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ALLOSTERIC PROPERTIES OF THE ISOENZYMES OF PYRUVATE KINASE FROM RAT KIDNEY CORTEX*

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SUMMARY

The pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40) isoenzymes from kidney cortex have been studied further. Pyruvate kinase type I which is the major component, shows homotropic cooperativity towards the substrate phosphoenol pyruvate, the effect is independent of pH. This isoenzyme is markedly inhibited by alanine and other amino acids but its activity is not modified by ATP or fructose 1,6-diphosphate. The kinetic behavior of this isoenzyme is then clearly different from that of the other pyruvate kinases from rat tissues.

Pyruvate kinase type II exhibits sigmoid kinetics with respect to phosphoenolpyruvate. The enzyme is activated by fructose 1,6-diphosphate and inhibited by ATP, alanine and cysteine. The allosteric properties of the enzyme are strongly affected by changes in the pH. It is concluded that this kidney cortex isoenzyme is very similar to the pyruvate kinase type L from liver.

INTRODUCTION

In a preliminary note we have reported the occurrence of two different forms of pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40) (types I and II) in rat kidney cortex[1]. Both isoenzymes could be resolved on DEAE-cellulose chromatography. Pyruvate kinase I was found to be the major component; it showed a kinetic behavior somewhat different from other pyruvate kinases of mammals (see refs 2 and 3).

The present paper reports further studies on the kinetic properties of both types of kidney cortex pyruvate kinase. The results presented here reinforce our pre-

Abbreviation: PEP, phosphoenolpyruvate.

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vious conclusion that the minor component (pyruvate kinase II) resembles very closely isoenzyme L of liver. Furthermore pyruvate kinase I has different kinetic properties as compared to both liver isoenzymes (L and M), hence it could belong to a third group of pyruvate kinases present in mammalian tissues.

MATERIALS AND METHODS

The enzymes were isolated from rat kidney cortex as already described[1]. The homogenate was centrifuged at 100 000 × *g* for 60 min. The clear supernatant fluid was purified by $(NH_4)_2SO_4$ fractionation between 30–45% (pyruvate kinase II) and 55–70% (pyruvate kinase I) saturation. Both isoenzymes were further purified by chromatography on DEAE- and CM-cellulose columns, respectively, as previously described for liver pyruvate kinases[4,5]. Pyruvate kinase I and pyruvate kinase II were purified in the last steps 20 and 15 times, respectively. Other materials and methods were as previously described[4].

RESULTS

The fractionation of both types of pyruvate kinase found in the 100 000 × *g* kidney cortex supernatant can be accomplished by chromatography on a DEAE-cellulose column. The major component (pyruvate kinase I) is eluted from the column with the equilibrating buffer (15 mM Tris–HCl, 250 mM sucrose, 1 mM EDTA and 1 mM dithiothreitol, adjusted to pH 7.5). It represents 70–80% of the total enzymic activity. The column was washed with the above-mentioned buffer and the other isoenzyme (pyruvate kinase II) was eluted with the same solution to which a linear gradient of KCl was superimposed. This second peak is eluted at 120 mM KCl. When the fractions obtained from this column were assayed at a low level of phosphoenolpyruvate (PEP) (0.8 mM) and in the presence or absence of fructose diphosphate (0.1 mM), only the second peak was activated by this metabolite.

Furthermore both activities are also separated by $(NH_4)_2SO_4$ fractionation (see Methods).

The elution profile of 100 000 × *g* kidney medulla supernatant shows only one peak of pyruvate kinase activity, which is not adsorbed to the column. When all the fractions were tested in the presence of fructose diphosphate (at high or low level of PEP) this enzyme activity was not stimulated by the metabolite.

Effect of PEP concentration on pyruvate kinase I and pyruvate kinase II activity

Fig. 1A shows plots of initial velocity of pyruvate kinase II against PEP concentrations in the absence or in the presence of ATP or both, ATP and fructose diphosphate. It can be observed that the enzyme exhibits a marked cooperative effect towards PEP. These substrate interactions become more pronounced in the presence of ATP. In fact the slopes of the Hill plot increased from 2.9 to 3.4, respectively (see inset of the same figure). Conversely, when 0.1 mM fructose diphosphate is added simultaneously with the inhibitor, the response to PEP concentration is transformed into a hyperbolic curve. A similar experiment performed with pyruvate kinase I shows that the enzyme also displays sigmoid kinetics with respect to PEP (Fig. 1B). However in contrast to the effects observed with the other isoenzyme (pyruvate

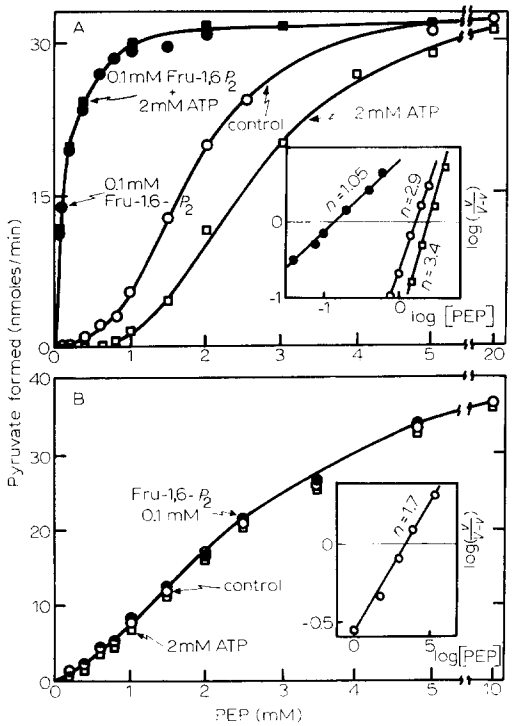

Fig. 1. A. Effect of PEP concentration on the reaction rate of pyruvate kinase II activity in the absence or in the presence of 2.0 mM ATP or 0.1 mM fructose 1 6-diphosphate. The incubation mixture was as follows: 100 mM Tris–maleate–tetramethylammonium hydroxide buffer, pH 7.5, 10 mM $MgCl_2$, 100 mM KCl, 2.5 mM ADP, 0.2 mM NADH, excess of commercial lactate dehydrogenase and different concentrations of PEP as indicated in the figure. The incubation was carried out at 30 °C. B. Influence of PEP concentration on the activity of pyruvate kinase I in the absence or presence of 2.0 mM ATP or 0.1 mM fructose 1,6-diphosphate, respectively.

kinase II), ATP and fructose diphosphate do not modify the kinetic behavior of pyruvate kinase I.

It has been found (see below) that several amino acids are inhibitors of both type of pyruvate kinase activities. Alanine was chosen as model effector for the following experiment. The effect of alanine or both alanine and fructose diphosphate on the PEP saturation curve of pyruvate kinase II and pyruvate kinase I is shown in Fig. 2. In the presence of this amino acid, the substrate rate relationships for pyruvate kinase II became more sigmoidal and the $S_{0.5}$ values for PEP increased. However, while fructose diphosphate reverses the alanine inhibition of pyruvate kinase II, this metabolite has no effect on the activity of the other isoenzyme.

Effect of different metabolites on pyruvate kinase I and pyruvate kinase II activity

Various amino acids were tested for their effect on both types of pyruvate kinase activities from kidney cortex. The levels of PEP used were near the K_m values for each enzyme. The data summarized in Table I show that the behavior of both isoenzymes against the amino acids is quite different. Pyruvate I kinase is inhibited by many amino acids. The other isoenzyme (pyruvate kinase II) is markedly inhibited

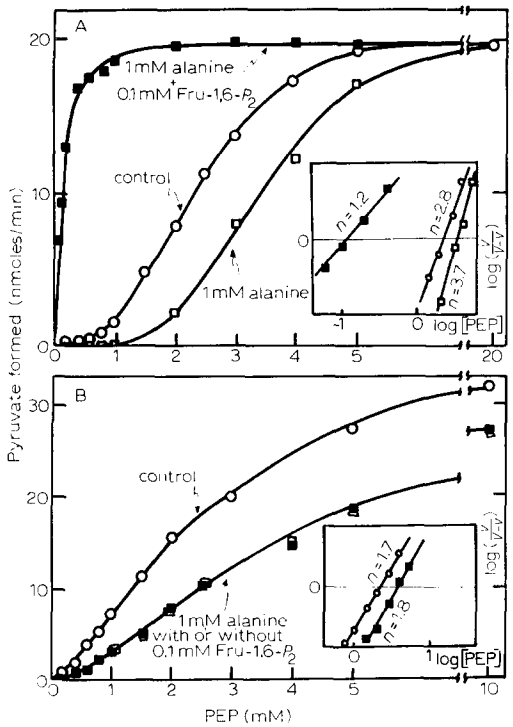

Fig. 2. A. Effect of PEP concentration on the reaction rate of pyruvate kinase II activity in the absence or in the presence of alanine and/or fructose 1,6-diphosphate as indicated on the curves. B. Influence of PEP on the activity of pyruvate kinase I in the absence or in the presence of alanine and/or fructose 1,6-diphosphate as indicated on the curves. Assay conditions in A and B were similar to those described in Fig. 1.

by alanine and cysteine and somewhat less by proline and valine. In this respect pyruvate kinase I and pyruvate kinase II are similar to liver M and L pyruvate kinases, respectively[3–6].

Due to the fact that pyruvate kinase I is inhibited by many amino acids, that its affinity for PEP is very low and that fructose diphosphate does not have any stimulatory effect, a number of other metabolites were tested to see whether they would modify its activity. The following compounds at a concentration of 5 mM had no significant effect: glucose 6-phosphate, 3-phosphoglyceric acid, fructose 6-phosphate, ribose 5-phosphate, carbamyl phosphate, phosphocreatine, glucose 1-phosphate, inorganic phosphate, fumarate, citrate, malate, succinate, α-glycerophosphate and β-glycerophosphate. The effect of these metabolites was tested at low or high level of PEP (0.5 and 2.5 mM) and with or without 1 mM phenylalanine.

The addition of Ca^{2+} at 2.5 or 5.0 mM concentration produces a marked decrease in the activity of both types of kidney cortex pyruvate kinase. This inhibition is partially reversed by raising the Mg^{2+} level as previously described for other pyruvate kinases. Furthermore in the presence of 2.5 mM Ca^{2+}, fructose diphosphate does not produce any stimulation in pyruvate kinase II activity even at 10 mM Mg^{2+}.

TABLE I

EFFECT OF DIFFERENT AMINO ACIDS ON THE ACTIVITY OF KIDNEY CORTEX PYRUVATE KINASE I AND PYRUVATE KINASE II

Experimental conditions were as described in Fig. 1 except for PEP concentration which was 2.5 and 1.7 mM in the assay of pyruvate kinase I and pyruvate kinase II, respectively. The figures represent percentage of enzyme activity.

Additions	*Concn (mM)*	*Pyruvate kinase I*	*Pyruvate kinase II**
Alanine	0.5	54	62.4
Alanine	2	21	31.8
Phenylalanine	0.5	32	—
Phenylalanine	2	10	85
Cysteine	5	55	12
Proline	5	38	38.5
Tryptophan	2.5	39	99
Isoleucine	5	41	94
Valine	2.5	44	63
Threonine	5	48	95
Tyrosine	2.5	84	95
Glycine	5	87	97.5
Histidine	5	73	94.5
Leucine	5	86	100
Glutamate	5	100	103
Aspartate	5	101	95
Serine	5	99	89

* The initial velocities in the absence of amino acids were 18 and 30 nmoles of pyruvate formed per min for pyruvate kinase I and pyruvate kinase II respectively.

Effect of pH on kinetic behavior of pyruvate kinase I and pyruvate kinase II

It has been demonstrated that the homotropic cooperative effect of isoenzyme L from liver is strongly dependent on the pH of the medium[4,7]. Preliminary experiments had shown that fructose diphosphate did not activate any of the isoenzymes of kidney cortex at acidic pH values and that in the alkaline range it produced a strong stimulation only on pyruvate kinase II activity[1]; for instance at pH 8.2 there was a 20-fold increase at 1 mM PEP. Therefore, it was of interest to study the kinetics of both isoenzymes at different pH values. It can be observed in Table II that the change in pH from 7.5 to 6.8 produces a marked effect on the substrate cooperative interactions of pyruvate kinase II. In contrast, the Hill-coefficient values for the other isoenzyme were practically unchanged in this pH range.

TABLE II

EFFECT OF pH ON THE PEP SATURATION CURVE OF PYRUVATE KINASE I AND PYRUVATE KINASE II

Assay conditions were as described in Fig. 1 except that the pH values of the incubation mixture were as indicated in the table.

pH	*Pyruvate kinase I*		*Pyruvate kinase II*	
	$S_{0.5}$ *(mM)*	n_H	$S_{0.5}$ *(mM)*	n_H
6.8	1.35	1.9	0.9	1.2
7.3	—	—	1.4	1.9
7.5	2.2	1.8	1.7	3.0

The effect of ATP on the activity of both types of pyruvate kinase at different pH values is shown in Fig. 3A. Two relevant features of these experiments should be pointed out. One is that pyruvate kinase I is not inhibited by ATP at the pH tested even at 5 mM (this result is not shown in the figure). The second feature is that this metabolite inhibits pyruvate kinase II activity at both pH values, but in the acid range cooperative effect is clearly observed ($n_H = 2$). In contrast, at pH 7.3 the ATP inhibition follows the Michaelis–Menten kinetics. Since it has been found (Table II) that the $S_{0.5}$ for PEP is dependent on pH, the concentration of PEP used was near the $S_{0.5}$ value at each particular pH. In Fig. 3B the percentage of pyruvate kinase II activity has been plotted against the inhibitor concentration at two different levels of PEP. At low substrate no cooperative effect of ATP was detectable. On the other hand at higher substrate levels, such as 4 mM, sigmoidal kinetics towards the inhibitor is clearly observed.

When alanine was used as inhibitor of pyruvate kinase II instead of ATP, the results were fairly similar. For example, the Hill coefficient value for this amino acid increases from 1.4 at pH 7.3 to 2.1 at pH 6.8.

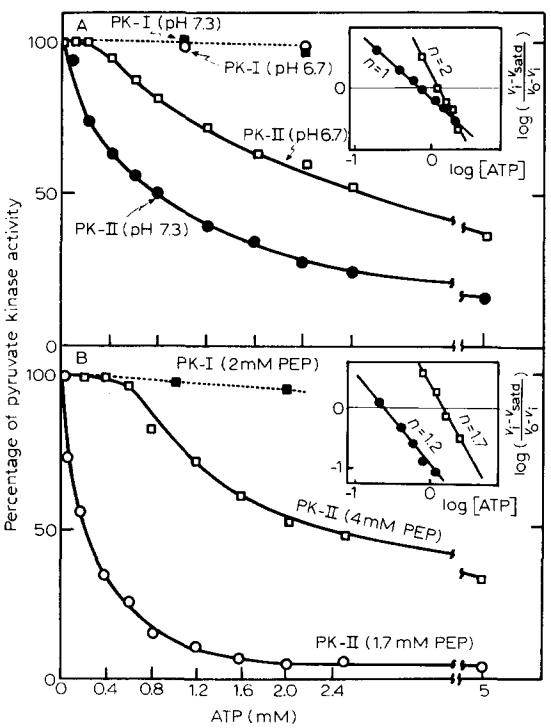

Fig. 3. A. Effect of ATP concentration on the reaction rate of both types of pyruvate kinase activity (PK-I and PK-II) at two different pH values as indicated. The experimental conditions were similar to those described in Fig. 1 except for the concentration of ADP (0.6 mM), the level of PEP (which was different in each experiment) and the pH values. In the assay of PK-I the concentration of PEP was 1.4 and 1.7 mM at pH 6.8 and 7.3, respectively. In the assay of pyruvate kinase II it was 0.9 and 1.4 mM at pH 6.8 and 7.3, respectively. B. Variation of pyruvate kinase I and pyruvate kinase II activities as a function of ATP concentration at different levels of PEP. Experimental conditions were as in A except for the pH value (7.5) and the concentration of PEP as indicated on the curves. Insets, Hill plots of the same data calculated as described elsewhere[7].

Effect of K^+ on pyruvate II kinase activity

Since the results presented here suggested that pyruvate kinase II has a kinetic behavior very similar to that of the isoenzyme L[4,7] from liver the properties of this kidney enzyme were further studied.

The kinetics of K^+ activation at 1.7 mM of PEP was studied in the presence or absence of fructose diphosphate (Fig. 4A). The enzyme shows a marked cooperative effect toward this cation. In the presence of fructose diphosphate or at high concentration of PEP (10 mM) the sigmoidal dependence on K^+ is transformed into a hyperbolic curve (see also Fig. 4B).

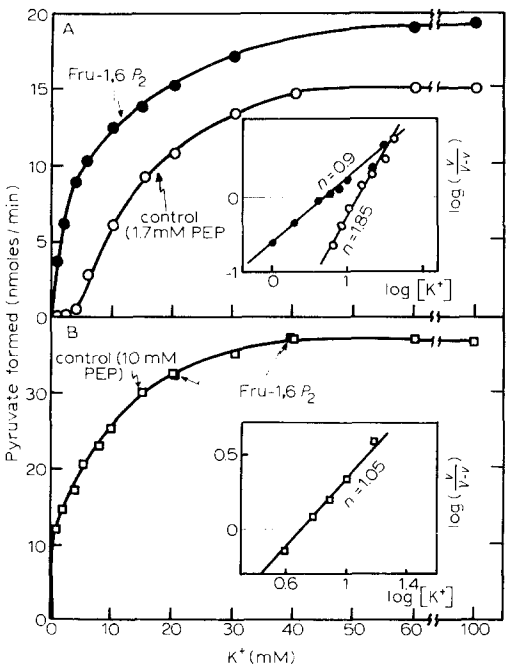

Fig. 4. Effect of K^+ concentration on the reaction rate of pyruvate kinase II at two different levels of PEP in the absence or in the presence of 0.1 mM of fructose 1,6-diphosphate. In Expts A and B, the concentration of PEP was 1.7 and 10 mM, respectively. Other experimental concentrations were similar to those described in Fig. 1 except that the K^+ concentration was varied as indicated.

DISCUSSION

Previous reports[1,8–13] have shown that liver and kidney cortex, the main gluconeogenic organs in mammals, contain at least two forms of pyruvate kinase which can be separated by electrophoresis in different systems. In our laboratory it has been found that the two different forms of pyruvate kinase from kidney cortex (pyruvate kinase I and pyruvate kinase II) can be fractionated by $(NH_4)_2SO_4$ precipitation and ion-exchange chromatography[1]. In the course of the purification by these methods, the behavior of pyruvate kinase I and pyruvate kinase II was shown to be similar to that of liver isoenzymes M and L, respectively[4,5]. The results described in this paper confirm our previous findings that pyruvate kinase I shares

properties of two hepatic isoenzymes[1]. The kinetics of enzyme activity as a function of PEP concentration has been found to exhibit a sigmoidal dependence, like the L isoenzyme from liver. It should be mentioned that these studies were performed with partially purified preparations of pyruvate kinase I free of enolase (EC 4.2.1.11), which indicates that the cooperative effect found is not an artifact. In contrast to the properties of the hepatic pyruvate kinase type L[7], the kinetic behavior of kidney isoenzyme (pyruvate kinase I) does not depend on pH and its activity is not affected by fructose diphosphate or ATP. In addition it is inhibited by several amino acids like the M isoenzyme from liver[5].

Alanine, chosen as a model inhibitor of pyruvate kinase I, slightly increases the homotropic cooperative effect of PEP and the inhibition is not counteracted by fructose diphosphate.

According to the chromatographic separation, pyruvate kinase I represents about 70–80% of the total pyruvate kinase activity of the kidney cortex. This fact would indicate that this isoenzyme is more directly related to the metabolic control of glycolysis and gluconeogenesis in this tissue. However the physiological significance of pyruvate kinase I is not clear due to the fact that $S_{0.5}$ value for PEP is high in comparison to the intracellular concentration of this metabolite[14,15]. Furthermore fructose diphosphate, which is a powerful activator of other pyruvate kinases[3,11,16–27] has no stimulatory effect on pyruvate kinase I activity.

These facts raise important questions regarding the function of this isoenzyme. On the other hand the presence in the cell of another compound which would enhance the affinity of this isoenzyme for PEP cannot be discarded. It should be mentioned that the addition of a boiled crude extract of kidney cortex to a partially purified preparation of pyruvate kinase I, at two levels of PEP (0.5 mM and 2.5 mM) does not increase its activity.

Preliminary experiments on a hypothetic interconversion between a form of pyruvate kinase I with a low affinity for PEP to a more active form have given negative results so far.

Previous work carried out in this laboratory indicates that the other isoenzyme from kidney cortex (pyruvate kinase II) is similar to Type L pyruvate kinase, which has been considered characteristic of liver. In the present paper we study this kidney isoenzyme in more detail and the following experimental facts reinforce the above conclusion: (1) The enzyme displays sigmoidal kinetics with respect to PEP; (2) ATP, alanine and fructose diphosphate have marked heterotropic effects on the homotropic cooperativity of the substrate; (3) the kinetic behavior of the enzyme towards PEP and the allosteric effectors is strongly dependent on pH within the physiological range; (4) at pH 7.5 there is a sigmoidal velocity response towards K^+. In the presence of fructose diphosphate or at saturating levels of PEP, the K^+ concentration graph gives a Michaelian curve.

The results presented in this paper are in agreement with those previously reported by Llorente *et al.*[21] if it is considered that pyruvate kinase II, the activity of which is modulated by ATP and fructose diphosphate, only represents a small fraction of the total kidney cortex pyruvate kinase.

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