Some Kinetic Properties of Liver Pyruvate Kinase (Type L)*

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HECTOR CARMINATTI,‡ LUIS JIMÉNEZ DE ASÚA,§ EDUARDO RECORDO,‡ SUSANA PASSERON,‡|| AND ENRIQUE ROBENGURTI||

From the Instituto de Investigaciones Bioquímicas “Fundación Campomar” and Facultad de Ciencias Exactas y Naturales, Ochobigo 2490, Buenos Aires (28), Argentina

SUMMARY

Type L pyruvate kinase from mouse liver has been studied. The enzyme is strongly inhibited by low concentrations of copper ions, and the inhibition is reversed by fructose 1,6-diphosphate. In the absence of inhibitor, fructose 1,6-diphosphate also increases the pyruvate kinase activity at pH values higher than 7. Furthermore, the Cu++ inhibition is pH and K+ concentration dependent.

Plots of reaction rates against phosphoenolpyruvate concentration give a sigmoid curve whereas a hyperbolic curve is obtained for the other substrate (adenosine diphosphate). When fructose 1,6-diphosphate is added, the response to phosphoenolpyruvate concentration is transformed to give a Michaelian curve and the affinity of the enzyme for this substrate increases. In the presence of Cu++, the Km for adenosine diphosphate is not substantially modified, but the Vmax decreases markedly. On the other hand, the apparent Ks for phosphoenolpyruvate is considerably increased by this cation.

The enzyme has a full requirement for Mg++ and a monovalent cation (K+ or NH4+). The activation of the enzyme with NH4+ has been found to be more efficient than with K+.

The physiological role of the Cu++-fructose 1,6-diphosphate interrelation in the balance between glycolysis and gluconeogenesis is discussed.

Glucoseogenesis is a process of considerable magnitude in mammals and occurs mainly, if not solely, in liver and kidney. The dynamic balance of the rate-limiting enzymes of glucoseogenesis and glycolysis plays a crucial role in determining the direction of carbohydrate metabolism (1).

There are at least two forms of pyruvate kinase (EC 2.7.1.40) in liver, tentatively named type M and type L (2). Although the activity of the latter varies with the nutritional state of the animal, the ratio of type L to type M under normal conditions is about 3 (2). This fact strongly suggests that the L isoenzyme is the more directly related to the metabolic control of glycolysis and glucoseogenesis, in this organ.

The activity of the type L pyruvate kinase is much higher than that of the opposite glucoseogenic enzymes, which catalyze the conversion of pyruvate to phosphoenolpyruvate. The latter is a strategic early step in the synthesis of glycogen and glucose from lactate and other precursors. The P-enolpyruvate which is formed at the expense of two high energy phosphate bonds (3) would be reconverted to pyruvate acid again if the pyruvate kinase activity were unchanged. The net reaction would amount to a short circuit in the energy metabolism of the cell. In order to prevent this wasteful cycle, it appears necessary to have a mechanism by which the type L pyruvate kinase is normally inhibited in the glucoseogenetic state.

In a previous communication from this laboratory, it has been reported that the L isoenzyme is strongly inhibited by Cu++ and that this inhibition is reversed by fructose-1,6-di-P, whereas the M isoenzyme is not affected by this cation (4).

The present paper reports further studies on the kinetic properties of the type L pyruvate kinase and the role of Cu++ in its regulation.

EXPERIMENTAL PROCEDURE

Materials—All chemicals were obtained commercially and were of the highest purity available. All the ADP samples, purchased from different laboratories, were contaminated with traces of Cu++ as shown by the spark emission spectrographic analysis.

Enzymatic Assay—Two assay procedures were used. In the first, pyruvate kinase activity was assayed by measuring the pyruvate formed from P-enolpyruvate in the presence of ADP (5). The standard reaction mixture contained 66.8 mM Tris-
Tris-HCl buffer (pH 7.5), 10 mM MgSO₄, 330 mM KCl, 2 mM ADP, 0.15 M. Incubations were carried out at 30°C, and the reaction before additions. Fructose-1,6-P, fructose 1,6-diphosphate, was used after lyophilization and storage at 5°C for 24 hours. Numbers on the curves, percentage of the rate of each sample activity was determined spectrophotometrically as described di-P (final concentration 0.2 mM) was added. Pyruvate kinase activity was determined spectrophotometrically as described under "Experimental Procedure." Curves A and B were obtained with a freshly prepared enzyme. In Curve C, the enzyme used was after lyophilization and storage at 5°C for 24 hours. Numbers on the curves, percentage of the rate of each sample before additions. Fructose-1,6-P, fructose 1,6-diphosphate.

HCl buffer (pH 7.5), 66.6 mM KCl, 1.33 mM P-enolpyruvate, 10 mM MgSO₄, 1.33 mM ADP, and enzyme in a total volume of 0.15 ml. Incubations were carried out at 30°C, and the reaction was stopped with 0.15 ml of 1% dinitrophenylhydrazine solution in 2 N HCl. In order to increase the sensitivity, the following modifications were introduced. The mixture with the dinitrophenylhydrazine was kept at 30°C for 5 min; then 0.05 ml of 10 N NaOH and 0.25 ml of ethanol were added, and the samples were mixed and centrifuged. The optical density of the supernatant fluid was measured at 590 nm.

One unit of enzyme is defined as the amount which catalyzes the formation of 1 μmole of pyruvate per min under these conditions.

In the second assay procedure, the enzyme system was coupled with lactate dehydrogenase, and the oxidation of NADH was followed at 340 nm in a Gilford model 2000 automatic recording spectrophotometer according to the method of Ocher and Pfleiderer (6).

**Enzyme Purification**—Enzyme was isolated from mouse liver as already described (4). After ammonium sulfate precipitation, the extract was dialyzed for 1 hour against 0.05 M Tris-HCl, pH 7.5. 0.5 mM mercaptoethanol 0.5 mM EDTA. This preparation was applied to a column (14 × 110 mm) of DEAE-cellulose (Serva) previously equilibrated with the same mixture. The column was then washed with 10 ml of the above mentioned buffer. The enzyme was eluted with the same solution, to which a stepwise KCl gradient was superimposed. Fractions of 2 ml were collected and assayed. The M and L isoenzymes were eluted at 0.025 M and 0.15 M KCl concentration, respectively. Protein was determined according to the method of Lowry et al. (7). Serum albumin was used as standard.

Approximately 10 to 20% of the initial enzyme activity was recovered in one or two fractions. The over-all purification varied with different preparations and usually ranged from 15- to 30-fold. In a typical preparation, the specific activities of the 100,000 × g supernatant, the ammonium sulfate fraction, and the DEAE-cellulose eluate were, respectively, 0.68, 1.3, and 19 units per mg of protein. Crude as well as purified L pyruvate kinase is highly susceptible to inactivation under a variety of experimental conditions. Therefore, in most of the experiments a freshly prepared extract was used. Although there was a certain variability in the behavior of the enzyme with storage, it usually lost its sensitivity to Cu++ very rapidly, as shown in Fig. 1.

**RESULTS**

In order to verify that the Cu++ effect on the L pyruvate kinase activity previously described (4) was on the initial velocity, the enzyme activity was measured by coupling the system with lactate dehydrogenase (see "Experimental Procedure"). As shown in Fig. 1, 0.02 mM Cu++ gave a 86% inhibition and addition of 0.2 mM fructose-1,6-di-P completely reversed this effect (Curve A). On the other hand, the enzyme after storage at 5°C for 24 hours was not affected by Cu++, but the addition of fructose-1,6-di-P gave a strong activation (Curve C). In the absence of Cu++, fructose-1,6-di-P greatly increased the activity of the fresh enzyme (Curve B). In the presence of inhibitor the fructose-1,6-di-P activation was smaller.

**Kinetic Constants for ADP and P-enolpyruvate**—Plots of 1/v against 1/ADP at a constant P-enolpyruvate concentration with or without added Cu++, fructose-1,6-di-P, or both are given in Fig. 2. The corresponding plots of 1/v against 1/P-enolpyruvate at a fixed ADP level are given in Figs. 3 and 4.

**Fig. 1.** Effect of Cu++ and fructose-1,6-di-P on L pyruvate kinase activity at pH 7.5. The DEAE-cellulose enzyme was incubated at 30°C in a final volume of 0.5 ml containing 100 mM Tris-HCl buffer (pH 7.5), 10 mM MgSO₄, 330 mM KCl, 2 mM ADP, 1 mM P-enolpyruvate, 0.2 mM NADH, 0.002 mg of serum albumin, and lactate dehydrogenase. At the times indicated by the arrows, CuSO₄ (final concentration 0.02 mM) or fructose-1,6-di-P (final concentration 0.2 mM) was added. Pyruvate kinase activity was determined spectrophotometrically as described under "Experimental Procedure." Curves A and B were obtained with a freshly prepared enzyme. In Curve C, the enzyme used was after lyophilization and storage at 5°C for 24 hours. Numbers on the curves, percentage of the rate of each sample before additions. Fructose-1,6-P, fructose 1,6-diphosphate.

**Fig. 2.** Effect of ADP concentration on the velocity of the L pyruvate kinase reaction under various conditions. Assay conditions were as described under "Experimental Procedure," but with different concentrations of ADP and 0.0066 mM Cu++ or 0.0066 mM fructose-1,6-di-P where indicated. Initial velocities were calculated from the time course plots and corrected according to the method of Algranati (8). In the A and B experiments, the enzyme used was a 20 to 45% ammonium sulfate fraction from two different preparations. Fructose-1,6-P, fructose 1,6-diphosphate.
enolpyruvate concentration, at a fixed ADP level, are sigmoidal in the presence or absence of Cu++. The reciprocal plots curve upward at low substrate concentration and the Hill plots have a slope higher than 1. Although the addition of Cu++ does not appreciably change the slope of the Hill plot (Table I), it greatly decreases the affinity of the enzyme for the substrate (P-enolpyruvate). Conversely, the addition of fructose-1,6-di-P (0.07 mM) alters the shape of the substrate saturation curve from a sigmoid to a hyperbolic pattern, increasing the affinity of the enzyme for P-enolpyruvate (Fig. 4). The apparent K_m values for P-enolpyruvate in the absence or presence of Cu++ are 0.67 and 2.7 mM. When fructose-1,6-di-P is added, the K_m decreases to 0.16 mM.

**TABLE I**

<table>
<thead>
<tr>
<th>Enzyme and additions</th>
<th>n</th>
<th>Apparent K_m (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Diethylaminoethyl cellulose fractiona</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>1.5</td>
<td>0.67</td>
</tr>
<tr>
<td>Cu++, 0.04 mM</td>
<td>1.4</td>
<td>2.7</td>
</tr>
<tr>
<td>Fructose-1,6-di-P, 0.07 mM</td>
<td>1.0</td>
<td>0.16</td>
</tr>
<tr>
<td>2. Ammonium sulfate fractionb</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDTA, 0.133 mM</td>
<td>1.8</td>
<td>1.2</td>
</tr>
<tr>
<td>EDTA, 0.005 mM</td>
<td>1.6</td>
<td>0.9</td>
</tr>
<tr>
<td>EDTA, 0.01 mM</td>
<td>1.9</td>
<td>0.85</td>
</tr>
<tr>
<td>EDTA, 2 mM</td>
<td>1.9</td>
<td>0.75</td>
</tr>
<tr>
<td>Cu++, 0.0060 mM</td>
<td>1.7</td>
<td>1.9</td>
</tr>
<tr>
<td>Cu++, 0.026 mM</td>
<td>1.7</td>
<td>2.8</td>
</tr>
</tbody>
</table>

a Calculated from the slopes of the plot of log (v/(V_max - v)) against log (P-enolpyruvate). The diethylaminoethyl cellulose enzyme was assayed under the conditions described in Fig. 3. The ammonium sulfate enzyme was assayed as described under "Experimental Procedure," but with varying P-enolpyruvate levels.

b The first two experiments were done with the same enzymatic preparation.

c In each experiment, the enzyme was a different preparation.

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1 The abbreviation used is: EDTA, ethylene glycol bis-(β-aminoethyl ether)-N,N'-tetraacetic acid.

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Fig. 3. Effect of P-enolpyruvate concentration on the velocity of the L pyruvate kinase reaction in the presence or absence of 0.04 mM Cu++. Assay conditions were as described in Fig. 1 except that the concentration of KCl was 66.6 mM and that of P-enolpyruvate was varied as indicated.

Fig. 4. Effect of P-enolpyruvate concentration on the velocity of the L pyruvate kinase reaction in the presence of 0.07 mM fructose-1,6-di-P. Assay conditions were as described in Fig. 3 except for the fructose-1,6-di-P.

The data of Fig. 2 show that the apparent K_m values of ADP are independent of the presence of Cu++ or fructose-1,6-di-P. Inverse plots of the rate as a function of substrate concentration give straight lines, thus showing Michaelian kinetics. In the absence of both effectors, the curve for initial velocity against substrate concentration is sigmoidal and the reciprocal plot is not linear. In this respect, it should be borne in mind that the ADP was contaminated with traces of Cu++; therefore the above effect could be due to the fact that the amount of inhibitor is gradually increasing with increasing substrate concentration. This explanation is substantiated by the linear Lineweaver-Burk plot at a constant Cu++ level (Curve A, right, in Fig. 2). Furthermore, a control run in the presence of 1 mM EGTA did not show this sigmoidal effect. The apparent K_m values for ADP at pH 7.5, in the presence or absence of either Cu++, fructose-1,6-di-P or both, calculated from the different curves are between 0.2 and 0.4 mM.

As shown in Fig. 3, the curves for initial velocity against P-
**Cation Requirements**—Muscle and brain pyruvate kinases have been shown to require both Mg\(^{2+}\) and either K\(^+\) or NH\(_4^+\) for maximum activity (9, 10). The same requirements have been found for the enzyme from liver.

The effect of substituting some monovalent cations for K\(^+\) on the activity of the L pyruvate kinase is shown in Table III. It can be seen that K\(^+\) cannot be replaced by Na\(^+\) or Li\(^+\). On the other hand, NH\(_4^+\) seems to be a better activator for the enzyme. Preliminary experiments indicate that NH\(_4^+\) alters the maximum velocity of the pyruvate kinase system. This effect could be due to the capacity of NH\(_4^+\) to form a complex with the endogenous Cu\(^{2+}\) of the system.

The addition of Na\(^+\) to a K\(^+\)-containing mixture does not have any effect on the activity of the enzyme. In the range of concentration tested, Li\(^+\) inhibits the reaction moderately.

**Table II**

_Effect of Cu\(^{2+}\) and fructose-1,6-di-P on pyruvate kinase activity with different nucleotides_

Assay conditions were as described in Fig. 1 except for the corresponding nucleotides, which were 3 mM in each case, and the K\(^+\), which was 133 mM.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ADP</td>
</tr>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>Cu(^{2+}), 0.06 mm</td>
<td>11</td>
</tr>
<tr>
<td>Fructose-1,6-di-P, 0.25 mm</td>
<td>145</td>
</tr>
<tr>
<td>Cu(^{2+}), 0.06 mm, and fructose-1,6-di-P, 0.25 mm</td>
<td>120</td>
</tr>
</tbody>
</table>

* The relative activities for each substrate, without any addition (ADP taken as 100%): IDP, 57; dADP, 47; GDP, 56; UDP, 44; CDP, 8.

* In other experiments with dADP, a 30% inhibition was found. These inconsistent results are under investigation.

**Table III**

_Effect of different monovalent cations on pyruvate kinase activity_

Conditions as indicated under “Experimental Procedure” except for K\(^+\) which was replaced by other cations where indicated. The enzyme was exhaustively dialyzed against 50 mM Tris-HCl buffer, pH 7.5, containing 0.5 mM mercaptoethanol before use. The incubation was at 30°C for 3 min. Pyruvate formed was measured as described under “Experimental Procedure.”

<table>
<thead>
<tr>
<th>Cations</th>
</tr>
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<tbody>
<tr>
<td>Na(^+), 133 mm</td>
</tr>
<tr>
<td>Li(^+), 133 mm</td>
</tr>
<tr>
<td>NH(_4^+), 133 mm</td>
</tr>
<tr>
<td>K(^+), 133 mm</td>
</tr>
<tr>
<td>K(^+), 66.5 mm</td>
</tr>
<tr>
<td>K(^+), 66.5 mm, + Li(^+), 66.5 mm</td>
</tr>
<tr>
<td>K(^+), 66.5 mm, + Na(^+), 66.5 mm</td>
</tr>
<tr>
<td>K(^+), 66.5 mm, + NH(_4^+), 66.5 mm</td>
</tr>
</tbody>
</table>

* All cations were added as chloride salts.
The requirement of the enzyme activity for monovalent cations is fulfilled only with \( \text{NH}_4^+ \) or \( \text{K}^+ \). The former has been found to be more efficient in the activation of the L pyruvate kinase. No effect was found when \( \text{Na}^+ \) was added to a medium containing optimum \( \text{K}^+ \) concentration.

The kinetics of the pyruvate kinase activity as a function of \( \text{P-enolpyruvate} \) concentration has been found to exhibit a sigmoidal dependence as shown in Fig. 3. This type of relationship suggests that cooperative effects act to facilitate the binding of successive molecules of \( \text{P-enolpyruvate} \) and is in agreement with the results already reported for the enzymes for liver (11) and yeast (12).

The cooperative homotropic effect for the substrate is apparently independent of the presence of \( \text{Cu}^{++} \) as indicated by the data of Table I. The values of \( n \) from the Hill plots were calculated from the slopes of the corresponding curves. The tabulated results illustrate that the cooperative effect of \( \text{P-enolpyruvate} \) appears in the absence or presence of \( \text{Cu}^{++} \) or of different metal-chelating agents. It should be noted, however, that the variations in \( n \) values reported, ranging from 1.4 to 1.9, may occur because a different enzyme preparation was used in each case. Results in Table I also suggest that the cooperative effect of \( \text{P-enolpyruvate} \) in the absence of inhibitor is not due to the \( \text{Cu}^{++} \) contamination of the commercial sample of \( \text{ADP} \) used. Furthermore, the addition of different levels of \( \text{Cu}^{++} \) does not change the slopes in the curves of the Hill plots.

The loss of the homotropic interaction when fructose-1,6-di-P was added to the system was clearly shown. The curve for reaction velocity against substrate concentration (\( \text{P-enolpyruvate} \)) in these conditions has now a Michaelis-Menten form; the reciprocal plot is linear, the Hill plot has a slope of unity, and there is an increase in the affinity of the enzyme for \( \text{P-enolpyruvate} \).

As a consequence of the very high activity of pyruvate kinase as compared to the opposing gluconeogenic enzymes, it has been assumed that some mechanism should work in liver by which pyruvate kinase may be inhibited in order to permit the operation of the gluconeogenic pathway. Weber et al. (13), have recently reported that octanoate strongly inhibited hepatic pyruvate kinase and assumed that the rapid action of fatty acid in promoting gluconeogenesis may be explained, at least in part, by the inhibition of the key enzymes of glycolysis.

Although the physiological significance of the \( \text{Cu}^{++} \) inhibition is presently obscure because the concentration of \( \text{Cu}^{++} \) available to interact with pyruvate kinase in the hepatic cell is unknown, it may be speculated that this cation normally maintains the enzyme activity at a low level. Small increases in the fructose-1,6-di-P concentration could switch on the pyruvate kinase activity and thus facilitate glycolysis. Furthermore, this explanation is somewhat emphasized by the fact that fructose-1,6-di-P is also a key metabolite in the regulation of another strategic step, namely the conversion of fructose-6-P to fructose-1,6-di-P and vice versa. It has been shown that, in liver, fructose-1,6-di-P is both a substrate and inhibitor for fructose diphosphatase (14), and both a product and activator of phosphofructokinase (15). This dual function of fructose-1,6-di-P could mean that a change in its level within the cell would control the rate and the direction of the flow of metabolites through this step in both senses. The results presented in this paper suggest that fructose-1,6-di-P could also regulate the activity of the L pyruvate kinase. It follows that, in liver,
fructose-1,6-di-P could dictate the balance between glycolysis and gluconeogenesis by controlling the reaction rate catalyzed by the three strategic enzymes, fructose diphosphatase, phosphofructokinase, and pyruvate kinase.

Acknowledgments—We wish to thank the members of the Instituto de Investigaciones Bioquímicas for helpful discussions and criticism. We are also indebted to Dr. Felipe Jiménez de Asúa, Centro de Investigaciones Oncológicas “Fundación Iturral,” Buenos Aires, for the animal supply, and to Dr. Federico M. Guitar for performing the spectrographic analyses.

REFERENCES

2. Note Added in Proof—After this manuscript had been submitted, the authors became aware of a paper by Tanaka, Harano, Sue, and Morimura (16) on the crystallization and characterization of two types of pyruvate kinase from rat tissues (liver and muscle). While the results obtained by these authors are essentially similar to those presented in this paper, some discrepancies may be noted. Tanaka et al. (16) found that ADP inhibits the I isoenzyme at concentrations higher than 0.5 mM. A similar effect has been obtained in this laboratory with some batches of commercial ADP, but it has been found to be caused by Cu++ contamination of this compound.

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