LACK OF PHOSPHORYLATABLE SITE AND SOME KINETIC PROPERTIES OF ERYTHROCYTE PYRUVATE KINASE FROM THE RAT

A modified L-type isozyme?

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The rat erythrocyte pyruvate kinase is not phosphorylated in vitro in the presence of cyclic AMP-dependent protein kinase and ATP. The enzyme binds, however, to anti-L-type pyruvate kinase–IgG–Sepharose. The kinetic properties of the rat erythrocyte pyruvate kinase strongly resemble those of subtilisin-modified L-type enzyme. The results indicate that erythrocyte pyruvate kinase is a proteolytically modified L-type enzyme.

1. Introduction

Pyruvate kinase (ATP: pyruvate phosphotransferase, EC 2.7.1.40) is known to exist as several isozymes [1]. There is no general agreement about the type of enzyme present in erythrocytes. The erythrocyte enzyme may be a hybrid between L-type and non-L-type subunits [2]. It may be a homotetramer with 4 identical L-type subunits [3,4]. The homotetramer could also be obtained after limited proteolysis of an enzyme composed of 2 L-type subunits and 2 subunits with a somewhat greater molecular weight [4].

L-type pyruvate kinase is the main isozyme in liver. It is also present in the kidney and intestine [5,6]. The regulation of its activity is under hormonal control [7]. Potent effectors of the activity of pyruvate kinase type L are fructose-1,6-diphosphate, ATP and alanine [8]. The L-type isozyme has been shown to be phosphorylated under the influence of cyclic AMP-dependent protein kinase in vitro [9]. This phosphorylation has also been observed in liver slices [10]. Phosphorylated pyruvate kinase has a lower affinity for phosphoenolpyruvate than the unphosphorylated enzyme [9,11]. An even more inhibited form of pyruvate kinase is obtained by proteolytic removal of a phosphorylated peptide with very low concentrations of subtilisin [12].

The present work demonstrates that partially purified erythrocyte pyruvate kinase from the rat, in contrast to liver pyruvate kinase, is not phosphorylated in vitro in the presence of cyclic AMP-dependent protein kinase and ATP. The enzyme binds, however, to anti-L-type pyruvate kinase–IgG–Sepharose. The kinetic properties of the rat erythrocyte pyruvate kinase strongly resemble those of subtilisin-modified L-type enzyme. The results indicate that erythrocyte pyruvate kinase is a proteolytically modified L-type enzyme.

2. Material and methods

Bovine serum albumin, fructose-1,6-diphosphate, dithiothreitol, NADH, phosphoenolpyruvate (PEP), ADP and ATP were purchased from Sigma. Rabbit lactate dehydrogenase was a product of Boehringer-Mannheim. DEAE–Sephadex A-50 was obtained from Pharmacia, Uppsala. [^{32}P]ATP was bought from New England Nuclear, Boston.

Protein determination was based on the $A^{1\%}_{1\ cm} = 10.0\ A_{280\ nm}$.

The catalytic subunit of cyclic AMP-stimulated protein kinase from rat liver was isolated as in [13].

Pyruvate kinase from rat liver was prepared as in [14]. Blood for preparation of erythrocyte pyruvate kinase was obtained by decapitation of anesthetized adult rats. The blood was collected in 0.9% NaCl containing 10 mM EDTA, and was left for 2 h to cool and sediment at 4°C. All subsequent steps were performed at 4°C. The blood cells were centrifuged at 3000 × *g* for 10 min, then washed 3 times with 0.9% NaCl. The erythrocytes were hemolysed in 2 vol. 0.5 mM dithiothreitol containing 0.5 mM EDTA under stirring for at least 2 h or overnight. The hemolysate was centri-

fuged at 17 500 × *g* for 45 min. The pellet was discarded. The supernatant was diluted with 2 vol. 30% (v/v) glycerol containing 0.1 mM dithiothreitol. An equal volume of DEAE–Sephadex A-50 equilibrated with 10 mM potassium phosphate, pH 7.0, was added to the diluted hemolysate and stirred for 30 min. The gel with absorbed protein was washed on a Büchner funnel with 10–20 vol. 10 mM potassium phosphate (pH 7.0), 30% (v/v) glycerol and 0.1 mM dithiothreitol until $A_{280} \simeq 0.1$ and then packed on a column. The DEAE–Sephadex column was eluted at pH 7.0 with a linear gradient from 100 mM potassium phosphate, pH 7.0, to 500 mM potassium phosphate containing 30% glycerol and 0.1 mM dithiothreitol. The total gradient consisted of 5 column vol. Fractions from the DEAE–Sephadex chromatography were analyzed for pyruvate kinase activity. The material that contained pyruvate kinase was pooled and concentrated to about 10 units pyruvate kinase/ml and stored at −18°C. For calculations, the specific activities of 290 units/mg [3] and 450 units/mg [14] were used for the erythrocyte and rat liver enzymes, respectively. L-Type pyruvate kinase that had been digested with low concentrations of subtilisin was obtained [12].

Pyruvate kinase from erythrocytes and from liver were phosphorylated at 1 mM [^{32}P]ATP (spec. act. ~ 30 cpm/pmol). The incubation medium had vol. 0.5 ml and contained 0.6 units pyruvate kinase, 22 mM potassium phosphate (pH 7.0), 6% (v/v) glycerol, 0.02 mM dithiothreitol and 10 mM magnesium acetate. The catalytic subunit from rat liver protein kinase was added in excess (3–4-times) of the amount of pyruvate kinase. The reaction was run for 30 min at 30°C and was terminated by the addition of 2 ml ice-cold 10% (w/v) trichloroacetic acid containing 50 mM H_3PO_4. The precipitates were spun down. The pellets were dissolved in 0.2 ml 0.5 M NaOH and reprecipitated with 2 ml 10% trichloroacetic acid containing 50 mM H_3PO_4. This was repeated 3 times. After the last centrifugation the pellets were dissolved in 0.5 ml 0.5 M NaOH and the radioactivity was measured as Čerenkov radiation [15].

Pyruvate kinase activity was measured according to a modification [6] of the methods in [16] during the purification and phosphorylation of the enzymes. The kinetic experiments were performed as in [11]. For each determination 0.03 unit of enzyme was used.

Protein kinase was assayed as in [14]. One unit of catalytic subunit from the protein kinase was defined as the activity that transfers 1 pmol phosphate from ATP into histone/min [14].

An immunoadsorbent column of Sepharose 6B, coupled with antibodies against L-type pyruvate kinase from the rat, was obtained [10]. The gel was equilibrated with 20 mM potassium phosphate buffer, pH 7.0, containing 0.9% NaCl. After the pyruvate kinase had become attached to the immunoadsorbent column the column was washed with the buffer containing 9% NaCl.

3. Results and discussion

Pyruvate kinase from erythrocytes and liver pyruvate kinase adhered to anti-L-type pyruvate kinase–IgG–Sepharose. Two columns, each containing 1 ml immunoadsorbent, were packed. About 10–30 μg pyruvate kinase from erythrocytes and liver, respectively, were applied to the 2 separate columns. About 80–100% of both enzyme forms adhered to the immunoadsorbent. On immunodiffusion in agar gels as in [17], the erythrocyte pyruvate kinase and the subtilisin-treated L-type pyruvate kinase appeared identical to L-type pyruvate kinase. The concentration of erythrocyte pyruvate kinase was very low in the experiment. This strongly indicates that the L-type and the erythrocyte pyruvate kinases are immunologically related, as has been shown by other investigators on the human enzymes [3,4].

If the erythrocyte pyruvate kinase is identical to the liver and kidney L-type isozyme it would be expected to be a substrate of cyclic AMP-dependent protein kinase. In order to test this hypothesis the enzyme was incubated with Mg^{2+}-[^{32}P]ATP plus the catalytic subunit from cyclic AMP-stimulated protein kinase from rat liver. No or low incorporation of ^{32}P into the erythrocyte pyruvate kinase was observed when the enzyme was incubated either alone or mixed with liver pyruvate kinase (table 1). When the enzymes were mixed only the liver pyruvate kinase was phosphorylated. The results suggest that the catalytic subunit of protein kinase was active and that no inhibitor of this enzyme was present in the preparation of the erythrocyte pyruvate kinase. To ascertain that erythrocyte pyruvate kinase was not a phosphorylated form of pyruvate kinase, it was incubated with

Table 1
Incorporation of ^{32}P into pyruvate kinase isozymes when incubated with Mg^{2+}-[^{32}P]ATP and catalytic subunit of cyclic AMP-dependent protein kinase

Sample	Enzyme subunit (pmol)	^{32}P Incorporated into enzyme (pmol)
Liver pyruvate kinase	100	94
Erythrocyte pyruvate kinase	160	0
Liver pyruvate kinase + Erythrocyte pyruvate kinase	50 80	56

Conditions as in section 2. The results of a representative experiment are given

phosphatase, which had no effect on the enzyme (data not given).

In order to further compare the liver and erythrocyte pyruvate kinase, some kinetic experiments were run. The activity of pyruvate kinase from rat liver and rat erythrocytes as a function of the concentration of PEP is presented in fig.1. The erythrocyte isozyme showed a lower affinity for PEP than the liver isozyme. The $K_{0.5}$ for PEP of the erythrocyte pyruvate kinase was 1.8 mM. For the liver enzyme $K_{0.5}$ for PEP was 0.4 mM. L-Type pyruvate kinase treated with low concentrations of subtilisin [12] loses its ability to be phosphorylated and exhibits a reduced affinity for PEP. The subtilisin-treated pyruvate kinase from liver has a $K_{0.5}$ for PEP of 1.8 mM, which is similar to the $K_{0.5}$ for PEP of the erythrocyte pyruvate kinase (fig.1).

The dependence of the two pyruvate kinases on pH is illustrated in fig.2. The concentration of PEP in these experiments was 0.2 mM. The enzyme activity of the erythrocyte enzyme was nearly the same for all pH values tested, while the activity of the liver enzyme increased at lower pH values. A pH curve similar to that of the erythrocyte pyruvate kinase was also obtained with the L-type pyruvate kinase after treatment with low concentrations of subtilisin [12].

In fig.3 the effect of fructose-1,6-diphosphate in

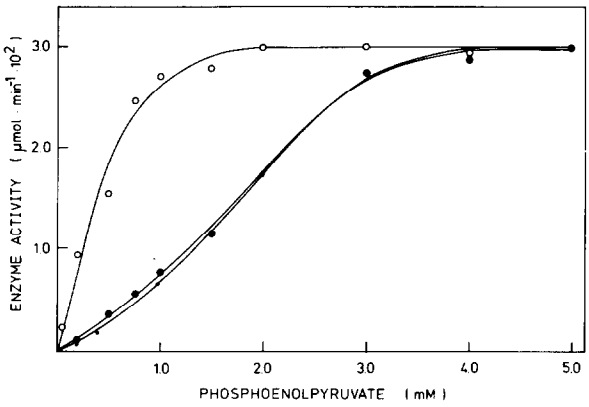

Fig.1. Enzyme activity of liver and erythrocyte pyruvate kinase at pH 7.3 and 30°C as a function of the concentration of phosphoenolpyruvate. No fructose-1,6-diphosphate or ATP was added. The assay conditions were as in section 2. (o——o) Liver pyruvate kinase. (•——•) Erythrocyte pyruvate kinase. (•——•) Liver pyruvate kinase treated with low concentrations of subtilisin.

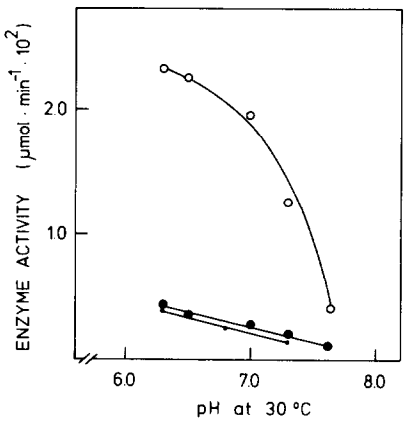

Fig.2. pH-dependence at 30°C of liver pyruvate kinase and the erythrocyte isozyme tested at 0.2 mM phosphoenolpyruvate and 2 mM ADP. (o——o) Liver pyruvate kinase. (•——•) Erythrocyte pyruvate kinase. (•——•) Liver pyruvate kinase treated with low concentrations of subtilisin.

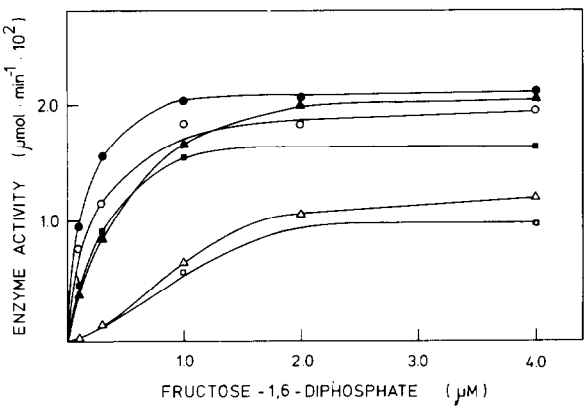

Fig.3. The activity of L-type pyruvate kinase and erythrocyte pyruvate kinase at pH 7.3 and 30°C as a function of fructose-1,6-diphosphate in the presence and absence of 2 mM ATP. Phosphoenolpyruvate conc. 0.2 mM. Open symbols and closed symbols represent the activity in the presence and absence of 2 mM ATP, respectively. (○——○, ●——●) Liver pyruvate kinase. (△——△, ▲——▲) Erythrocyte pyruvate kinase. (□——□, ■——■) Liver pyruvate kinase treated with low concentrations of subtilisin.

the presence and absence of 2 mM ATP is shown for the 3 pyruvate kinases. $K_{0.5}$ for the erythrocyte enzyme and the liver enzyme were 0.4 μM and 0.1 μM fructose-1,6-diphosphate, respectively, in the absence of ATP and 0.9 μM and 0.2 μM in the presence of ATP. The ATP inhibition could not be overcome by high concentrations of fructose-1,6-diphosphate with any of the enzyme forms. The dependence of the erythrocyte pyruvate kinase on fructose-1,6-diphosphate in the presence and absence of 2 mM ATP was very similar to the dependence on these effectors of the L-type pyruvate kinase modified with low concentrations of subtilisin [12]. The profile of the curves appears to be the same. Furthermose, the same $K_{0.5}$ values for fructose-1,6-diphosphate were obtained.

Pyruvate kinase from the liver and that from erythrocytes seem to be immunologically related or identical, as shown here and [3]. In spite of this observation the erythrocyte pyruvate kinase cannot be phosphorylated in the presence of catalytic subunit from cyclic AMP-stimulated protein kinase from rat liver in our system. In vitro the existence of one form of L-type pyruvate kinase that has lost its ability to be phosphorylated is known. This is an enzyme that has been digested with very low concentrations of subtilisin [12]. The removal of the phosphorylatable peptide changed the affinity of the enzyme for the substrate PEP without affecting its V_{max} [12].

The erythrocyte pyruvate kinase might be an L-type pyruvate kinase that has been modified by proteolysis in the cell. The kinetic and pH dependence studies in this report strongly support this idea. In all kinetic experiments the properties of the erythrocyte isozyme were very similar to those of liver pyruvate kinase modified by subtilisin. This should then be the first report of the existence of such a modified enzyme in the cell. Concerning human erythrocyte pyruvate kinase proteolytic modification with trypsin in vitro was reported [4].

The fact that the proteolytic activity of a hemolysate towards phosphorylated pyruvate kinase is much greater than that of a cell sap from rat liver provides good support for this theory (P. Ekman and U. D. E., to be published).

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