erythrocyte pyruvate kinase is genetically identical to L-type enzyme from liver.

The pyruvate kinase enzyme could be synthesized in a 'proenzymatic' form requiring a partial proteolysis to reach whole activity. This phenomenon could be especially active in liver.

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