A monomeric form of pyruvate kinase in human pyruvate kinase deficiency

(enzymopathy/red blood cell/allosteric protein/enzyme kinetics)

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ABSTRACT A mutant pyruvate kinase (ATP:pyruvate 2-O-phosphotransferase, EC 2.7.1.40) from human erythrocytes which is easily separated into monomers and dimers by gel chromatography is described. The mutant enzyme shows almost the same pH optimum and thermostability as normal enzyme, but has a decreased stability on shaking with air, a decreased  Km for phosphoenolpyruvate and a loss of allosteric properties. The apparent  Km values for phosphoenolpyruvate of tetramers and monomers were the same. The tetrameric enzyme was slightly activated by fructose-1,6-diphosphate but the monomeric form was not. The tetrameric enzyme was found to dissociate spontaneously to dimeric and monomeric forms.

Erythrocyte pyruvate kinase (PK, ATP:pyruvate 2-O-phosphotransferase, EC 2.7.1.40), is one of the key enzymes in the Embden-Meyerhof pathway of glycolysis (1). It has the well-defined properties of a rate-controlling enzyme, showing a sigmoidal velocity curve with respect to its substrate phosphoenolpyruvate (P-enolpyruvate) and having fructose-1,6-bisphosphate (Fru-1,6-P2) as a positive allosteric effector. It has a molecular weight of approximately 200,000 (2) and is assumed to have four subunits.

A marked reduction in activity of this enzyme is associated with a defect in energy metabolism of erythrocytes and results in a hemolytic disorder (3). In PK deficiency, one of the most common genetic defects of the glycolytic pathway in human erythrocytes (4), there is a shortened erythrocyte survival, increased 2,3-diphosphoglycerate, and a right-shifted hemoglobin oxygen dissociation curve (5).

This study deals with an abnormal pyruvate kinase from human erythrocytes which has no allosterism and which dissociates easily into monomers. The enzyme was isolated from a patient with PK deficiency and severe hemolytic anemia. The abnormal instability and kinetic properties of the mutant PK demonstrate the importance of tetramer formation in the normal enzyme.

MATERIALS AND METHODS

Chemicals. ADP, Fru-1,6-P2, NADH, and other chemicals were obtained from the Sigma Chemical Co. P-enolpyruvate and enzymes were purchased from Boehringer Mannheim. ACA-34 (Ultrigel) is a product of LKB Instruments, Inc.

Pyruvate Kinase Enzyme Assay. Heparinized blood obtained from normal subjects and a 21-year-old splenectomized patient with PK deficiency (hemoglobin level 8.2 g/dl, reticulocytes represented 58% of total red blood cells) was centrifuged at 12,000 X g for 2 min at room temperature in an Eppendorf Micro centrifuge. The cells were washed several times with isotonic saline. After each washing, the buffy coat and the top layer of erythrocytes were carefully removed. The erythrocytes were hemolyzed by mixing with 20 volumes of 5 mM phosphate buffer at pH 7.5, containing 0.5 mM EDTA (T. Asakura, K. Minakata, K. Adachi, M. O. Russell, and E. Schwartz, J. Clin. Invest., in press). After standing for 10 min in an ice bath, the mixture was centrifuged at 15,000 X g for 10 min at 4° and the supernatant solution was used for the enzyme assay. The enzyme activities were measured according to the method of Beutler (6). Standard assays for PK activity were conducted in the following incubation mixture in a total volume of 2 ml: 1.5 mM phosphoenolpyruvate, 0.4 mM ADP, 0.1 M KCl, 10 mM MgCl, 0.2 mM NADH, 10 units of lactate dehydrogenase, 0.1-1.0 ml of hemolysate, and 100 mM Tris-Cl buffer at pH 8.0. One unit of lactate dehydrogenase activity (Boehringer) will reduce 1.0 mmole of pyruvate to lactate per min at pH 7.0 at 25°. The reaction was initiated by the addition of P-enolpyruvate. All assays were performed at room temperature (22°) with an Aminco DW2 UV-VIS spectrophotometer (full absorbance scale 0.05 or 0.1), and the activity was recorded on a Hitachi Perkin Elmer Recorder 56.

Kinetic studies were performed on the native hemolysate and on hemoglobin-free enzymes that had been partially purified by ACA-34 gel chromatography (2, 5). A unit of activity is defined as the amount of enzyme required to oxidize 1 mmole of NADH per min in the standard assay.

Thermostability Tests. Thermostability tests were carried out by the method of Blume et al. (7). Crude hemolysate with a protein content of 6 mg/ml prepared in 75 mM 2-[bis(2-hydroxyethyl)amino]-2-(hydroxyethyl)-1,3-propanediol buffer at pH 7.4 was incubated at 55°. At 0, 20, 60, and 120 min, aliquots (0.5 ml) were chilled for 5 min before being centrifuged for 10 min at 12,000 X g. The supernatant was then assayed for PK activity.

Mechanical Shaking Test. Shaking experiments were carried out in a 10 X 40 mm vial at room temperature using a TCS shaker (8). Two milliliters of the hemolysate containing 12 mg of Hb in 75 mM 2-[bis(2-hydroxyethyl)amino]-2-(hydroxyethyl)-1,3-propanediol buffer at pH 7.4 were shaken at a frequency of 28 Hz for various time periods. After shaking, the precipitate was removed by centrifugation and the supernatant was used for the assay of PK activity.

Molecular Weight Determination. The molecular weight of normal and abnormal PK was determined by gel filtration on ACA-34 using hemoglobin (65,000), rabbit muscle aldolase (158,000), and rabbit muscle pyruvate kinase (237,000) as molecular weight standards. The column (1.5 X 99 cm) was equilibrated with a buffer containing 0.1 M KCl, 1 mM 2-mercaptoethanol, and 50 mM Tris-Cl at pH 7.5. One milliliter of crude hemolysate (1:2 hemolysis) was placed on the column and eluted with the same buffer at 4°. The enzyme activity of the eluate was determined by using 0.2-1.0 ml of each fraction as described above.
RESULTS

Characterization of PK

Kinetics. PK activities of the patient's and controls' hemolysates were studied with various P-enolpyruvate, ADP, and Fru-1,6-P$_2$ concentrations. The total PK activity of the patient's hemolysate was only 5–7% of that of the normal values. The apparent $K_m$ for P-enolpyruvate of the patient's enzyme was 0.07 mM, in contrast to 1.86 mM for the normal enzyme. The mutant enzyme showed a hyperbolic curve in the absence of Fru-1,6-P$_2$ rather than the sigmoidal curve of a normal enzyme (Fig. 1). Although Fru-1,6-P$_2$ induces a transition from sigmoidal to hyperbolic kinetics (9), Fru-1,6-P$_2$ had little effect on the patient's enzyme (Fig. 1). The apparent $K_m$ for ADP of the mutant enzyme was 0.15 mM, the same as that of normal enzyme (Fig. 2). These values were not affected by Fru-1,6-P$_2$.

Effect of pH. The pH optima of some types of mutant PK have been reported to be different from that of normal PK (10, 11). Paglia and Valentine (12) reported that the pH optimum of PK in old cells becomes slightly alkaline compared to that of young cells. The pH dependency of observed $V_{max}$ in the absence and presence of Fru-1,6-P$_2$ is shown in Fig. 3. The pH maximum of both enzyme activities was not substantially altered. The stimulation by Fru-1,6-P$_2$ of both enzymes was found only above pH 7.

Thermostability and Mechanical Stability. It has been reported that mutant PK from some patients is heat labile (13). Comparison of heat stability of our mutant enzyme with that of the normal enzyme is shown in Fig. 4. Although the patient's enzyme was slightly more unstable than normal enzyme, the difference is not as great as previously reported for other patients' PK deficiency (7). The stability of the mutant PK was further studied using a mechanical shaking method. Extensive studies of the mechanism of precipitation, including the effects of deoxygenation (8) and organic phosphates (14) on the rate of denaturation of hemoglobin, suggest that the rate of mechanical precipitation depends on protein conformation in solution (14). As shown in Fig. 5, the patient's PK activity decreased more rapidly than that of normal subjects, suggesting that this abnormal PK is slightly more unstable than normal enzymes. Upon the addition of Fru-1,6-P$_2$, both enzymes become more stable, probably due to the conformational changes which occur upon binding of Fru-1,6-P$_2$ to the enzyme.

Molecular Weight Studies. The molecular weight of the mutant enzyme was estimated by ACA-94 gel filtration. As shown in Fig. 6, two peaks of PK activity appeared. The first peak elutes before hemoglobin and the second smaller peak after hemoglobin. The molecular weight of the first peak (fraction 61) corresponds to 220,000 and that of the second peak (fraction 86) to 47,000. The molecular weight of the protein eluted at the first peak is similar to that of muscle PK. A similar experiment with hemolysate obtained from a control showed only one peak before hemoglobin (Fig. 6). This was in the same position as that of the first peak of the patient's enzyme.

Characterization of the two PK enzyme fractions from the hemolysate of the patient

The tubes comprising the two peaks (Fig. 6, tubes 55–67 and 82–86) were collected separately and concentrated with a collodion bag to a final volume of 1–2 ml (protein concentration 1–2 mg/ml) at 0°. Kinetic studies of these fractions showed that the apparent $K_m$ (P-enolpyruvate) of the first peak enzyme (tubes 55–67) was 0.07 mM, identical to that obtained from hemolysate (Fig. 1). The enzyme activity was slightly activated by Fru-1,6-P$_2$. The enzyme in the second fraction had the same apparent $K_m$ value for P-enolpyruvate. However, this enzyme was not affected by the addition of Fru-1,6-P$_2$.
FIG. 5. The stability by mechanical shaking of the activity of pyruvate kinase in normal and patient hemolysate with and without Fru-1,6-P\(_2\). Two milliliters of the hemolysate which contain 12 mg of Hb in 75 mM 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol buffer at pH 7.4 were shaken at a frequency of 28 Hz with a TCS shaker at room temperature (31\(^\circ\)C). After shaking, the precipitate was removed by centrifugation and the supernatant was used for the assay of PK activity. Further conditions are described in the text.

To examine the possibility of the enzyme with a high molecular weight dissociating into subunits, we collected fractions 55-67, concentrated, and reapplied to the same ACA-34 column. In this case, three clear peaks with PK activity appeared (Fig. 7). Peaks I, II, and III correspond to molecular weights of 230,000, 124,000, and 47,000, respectively. Rechromatography of normal PK showed a single peak at the original elution position corresponding to molecular weight of 230,000. These results clearly indicate that the tetrameric enzyme obtained from the patient readily dissociates into dimeric and monomeric forms. The preliminary studies show that the monomeric fraction reassociated into tetramers.

**DISCUSSION**

PK-deficiency hemolytic anemia comprises a heterogeneous group of disorders characterized by both quantitative and qualitative abnormalities. Munro and Miller (15) have categorized mutant enzymes as exhibiting (i) decreased activity and normal apparent \(K_m\) for \(P\)-enolpyruvate (16, 17), (ii) decreased activity and decreased apparent \(K_m\) for \(P\)-enolpyruvate (18, 19), or (iii) slightly decreased or normal activity and increased apparent \(K_m\) for \(P\)-enolpyruvate (20, 21). Staal et al. (13) identified one mutant enzyme with decreased activity without allosteric properties. The mutant enzyme discussed in this paper appears to belong to type (ii), but it is different from those previously reported by Staal et al. (13) in that it easily dissociates into monomers and dimers.

The term pyruvate kinase deficiency does not indicate whether the low activity is due to a qualitative or quantitative difference from normal PK. Although it is not clear if the total number of pyruvate kinase molecules in our patient differs from those present in normal blood, it is apparent that there is not only a decrease in total activity but a qualitative difference in the kinetic properties.

Pyruvate kinase has structural similarities to hemoglobin. Not only do both hemoglobin and PK consist of tetrameric subunits, but they exist in either of two physical conformations analogous to the R and T forms described by Monod et al. (22).

The molecular weight of purified human erythrocyte PK has been variously reported to be 195,000 (23, 205,000 (2), and 237,000 (24). Blume et al. (23) reported that the molecular weight of a variant PK, determined by chromatography on Sephadex G-200, was 195,000. No dimer or monomer forms were detected. We found that the tetramer (230,000 molecular weight) of our mutant enzyme easily dissociated into dimers and monomers and preliminary studies show that the subunits reassociate into tetramers, thereby indicating that the enzyme is in the following equilibrium:

\[
\text{Tetramer} \rightleftharpoons \text{Dimer} \rightleftharpoons \text{Monomer}
\]

FIG. 6. Gel chromatography of the pyruvate kinase in normal and patient hemolysate by ACA-34. The hemolysate (1:2) (1.0 ml) was placed on the column (1.5 x 99 cm) and eluted with 0.1 M KCl, 1 mM 2-mercaptoethanol, and 50 mM Tris-HCl buffer at pH 7.5 (one fraction equals 1.86 ml). The conditions of measurement of pyruvate kinase activity are described in the text.

FIG. 7. Repeat gel chromatography of the pyruvate kinase of fractions 55-67 by ACA-34. The fractions (55-67) were collected, concentrated, and reapplied to the ACA-34 column. The other conditions are the same as those of Fig. 6.
Since the enzyme activity of the patient’s PK put on the column was recovered totally before and after chromatography, the tetramer of this mutant enzyme may be a simple aggregation of monomeric units. This assumption would explain the low activity and the loss of allosteric properties of this mutant enzyme. It was found that Fru-1,6-P2 slightly affects the tetramer of this mutant enzyme but not the monomer. The results of the mechanical shaking test also showed that this mutant enzyme is stabilized by the addition of Fru-1,6-P2.

In normal PK, the monomer which has low $K_m$ value for P-enolpyruvate appears to change conformation in the tetrameric form, resulting in an increase in $K_m$ values. However, the apparently decreased $K_m$ for P-enolpyruvate of this mutant enzyme was the same in both its tetrameric and monomeric forms. Our results indicate that the binding site for P-enolpyruvate of this mutant enzyme tetramer may be the same as the monomer. Although the monomeric form prepared by treatment of muscle and erythrocyte PK with urea is inactive (25), the monomeric form found in our patient has a low but nevertheless distinct enzyme activity. Ibsen et al. (24) also reported that the subunits of normal human erythrocyte PK obtained by sonic disruption had PK activity, although the activity was low. The low activity associated with this unique monomeric PK is clearly differentiated from the quantitatively higher activity of the tetrameric form found in normal PK. The conformational change that occurs by the aggregation of subunits or by the addition of P-enolpyruvate and Fru-1,6-P2 may play an important regulatory role in the activity of this glycolytic enzyme.

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