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SOME KINETIC PROPERTIES OF THE ALLOSTERIC M-TYPE PYRUVATE KINASE FROM RAT LIVER; INFLUENCE OF pH AND THE NATURE OF AMINO ACID INHIBITION

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SUMMARY

1. The influence of the pH on the activity of the M-type pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40) from rat liver has been studied. The $K_{0.5}$ for the substrate phosphoenolpyruvate was pH dependent and above pH 7.25 sigmoidal curves have been obtained. Fructose-1,6-diphosphate was able to convert these curves into a hyperbolic relationship.

2. It was found that alanine acts as an allosteric inhibitor. The inhibition could be fully abolished by the addition of fructose-1,6-diphosphate. The alanine-inhibition is dependent on the pH and on the phosphoenolpyruvate concentration.

3. It is concluded that most of the properties can be explained by the model of Monod, Wyman and Changeux.

INTRODUCTION

It is generally accepted that the mammalian glycolytic enzyme pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40) exists in two different forms[1]: L(liver) type and M(muscle) type. The L-type pyruvate kinase is present in liver, erythrocytes and kidney[2] and the enzymatic activity of this form is allosterically modulated by phosphoenolpyruvate (PEP), K^+, fructose-1,6-diphosphate (Fru-1,6-P_2), glucose-1,6-diphosphate, phosphorylated hexoses, ATP and alanine[3–7]. The M-type is present in muscle, brain, heart, liver, kidney and leucocytes[2,8]. The M-type was called a non-allosteric enzyme, as could be concluded from the Michaelis–Menten kinetics. Recently Jiménez de Asúa *et al.*[9] showed that the M-types from liver and muscle can be inhibited by amino acids. From these inhibition studies they concluded that there are at least two different forms of M-type pyruvate kinase. From our studies with M-type pyruvate kinase from leucocytes[8] it was concluded that this

Abbreviations: PEP, phosphoenolpyruvate; Fru-1,6-P_2, fructose-1,6-diphosphate.

enzyme shows cooperative interaction towards PEP in the presence of the inhibitors alanine and phenylalanine, Fru-1,6-P_2 completely reversed this inhibition and normal Michaelis–Menten kinetics were obtained. On the basis of the similarity in inhibition by amino acids of the M-type pyruvate kinase of leucocytes and the M-type of liver, it was suggested that these M-types are quite similar. However Jiménez de Asúa *et al.*[9] were not able to show cooperative interaction between the PEP binding sites in the presence of amino acids. Therefore, we studied the nature of the amino acid inhibition of this enzyme at different pH values, especially in connection with the earlier proposed R $\rightleftharpoons$ T model for the M-type pyruvate kinase[8].

For comparison with the properties of the L-type pyruvate kinase from rat liver, we also isolated L-type by $(NH_4)_2SO_4$ precipitation of the cytosol between 20 and 45% saturation. The M-type, on the other hand, is isolated in the 55–70% $(NH_4)_2SO_4$ fraction. The properties obtained with the L-type were found to be similar to the results obtained by Rozengurt *et al.*[10] (compare also Van Berkel *et al.*[11]). This makes it possible to compare our results obtained with the M-type with the properties of the L-type described by Rozengurt *et al.*[10].

MATERIALS AND METHODS

Type L pyruvate kinase was isolated from rat liver according to the isolation procedure described earlier[5]. Type M pyruvate kinase was isolated by the method of Passeron *et al.*[12], except that during this procedure 1 mM mercaptoethanol was omitted and the final $(NH_4)_2SO_4$ precipitation (between 55 and 70%) was dissolved in 0.25 M Tris–HCl of pH 7.25, 7.5, 7.75, 8.0 or 8.5, as indicated in the legends. The purification was about 10 to 12-fold. For the assay at pH 5.9 or 6.5 the enzyme was dissolved in 0.1 M Tris–maleate buffer of the corresponding pH.

Pyruvate kinase was assayed by following the decrease in absorbance at 340 nm in the coupled reaction with lactate dehydrogenase at room temperature according to Valentine and Tanaka[13]. The triethanol–HCl buffer (0.4 M, pH 7.5) was replaced by Tris–HCl buffer (0.25 M, pH 7.25, 7.5, 7.75, 8.0 or 8.5, as indicated in the figures). For the assay at pH 5.9 and 6.5 a 0.1 M Tris–maleate buffer was used.

ADP, ATP, PEP, NADH, Fru-1,6-P_2 and oxidized glutathione were obtained from Boehringer (Mannheim, Germany). L-Alanine was obtained from Merck (Darmstadt, Germany). Reduced glutathione was obtained from Sigma Chemical Co. All other reagents were of analytical grade purity.

RESULTS

Fig. 1 shows the dependence of the liver M-type pyruvate kinase on the PEP concentration at an ADP concentration of 2 mM at pH 7.5 and pH 8.0 before and after the addition of Fru-1,6-P_2. The activity at pH 7.5 shows only a slight sigmoidal response on increasing the PEP concentration ($n = 1.3$), suggesting that the cooperative interaction between the PEP binding sites at this pH, at least under these conditions, is very slight. With the addition of Fru-1,6-P_2 we only find a significant increase of the activity at very low PEP concentrations (up to 0.05 mM). Fru-1,6-P_2 lowers the n value, calculated from the Hill plot (insert Fig. 1), from 1.3 to 1.0. At pH 8.0 the relation between the activity and the PEP concentration is clearly

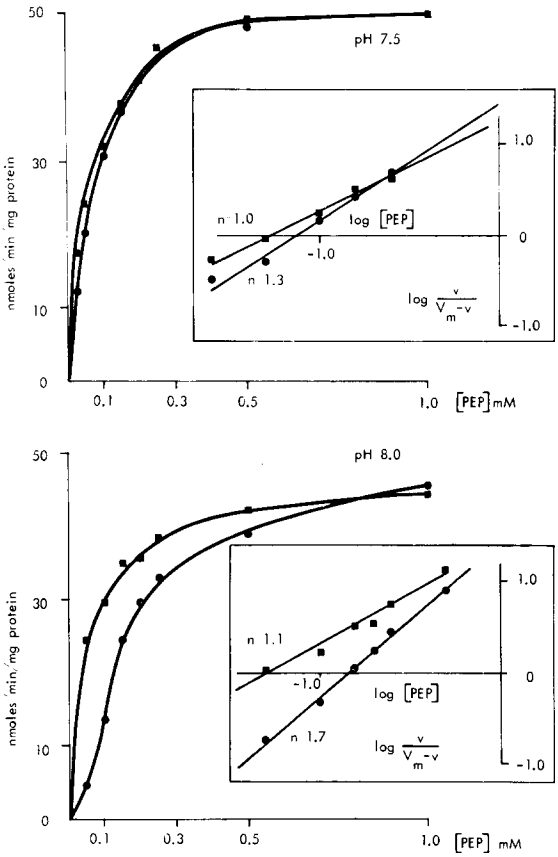

Fig. 1. M-type pyruvate kinase activity *vs* PEP concentration at pH 7.5 and pH 8.0 with 2 mM ADP. ●—●, control; ■—■, with 0.5 mM Fru-1,6-P_2. The inserts are the Hill plots of the values obtained. The calculated Hill coefficients (n) are indicated.

sigmoidal. The n value is raised from 1.3 at pH 7.5 till 1.7 at pH 8.0. Also the $K_{0.5}$ value for PEP has markedly increased from 0.06 mM at pH 7.5 to 0.14 mM at pH 8.0. At this pH Fru-1,6-P_2 markedly stimulates the enzymatic activity at low PEP concentrations and lowers the n value to $n = 1.1$, which allows the conclusion that

TABLE I

EFFECT OF pH ON THE PEP SATURATION CURVE OF THE M-TYPE PYRUVATE KINASE FROM RAT LIVER

Assay conditions as indicated in the legend to Fig. 1. n_H signifies Hill coefficient.

pH	$K_{0.5}$ *(mM)*	n_H
5.9	0.05	1.13
6.5	0.06	1.14
7.25	0.07	1.13
7.5	0.06	1.28
7.75	0.11	1.38
8.0	0.14	1.69
8.5	0.14	1.73

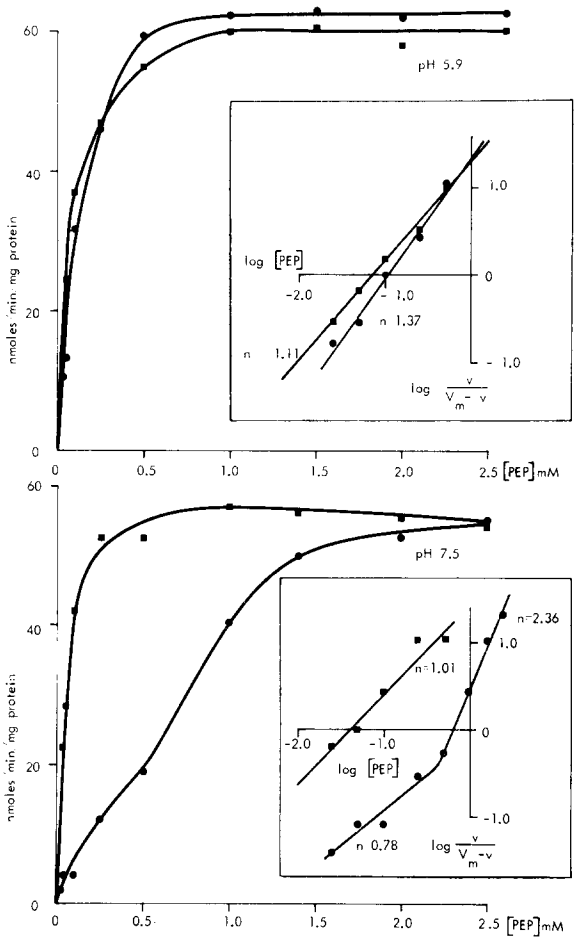

Fig. 2. M-type pyruvate kinase activity *vs* PEP concentration at pH 5.9 and pH 7.5 with 2 mM ADP. ●—●, 1 mM alanine present; ■—■, both 1 mM alanine and 0.5 mM Fru-1,6-P_2 present. The inserts are the Hill plots of the values obtained. The calculated Hill coefficients (n) are indicated.

the decrease in the $K_{0.5}$ value is accompanied by a loss of cooperative interaction between the PEP binding sites. Since alterations in the pH may play a regulatory role *in vivo*, we investigated the effect of the pH on the allosteric behaviour of the enzyme more closely. Table I shows that no cooperativity is observed when the pH is equal to or less than 7.25. However, when the pH is raised from 7.25 to 8.0 the cooperativity increases, and this is accompanied by an increase in the $K_{0.5}$ value. By plotting the p$K_{0.5}$ against the pH, according to Dixon and Webb[14], a pK_a value for the enzyme of about 7.5 is calculated. Similar calculations for the L-type pyruvate kinase from rat liver lead to a pK_a value of 6.9 (ref. 10). According to Wieker and Hess[15] this method of Dixon and Webb[14] can lead to erroneous results with allosteric enzymes, if the dissociation constants are not sufficiently separated from each other. However, this objection is not valid in this case, because both with L- and M-type only one pK_a value is found. Therefore, we can compare the pK_a value found for the M-type with the value found by Rozengurt *et al.*[10] for the L-type.

It has been reported that the alanine inhibition of the M-type pyruvate kinase from rat liver was pH independent[9]. Since the alanine inhibition of the M-type from leucocytes was found to be pH dependent[8], we investigated the influence of pH on the alanine inhibition of our preparation of the M-type from liver. Fig. 2 shows the activity as a function of the PEP concentration in the presence of 1 mM alanine before and after the addition of Fru-1,6-P_2 at pH 5.9 and pH 7.5. At pH 5.9 in the presence of 1 mM alanine the $K_{0.5}$ for PEP has hardly increased and the n value obtained is 1.37. With the addition of Fru-1,6-P_2 the n value has decreased to 1.11, suggesting that the small degree of cooperativity is lost. At pH 7.5 the alanine inhibition is more pronounced and alanine (1 mM) increases the $K_{0.5}$ value for PEP from 0.06 mM to 0.70 mM. Also at this pH Fru-1,6-P_2 counteracts the cooperativity between the PEP binding sites. At low PEP concentration (up to 0.25 mM) in the presence of 1 mM alanine a n value of 0.78 is found, which might indicate negative cooperativity[16]. This n value < 1.0 at low PEP concentration was quite reproducible. With four different preparations at pH 7.5 we found n values between 0.7 and 0.8. At higher concentrations of PEP the n value is about 2.4, which indicates a positive cooperativity. Table II summarizes the $K_{0.5}$ and n values for PEP in the

TABLE II

EFFECT OF pH ON THE PEP SATURATION CURVE OF THE M-TYPE PYRUVATE KINASE FROM RAT LIVER IN THE PRESENCE OF 1 mM ALANINE

Assay conditions as indicated in the legend to Fig. 2. n_{HI} signifies Hill coefficient at low PEP concentrations. n_{HII} signifies Hill coefficient at higher PEP concentrations (*cf.* Fig. 2).

pH	$K_{0.5}$ *(mM)*	n_{HI}	n_{HII}
5.9	0.07	—	1.37
6.5	0.15	0.89	1.89
7.25	0.72	0.80	2.56
7.5	0.68	0.78	2.36
7.75	1.65	0.55	2.45
8.0	1.70	0.53	2.60
8.5	1.76	0.59	2.58

presence of 1 mM alanine at different pH values. From this table it can be concluded that also in the presence of 1 mM alanine the $K_{0.5}$ for PEP rapidly increases between pH 7.5 and pH 8.0, suggesting that the same ionizing group as obtained in the absence of 1 mM alanine influences the activity. A further increase in pH does not alter the $K_{0.5}$ value, as is also the case when alanine is absent (*cf.* Table I).

Fig. 3 shows the influence of alanine at 0.1 mM PEP at pH 7.5 and pH 5.9. At pH 7.5 the shape of the alanine inhibition curve is hyperbolic, which is in agreement with Jiménez de Asúa *et al.*[9]. At alanine concentrations up to 2.5 mM, Fru-1,6-P_2 is able to restore the activity completely. At higher concentrations of alanine the restoration of activity is incomplete. When the reaction is carried out at pH 5.9, the plot obtained is sigmoidal. When the n value is measured according to the method of Jensen and Nester[17], a value of 2.01 is obtained. At this pH, the activity is completely restored with the addition of Fru-1,6-P_2. From this plot it can also be concluded, that alanine inhibition is pH dependent, which is at variance with the conclusion reached by Jiménez de Asúa *et al.*[9]. It appears that the pH dependence is

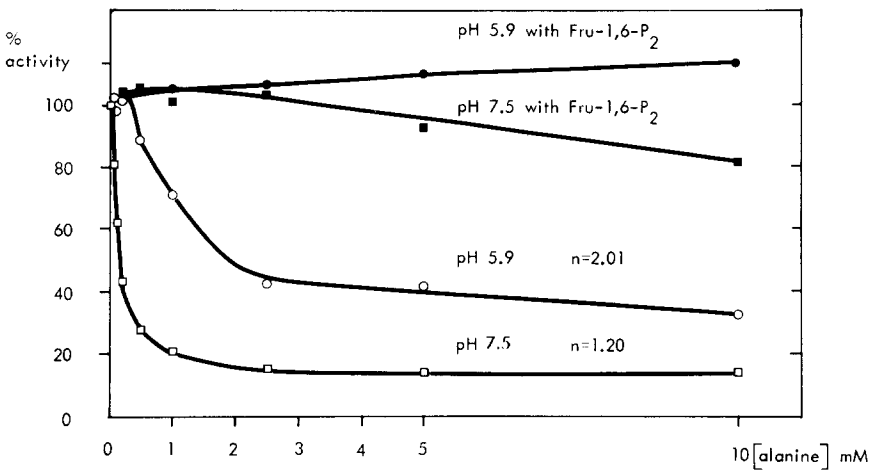

Fig. 3. The influence of alanine on the activity of liver M-type pyruvate kinase at 0.1 mM PEP 2 mM ADP and pH 5.9 or 7.5. □—□, reaction at pH 7.5; ■—■, reaction at pH 7.5 in the presence of 0.5 mM Fru-1,6-P_2; ○—○, reaction at pH 5.9; ●—●, reaction at pH 5.9 in the presence of 0.5 mM Fru-1,6-P_2. The calculated Hill coefficients (n) are indicated.

influenced by the concentration of alanine used. At 0.2 mM alanine and pH 7.5 an inhibition of 57% is found whereas 0.2 mM alanine is not inhibitory at all at pH 5.9.

In Fig. 4 the alanine inhibition curve at 1 mM PEP is shown. It can be observed that the alanine inhibition curve at 1 mM PEP and pH 7.5 is sigmoidal. A n value of 2.35 is found. When we compare this with the n value of 1.20 obtained at 0.1 mM PEP, we can conclude that there is a antagonistic interaction between the PEP-and the alanine binding sites. At pH 5.9 and 1 mM PEP alanine does not inhibit the enzymatic activity up to concentrations of 5 mM. Only at 10 mM alanine a small

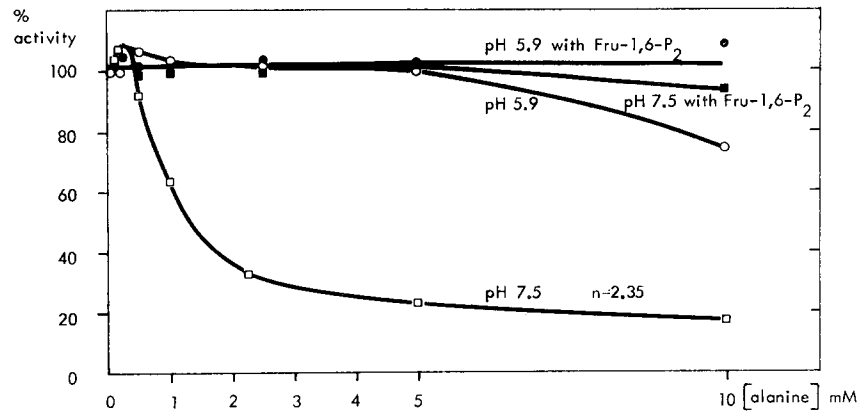

Fig. 4. The influence of alanine on the activity of liver M-type pyruvate kinase at 1.0 mM PEP, 2 mM ADP and pH 5.9 or pH 7.5. □—□, reaction at pH 7.5; ■—■, reaction at pH 7.5 in the presence of 0.5 mM Fru-1,6-P_2; ○—○, reaction at pH 5.9; ●—●, reaction at pH 5.9 in the presence of 0.5 mM Fru-1,6-P_2. The calculated Hill coefficient (n) is indicated.

inhibition can be observed. Both at pH 7.5 and pH 5.9 the restoration of activity by the addition of Fru-1,6-P_2 is complete.

These results are in contrast with the results of Imamura *et al.*[18], who found that with the highly purified enzyme there was no restoration of the activity when they added Fru-1,6-P_2 to the alanine-inhibited enzyme. From their results they concluded that the mechanism of inhibition by alanine for the L- and M-type is different. However, with the freshly isolated M-type the effect of alanine on the pyruvate kinase activity is quite similar to the results described by Schoner *et al.*[19] for the L-type. Since ATP affects the L-type pyruvate kinase activity in the same way as alanine[7,19], we also investigated the influence of pH on the ATP inhibition with the M-type. Fig. 5 shows the action of ATP at a PEP concentration of 0.1 mM and this can be compared with Fig. 3. A comparison of both figures shows that

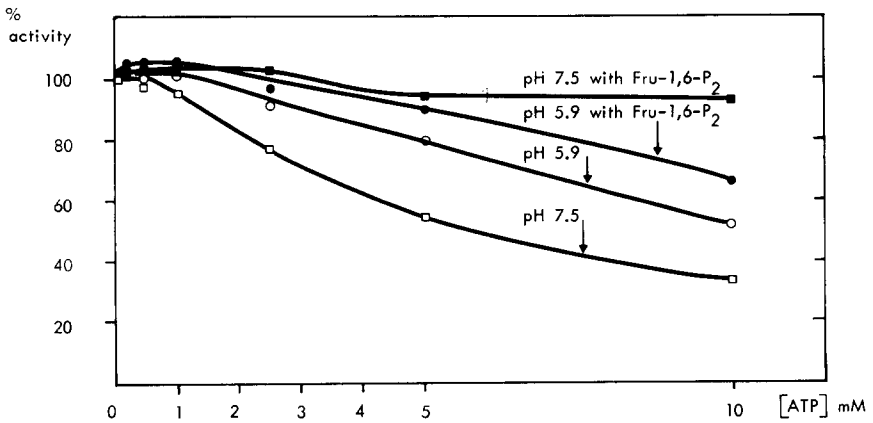

Fig. 5. The influence of ATP on the activity of liver M-type pyruvate kinase at 0.1 mM PEP, 2 mM ADP and pH 5.9 or pH 7.5. □—□, reaction at pH 7.5; ■—■, reaction at pH 7.5 in the presence of 0.5 mM Fru-1,6-P_2. ○—○, reaction at pH 5.9; ●—●, reaction at pH 5.9 in the presence of 0.5 mM Fru-1,6-P_2.

the mechanism of ATP inhibition differs from that of alanine. The ATP inhibition is slightly pH dependent and the restoration of activity by the addition of Fru-1,6-P_2 is only partial, especially at pH 5.9.

Because Imamura *et al.*[18] observed no restoration of activity with their purified M-type pyruvate kinase from rat liver when Fru-1,6-P_2 was added to the alanine-inhibited enzyme, we investigated some possibilities for these differences. As reported earlier[11], the L-type pyruvate kinase from rat liver can exist in two forms which can be reversibly interconverted by sulphydril reagents. Such an interconversion could also be important for the M-type. Therefore, the effect of oxidizing reagents on this M-type from liver was studied. By incubating the M-type at 4 °C overnight in the presence of 2.5 mM oxidized glutathione, an enzyme with the same V as the freshly prepared M-type was obtained. However, the $K_{0.5}$ for PEP is raised and the alanine inhibition is not affected by the addition of Fru-1,6-P_2 (Table III). In contrast to the results obtained with the L-type from rat liver[11], the oxidation of thiol groups is not reversible. Neither reduced glutathione, nor mercaptoethanol was able to convert this "oxidized" enzyme to the freshly prepared form. There is a possibility that our

TABLE III

COMPARISON OF KINETIC PARAMETERS OF THE FRESHLY PREPARED-AND "OXIDIZED" M-TYPE PYRUVATE KINASE FROM RAT LIVER

The assay is performed at pH 8.0; further conditions as indicated in the legend to Fig. 1.

	Freshly prepared enzyme	*"Oxidized" enzyme*
$K_{0.5}$ for PEP (mM)	0.14	0.30
K_m for PEP (mM) in the presence of 0.5 mM Fru-1,6-P_2	0.03	0.23
$K_{0.5}$ for PEP (mM) in the presence of 1 mM alanine	1.7	1.7
Reversal of alanine (1 mM) inhibition by Fru-1,6-P_2 (0.5 mM)	Complete	None

partially purified preparation of the M-type is also affected by the isolation procedure. At pH 8.0, however, it is possible to measure the $K_{0.5}$ of the M-type for PEP in a crude liver homogenate and a value of 0.15 mM has been obtained. This value agrees with the value of 0.14 mM obtained with the partially purified M-type, which suggests that our M-type preparation is identical with the enzyme present in the crude liver homogenate.

DISCUSSION

By comparing the inhibition of the pyruvate kinase activities of the M-type of leucocytes and the M-type of liver by amino acids it was concluded[8] that these M-types were similar. However, the data of Imamura *et al.*[18] suggested that there were differences. They found that, in contrast to the leucocyte enzyme, Fru-1,6-P_2 was not able to overcome the alanine inhibition. Moreover, Jiménez de Asúa *et al.*[9] were not able to show cooperative interaction between the binding sites for PEP in the presence of alanine and showed a hyperbolic inhibition curve by the amino acids alanine and phenylalanine. With the leucocyte enzyme, however, the alanine inhibition was of an allosteric nature and alanine induced cooperative interactions between the PEP binding sites[8]. Moreover, the alanine inhibition was pH dependent, whereas for the liver M-type only a slight pH dependence has been reported[9].

From the presented kinetic data, obtained with the freshly prepared enzyme, it must be concluded that at the higher pH values the M-type of liver can show cooperative interaction between the binding sites for PEP. This interaction is abolished in the presence of the positive effector Fru-1,6-P_2. The presence of 1 mM alanine can raise the $K_{0.5}$ value for PEP and also under these conditions Fru-1,6-P_2 transforms the obtained curve into a hyperbolic relationship towards PEP concentration. It is clear that the allosteric properties are markedly influenced by the pH. At a pH lower than 7.5, hyperbolic curves are obtained; Fru-1,6-P_2 does not affect the activity. An increase in the pH to 8.0 introduces cooperative interactions and the $K_{0.5}$ value for PEP increases markedly. Above pH 8.0 a further increase in pH has no further affect on the allosteric properties. These effects are qualitatively quite similar to the results obtained with the L-type. There are, however, quantitative differences: (1) The pK_a, possibly associated with the cooperative interaction for

PEP, is about 6.9 for the L-type, whereas for the M-type a value of about 7.5 can be calculated; (2) The Hill coefficient of the L-type increases from 1.0 to 3.0, when the pH is raised from 5.9 to 8.35. The increase of the n value is accompanied by an increase in $K_{0.5}$ for PEP from 0.3 to 2.3 mM. The Hill coefficient of the M-type raises only till 1.7, which means that the homotropic cooperative interaction of PEP with M-type pyruvate kinase is lower than with L-type pyruvate kinase. Also the increase in $K_{0.5}$ from the M-type for PEP varies only from 0.06 mM to 0.14 mM. This shows that the M-type has a higher affinity for PEP than the L-type.

The influence of the pH on the alanine inhibition seems to be of the same nature as that found with the L-type[19]. At pH 7.5 the inhibition is more pronounced than at pH 5.9. PEP is able to overcome the alanine inhibition and Fru-1,6-P_2 acts as an allosteric antagonist. These properties are in complete agreement with the two-state R $\rightleftharpoons$ T model of Monod *et al.*[20] applied originally to the allosteric L-type liver pyruvate kinase by Rozengurt *et al.*[10], which we have extended to the M-type pyruvate kinase by our earlier studies on the leucocyte enzyme[8]. There are, however, discrepancies as is also the case with the L-type pyruvate kinase[21,22]. One of the main discrepancies is the effect of ATP on the M-type pyruvate kinase. As has been reported earlier[9,18] the ATP inhibition can be partly reversed by Mg^{2+}. From our data we can also conclude that the ATP inhibition is different from the alanine inhibition, which can be an unique property for the M-type. The n values observed in the presence of alanine at low PEP concentrations are also difficult to interprete on the basis of the R $\rightleftharpoons$ T model at this moment. n values lower than unity have been shown earlier with yeast pyruvate kinase by Haeckel *et al.*[21], who observed n values of 0.7 at low ADP and K^+ concentrations. With the L-type from liver at low PEP concentrations such values have also been found[22]. We cannot conclude at the moment, whether these low n values are a consequence of intramolecular interactions between the binding sites for PEP (*cf.* ref. 16) or of the existence of two interconvertible forms of M-type pyruvate kinase, as has been suggested by Imamura *et al.*[18]. These two forms should then possess different affinities for alanine or PEP. However, we were not able to show such interconversions by thiol reagents, which makes it likely that, if such forms exist, the interconversion cannot be due to oxidation or reduction of the –SH groups.

The fact that the allosteric nature has not been detected earlier except by Imamura *et al.*[18], may be due to the fact that most authors[23,24] test the enzymatic activity at about pH 7.4. Indeed, at this pH it is very difficult to detect the allosteric interaction, because Fru-1,6-P_2 stimulates the enzyme only at very low PEP concentrations (less than 0.05 mM). Furthermore, high purification of the enzyme leads to a great loss of activity[9,18,23] and can lead to a modification of the enzyme. Some alteration of the thiol groups may be involved in this modification, as can be concluded from the properties of the "oxidized" enzyme. Comparison of the M-types of liver and leucocytes leads to the conclusion that these M-types are very similar. However, some differences are obvious. We do not observe a clear allosteric behaviour towards the substrate PEP with the leucocyte enzyme and in the whole pH range from 5.9 to 8.5 a $K_{0.5}$ value of 0.05 mM has been observed. Higher concentrations of alanine are needed to inhibit the leucocyte enzyme to the same extent as the M-type from rat liver and the $K_{0.5}$ value for PEP in the presence of 1 mM alanine at pH 7.5 is 0.2 mM for the leucocyte enzyme, whereas with the M-type from liver a

value of 0.68 is obtained. It is likely that these differences are due to a different allosteric constant (L).

The metabolic implication of the possibility to regulate the M-type pyruvate kinase is not obvious. In liver the M-type pyruvate kinase is located in the non-parenchymal cells[24,25] and little is known about regulation in these cell types. An important common property between non-parenchymal cells from rat liver and leucocytes is the high rate of phagocytosis of both cell types. During phagocytosis carbohydrate metabolism is highly activated[26] and allosteric stimulation of pyruvate kinase activity may be part of this activation process.

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