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SOME KINETIC DIFFERENCES BETWEEN THE M ISOENZYMES OF PYRUVATE KINASE FROM LIVER AND MUSCLE

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SUMMARY

The kinetic properties of type M pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40) from rat liver have been studied. Similarly to the muscle enzyme, the plots of initial velocities *versus* phosphoenolpyruvate and ADP concentrations give hyperbolic curves. In contrast, the behavior of the liver enzyme toward amino acids differs from that of muscle.

In a previous paper it has been found that the muscle enzyme is inhibited only by phenylalanine. This inhibition is pH dependent and is reversed by cysteine, serine or alanine. The liver enzyme is inhibited not only by phenylalanine but also by alanine, tryptophan, valine, tyrosine, proline and threonine. The inhibition by phenylalanine and alanine of the liver pyruvate kinase Type M is of the mixed type with respect to phosphoenol pyruvate and is only slightly dependent on pH.

Thus on the basis of kinetic properties, at least two forms of pyruvate kinase Type M can be distinguished in rat tissues.

INTRODUCTION

Recent reports[1–3] have shown that there are at least two forms of pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40) in liver, designated Type M and Type L. The level of the latter is under hormonal and dietary regulation and its activity is allosterically modulated by phosphoenolpyruvate, K^+, fructose-1,6-diphosphate, ATP, alanine and pH[3–13]. Although the hepatic M isoenzyme is immunologically indistinguishable from the muscle enzyme[3], they have different electrophoretic mobilities[6,14]. Nevertheless it is still uncertain whether these two pyruvate kinases have similar kinetic properties[3]. Recently, it has been found that the pyruvate kinase from muscle exhibits kinetic properties of allosteric type[15]. The enzyme displays homotropic cooperative effect with respect to the inhibitor phenylalanine, and the inhibition is reversed by cysteine, serine and alanine. Therefore it was of interest to investigate whether the M isoenzyme from liver has similar kinetic properties.

This paper presents evidences showing that both isoenzymes have different kinetic behavior toward several amino acids.

MATERIALS AND METHODS

Pyruvate kinase type M was isolated from liver of Wistar rats maintained on a laboratory diet and water *ad libitum*. The tissue was homogenized with 1.5 vol. of cold 25 mM Tris–HCl–1 mM EDTA–250 mM sucrose (pH 7.5), in a glass homogenizer. The extract (130 ml) was centrifuged at 100 000 × *g* for 60 min. The supernatant was purified by $(NH_4)_2SO_4$ fractionation between 55 and 70% saturation. The precipitate was dissolved in a small volume of 10 mM Tris–maleate–1 mM EDTA–1 mM dithiothreitol (pH 6.0), and passed through a Sephadex G-25 column previously washed with the same buffer mixture. This preparation (200 mg of protein) was applied to a column of CM-cellulose (18 mm × 200 mm) treated as previously described[16] and equilibrated with the above-mentioned mixture. The column was then washed with 100 ml of the equilibrating buffer, and the enzyme was eluted with the same solution to which a linear gradient of KCl (0–160 mM) was superimposed. Type M pyruvate kinase came out at about 70 mM salt concentration. The last purification step led to a great loss of activity. However, a better recovery was obtained when the column was developed very rapidly. The flow used in most of the chromatographic runs was adjusted to 6–8 ml/min. The preparations obtained were free of enolase, pyruvate kinase Type L, and NADH oxidase activity. Since the CM-cellulose enzyme was highly susceptible to inactivation a fresh preparation was used in most of the experiments. The purification was about 8–10-fold. Similar results were obtained when the starting material was perfused liver. Pyruvate kinase and enolase activities were measured as reported elsewhere[17]. Other methods and materials were as previously described[9].

RESULTS

In order to test whether the kinetic properties of both M enzymes toward substrates were similar, the K_m values for phosphoenolpyruvate and ADP were determined for the liver enzyme. Fig. 1 shows double reciprocal plots of initial velocity *versus* phosphoenolpyruvate concentration with ADP as the non-variable substrate. At different levels of ADP, the relationship between $1/v$ and the reciprocal of the phosphoenolpyruvate concentration yielded a family of lines which have a common intersection on the substrate axis. The same pattern (Fig. 1B) was obtained at different concentrations of phosphoenolpyruvate when ADP was the variable substrate. The Michaelis constant values determined by these plots were 0.18 and 0.7 mM for phosphoenolpyruvate and ADP, respectively (Fig. 1A and B). These patterns of initial velocities are similar to those reported for the muscle enzyme[18]. They are consistent with a sequential mechanism in which the substrates are added to the enzyme in a rapid random reaction[19].

A number of other metabolites were tested to see whether they would modify the enzyme activity. The following compounds at a concentration of 1 to 5 mM had no significant effect: glucose 1-phosphate, glucose 6-phosphate, fructose-1,6-diphosphate, dihydroxyacetone phosphate, 3-phosphoglycerate, AMP, cyclic 3′,5′-AMP,

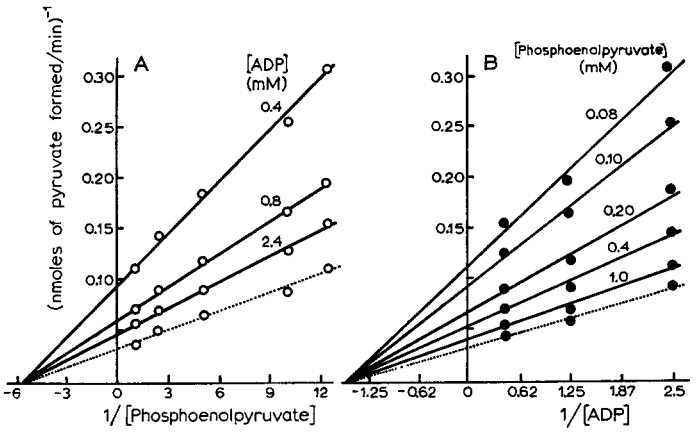

Fig. 1A. Effect of phosphoenolpyruvate concentration on the activity of the liver pyruvate kinase (Type M) at different levels of ADP. B. Effect of ADP concentration on the enzyme activity at different levels of phosphoenolpyruvate. The CM-cellulose enzyme was incubated at 30° with 100 mM TES–TMA (*N*-tris (hydroxymethyl)methyl-2-aminoethanesulfonic acid–tetramethyl ammonium hydroxide) buffer, pH 7.5, 100 mM KCl, 8 mM $MgCl_2$, 0.2 mM NADH and excess of commercial lactate dehydrogenase (Sigma). The concentrations of phosphoenolpyruvate and ADP were varied as indicated. The final volume was 0.5 ml. Dotted lines represent the reciprocal of the apparent maximal velocity (ordenate intercept) as a function of the non-variable substrate concentration.

UMP, UDP-glucose and P_i. On the other hand, ATP, GTP, UTP and citrate tested at 5 mM inhibited 40–60% the enzyme activity. The data presented in Table I indicate that the effect of citrate and ATP is largely due to the formation of complexes with Mg^{2+}, since the inhibitions were almost overcome by raising the metal ion concentration. Thus, the behavior of liver type M pyruvate kinase with respect to ATP is very similar to that observed for the muscle enzyme[20,21]. Table I also shows that low concentrations of Ca^{2+} produced a marked decrease in enzyme activity and that this inhibition is rather competitive with respect to Mg^{2+}, as it was

TABLE I

EFFECT OF Ca^{2+}, ATP AND CITRATE ON LIVER PYRUVATE KINASE (TYPE M) ACTIVITY AT DIFFERENT LEVELS OF Mg^{2+}

Experimental conditions as described in Fig. 1 except for ADP and phosphoenolpyruvate concentrations which were 0.5 mM and 0.15 mM, respectively. Mg^{2+} was varied as indicated. This cation and Ca^{2+} were added as chloride salts. The figures represent percentage of enzyme activity.

Mg^{2+} (mM)	*Additions*						
	None	*Ca^{2+}*		*ATP*		*Citrate*	
		1 mM	*5 mM*	*1 mM*	*5 mM*	*1 mM*	*5 mM*
0.5	100 (5.2)*	42	4	48	2	70	6
2.5	100 (13.2)	58	31	84	20	92	62
10	100 (13.4)	95	55	97	78	96	100

* Figures in brackets represent the initial velocities in the absence of additions expressed as mμmoles of pyruvate formed per min.

TABLE II

EFFECT OF DIFFERENT AMINO ACIDS ON THE ACTIVITY OF THE M ISOENZYME OF PYRUVATE KINASE FROM MUSCLE AND LIVER

Experimental conditions were as described in Fig. 1 except for phosphoenolpyruvate and ADP concentrations which were 0.15 mM and 1.5 mM, respectively. In the assay of muscle enzyme, the phosphoenolpyruvate concentration was 0.1 mM. The figures represent percentage of enzyme activity Glycine, aspartate and arginine at 5.0 mM had no significant effect.

Additions	*Concentration (mM)*	*Liver* type M*	*Muscle* type M*
None		100	100
Phenylalanine	1	41	95
Phenylalanine	5	8	21
Alanine	1	53	100
Alanine	5	45	103
Valine	5	56	100
Threonine	5	53	98
Proline	5	50	102
Tyrosine	2.5	49	103
Tryptophan	5	31	94
Glutamate	5	73	76

* The initial velocities in the absence of amino acids were 8.0 and 33.0 nmoles of pyruvate formed per min for the liver and muscle type M enzyme, respectively.

previously described for other pyruvate kinases[22]. The Ca^{2+} effect is interesting since it has been observed that phosphofructokinase and hexokinase are also inhibited by this cation[23,24].

Various amino acids were tested for their effect on M type pyruvate kinase from liver. The results depicted in Table II also include, for comparison, the effect of the same compounds on the muscle enzyme. The levels of phosphoenolpyruvate used were near the K_m values for each enzyme. Two relevant features of these data should be pointed out. One is that both isoenzymes are differentially inhibited by some amino acids. Alanine, threonine, valine, proline, tyrosine and tryptophan markedly decrease the activity of the hepatic enzyme. In contrast, these effects were not observed with the muscle pyruvate kinase. The second feature is that the only amino acid which showed a marked effect on both enzymes is phenylalanine. However, a greater response was obtained with the liver enzyme when low concentrations of this compound were used. Furthermore, other amino acids tested on both isoenzymes had no significant effect. For example serine and cysteine, which are structurally related to alanine, do not inhibit appreciably the enzyme from liver. It should be mentioned that VIJAYVARIGIYA *et al.*[25] have recently reported that phenylalanine inhibits the pyruvate kinase from muscle.

The results of Table II suggest that at least two forms of pyruvate kinase Type M exist in rat tissues, one which is inhibited by several amino acids and the other which is unaffected by them. In order to investigate whether there are other differences between both M type isoenzymes (muscle and liver), the inhibition by amino acids on the latter was further studied.

The effect of alanine or phenylalanine on the phosphoenolpyruvate saturation curve for the liver M isoenzyme, is shown in Fig. 2. Inverse plots of the rate as a

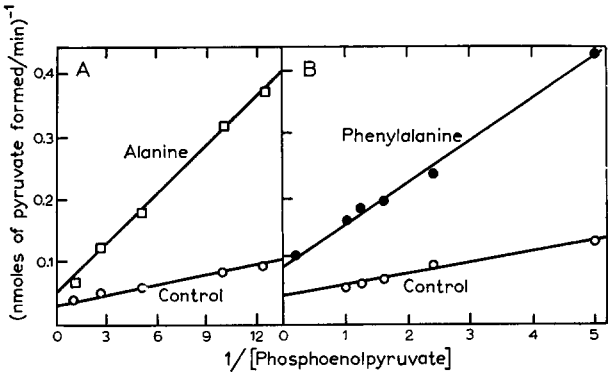

Fig. 2A and B. Effect of phosphoenolpyruvate concentration on the activity of the liver pyruvate kinase (Type M) in the absence and in the presence of alanine (1.5 mM) or phenylalanine (2.0 mM) respectively, as indicated in the figure. Assay conditions were as described in Fig. 1 except that the concentration of ADP was 1.5 mM.

function of substrate concentration give straight lines, both in the absence or in the presence of the inhibitors, thus showing Michaelis–Menten kinetics. In the presence of alanine or phenylalanine, the apparent affinity for phosphoenolpyruvate and the v_{max} decrease.

Curves of enzyme activity as a function of alanine or phenylalanine are given in Fig. 3. It can be seen that the shape of the inhibition curves is hyperbolic. The concentrations of alanine and phenylalanine which give half maximal inhibition were 0.06 mM and 0.11 mM, respectively. It should be mentioned that under similar assay conditions, the muscle pyruvate kinase clearly exhibits a homotropic co-operative effect toward phenylalanine, and the $I_{0.5}$ value is about one order of magnitude greater than that obtained for the liver enzyme[26]. Furthermore, the inhibition curve by alanine reaches a plateau at 1 mM and the enzyme activity is not further decreased, even at 10 mM of this metabolite. The partial nature of the alanine inhibition suggests that it is not competing with the substrates for a common binding site.

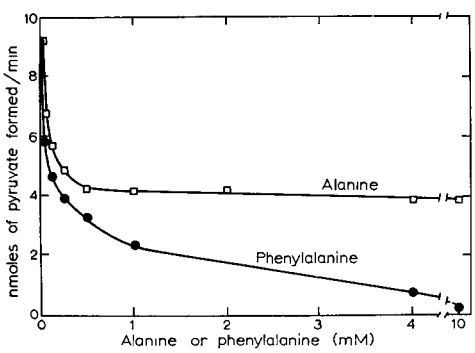

Fig. 3. Effect of alanine and phenylalanine concentration on the reaction rate of liver pyruvate kinase (Type M). Assay conditions were as described in Table II.

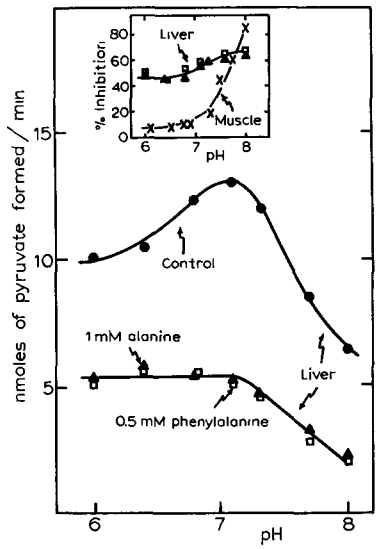

Fig. 4. Effect of pH on the activity of the liver pyruvate kinase (Type M) in the absence and in the presence of alanine or phenylalanine. MES–TMA (2-(*N*-morpholino)ethanesulfonic acid–tetramethyl ammonium hydroxide) and TES–TMA (*N*-tris(hydroxymethyl)methyl-2-amino-ethanesulfonic acid–tetramethyl ammonium hydroxyde) were used at 100 mM as buffers the former for determinations in the acid range and the other for pH values above 7. All other conditions were as described in Table II. The addition of alanine or phenylalanine was indicated on the graph. Inset: Percentage of inhibition of the M isoenzyme from liver and muscle by amino acids at different pH values. Effect of phenylalanine on the muscle enzyme (×—×). Effect of alanine (▲—▲) or phenylalanine (□—□) on the liver enzyme.

It was previously demonstrated that the phenylalanine inhibition on the muscle pyruvate kinase is strongly dependent on pH[15,26]. Therefore it was of interest to study the effect of alanine and phenylalanine on the hepatic M isoenzyme at different pH values. The results presented in Fig. 4 show that, in this case, the inhibitions are only slightly dependent on pH. In the inset of Fig. 4, it is also presented, for com-

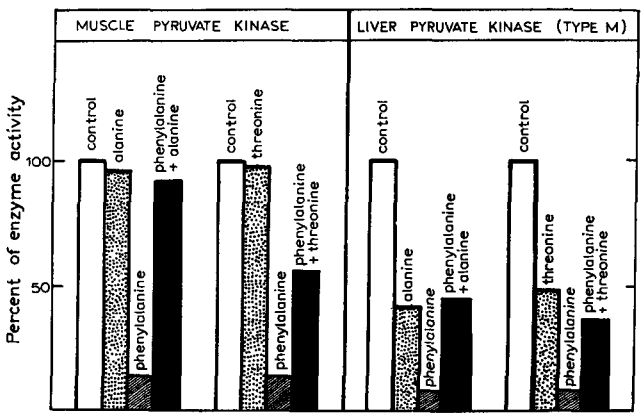

Fig. 5. Effect of alanine or threonine on the phenylalanine inhibition of the M isoenzymes from muscle and liver as indicated in the figure. Assay conditions as described in Table II.

TABLE III

EFFECT OF SERINE AND CYSTEINE ON THE INHIBITION OF THE HEPATIC PYRUVATE KINASE TYPE M BY SOME AMINO ACIDS

Assay conditions were as described in Table II. The figures represent percentage of enzyme activity.

Additions	*Concentration (mM)*	—	*2 mM serine*	*2 mM cysteine*
None		100*	103	93
Alanine	10	43	64	64
Phenylalanine	5	8	54	70
Valine	5	56	72	72
Valine	10	29	79	72
Threonine	5	54	68	65
Threonine	10	49	65	79
Proline	5	50	86	61

* The initial velocity in the absence of amino acids was 7.0 nmoles of pyruvate formed per min.

parative purposes, the effect of pH on the phenylalanine inhibition of muscle pyruvate kinase. It can be observed that the effect of this amino acid at pH values lower than 7.0 is another distinctive property between these M isoenzymes.

As it is shown in Fig. 5, alanine reverses the inhibition by phenylalanine on both enzymes. However in the case of liver enzyme, due to the fact that alanine is also an inhibitor, the activity obtained is almost equal to that observed when alanine is the only amino acid present.

Serine and cysteine which have no effect on the activity of the liver M isoenzyme (Table II) partially reverse the inhibitory effect of the amino acids listed in Table III.

DISCUSSION

It has been suggested, on a immunochemical basis, that the pyruvate kinase Type M from liver and muscle could be similar[3]. On the other hand, it has been reported that these isoenzymes can be separated by electrophoresis in different systems[2,6,14]. The results presented in this paper indicate that these enzymes exhibit different kinetic responses to several amino acids. It can be concluded that there are, at least, two different forms of the isoenzyme M in rat tissues. Although cooperative effects were not observed with the type M pyruvate kinase from liver under the experimental conditions employed, the inhibition by amino acids seems to be of allosteric nature, as is the case with other pyruvate kinases[11,15,25]. In favor of this suggestion are the following facts: (1) the inhibitory compounds are not structurally analogs of the substrates, except alanine which is not very different from phosphoenolpyruvate; (2) some amino acids, including alanine, do not inhibit completely even at high concentrations. This fact is indicative of the presence of separate binding sites for the inhibitor and substrates; (3) cysteine and serine partially reverse the inhibitions by amino acids. Furthermore, the results obtained when combinations of inhibitors were tested (see Fig. 5) can be interpreted, considering that alanine and

threonine displace phenylalanine from a common allosteric site and then produce their own inhibitory effects.

The kinetic differences observed here could represent two distinct regulatory mechanisms operating in liver and muscle. In the former tissue it is expected to have a considerably different set of metabolic controls than in muscle because it has the ability to carry out gluconeogenesis.

Although the ratio of pyruvate kinase Type L to Type M under normal conditions is about 3, with fasted or diabetic animals and with those fed on high protein diet, this ratio decreased markedly[3]. Thus, under these physiological and pathological conditions the isoenzyme M activity should not be overlooked. Therefore it is plausible to assume that the M type of pyruvate kinase is also under metabolic regulation like the L enzyme.

Although under the gluconeogenic conditions mentioned above, the hepatic levels of alanine and phenylalanine decrease from 1.23 and 0.15 to 0.46 and 0.07 moles/g of wet tissue, respectively[27,28], it is noteworthy that even these low concentrations still produce a marked inhibition of the enzyme activity. Moreover serine which partially reverses these inhibitions (Table III) experiences a large decrease in its level (from 1.65 to 0.32 mole/g of wet tissue) in diabetic animals[28].

It should be mentioned that the physiological implications drawn from kinetic studies *in vitro* are subject to a great many limitations[29]. However, it is tempting to speculate upon the possibility that the changes in the ratio of amino acid inhibitors and reactivators could modulate the activity of the isoenzyme M in the hepatic cell. In favor of this, is the fact that SCHONER *et al.*[11] have recently studied in more detail the alanine inhibition of the hepatic L isoenzyme, and they suggest that this inhibition is an important regulatory mechanism for the control of gluconeogenesis. A similar conclusion was drawn in a recent paper of LLORENTE *et al.*[12].

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