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SOME KINETIC PROPERTIES OF M_2-TYPE PYRUVATE KINASE FROM RAT LIVER AT PHYSIOLOGICAL Mg^{2+} CONCENTRATION

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SUMMARY

1. The influence of the free Mg^{2+} concentration on the kinetic parameters of M_2-type pyruvate kinase (ATP: pyruvate phosphotransferase, EC 2.7.1.40) from rat liver, isolated in the presence of the physiological free Mg^{2+} concentration, is investigated.

2. At 1 mM Mg^{2+}_{free} and pH 7.5 the affinity of the enzyme for the substrate phosphoenolpyruvate is decreased as compared to 10 mM Mg^{2+}_{free}, whereas the affinity for the second substrate ADP does not change. The decrease in affinity for phosphoenolpyruvate at low free Mg^{2+} concentrations appears to be the consequence of the presence of a low affinity form of the enzyme.

3. It is shown that the ATP inhibition is mainly due to Mg^{2+} binding and that it is probably not of physiological importance.

4. The enzyme possesses a high affinity for Fru-1,6-P_2 (half maximal activation at 1 μM) which is decreased by the addition of alanine. The affinity of the enzyme for Fru-1,6-P_2 at 0.1 mM phosphoenolpyruvate is not influenced by the free Mg^{2+} concentration. In the presence of alanine the affinity for Fru-1,6-P_2 is increased by increasing the phosphoenolpyruvate concentration.

5. It is concluded that at physiological concentrations of alanine, Mg^{2+}, ADP and phosphoenolpyruvate the M_2-type pyruvate kinase activity is mainly dependent upon the Fru-1,6-P_2 concentration.

INTRODUCTION

Recent data have shown that there are at least three non-interconvertible rat pyruvate kinases [1–3]. The L-type pyruvate kinase is present in liver, erythrocytes and kidney [1] and the enzymatic activity of this form is allosterically modulated by phosphoenolpyruvate, K^+, fructose 1,6-diphosphate (Fru-1,6-P_2), glucose 1,6-diphosphate (Glc-1,6-P_2), phosphorylated hexoses, ATP and alanine [4–8]. The presence of this form of pyruvate kinase in a cell seems to be obligatory for gluconeogenesis. The M_1-type is present in muscle and brain [9] and possesses fewer regulatory properties. The third type of pyruvate kinase, called M_2-type by Imamura and

Tanaka [1]*, possesses intermediate regulatory properties. The occurrence of M_2-type pyruvate kinase has now been clearly shown for adipose tissue [2, 10, 11], kidney [2, 3], leucocytes [12], liver [1, 13] and hepatomas [1, 14]. These studies have made it clear that at pH 7.5 this type of pyruvate kinase has a high affinity for the substrate *P*-enolpyruvate ($K_{0.5}$ approx. 0.05 mM) compared to the L-type. It was shown that this enzyme can exhibit cooperative interactions towards *P*-enolpyruvate especially in the presence of the allosteric inhibitors alanine and phenylalanine. Fru-1,6-P_2 completely reversed this inhibition and normal Michaelis–Menten kinetics are obtained.

Purification of M_2-type pyruvate kinase can easily modify the enzyme as has been discussed earlier [13]. For the enzyme from adipose tissue, Pogson [10] has shown that the presence of the chelator EDTA in the extraction buffer gives an enzyme with a completely different kinetic behaviour (called by Pogson Pyk-A [10]). The effect of EDTA suggests a possible important function of bivalent cations in the active enzyme. Recently it was found in studies with cultured liver cells that culturing in the absence of glucose yields a pyruvate kinase with properties similar to the Pyk-A of adipose tissue [10, 14]. Although the absence of glucose is rather unphysiological for liver cells and although it is known that cell culture may change enzyme patterns (for example the normally present L-type pyruvate kinase is replaced by M_2-type), this might indicate that the Pyk-A form of M_2-type pyruvate kinase is not only an isolation artifact. For reason that the M_2-type isolated from normal liver shows intersecting lines in the Hill plots (see ref. 13) we were interested whether this phenomenon could be related to the presence of the Pyk-A form. In this study the enzyme was isolated from normal rat liver in the presence of the physiological concentration of free Mg^{2+} (1 mM) [15], and the kinetic properties under physiological conditions were investigated.

MATERIALS AND METHODS

M_2-type pyruvate kinase was isolated as described earlier [13], except that during the isolation procedure 1 mM $MgCl_2$ was present.

Pyruvate kinase was assayed by following the decrease in absorbance at 340 nm in the coupled reaction with lactate dehydrogenase at 23 °C according to Valentine and Tanaka [16]. Duplicates were run with twice the amount of lactate dehydrogenase to exclude possible limitations by this enzyme reaction. The assay mixture contained: 25 mM Tris–HCl, pH 7.5 or 8.0, 100 mM KCl, 1 mM ADP, 0.12 mM NADH, 0.1–0.2 mg lactate dehydrogenase and *P*-enolpyruvate concentrations as indicated in the legends to the figures. For the assay at pH 5.9, 0.1 M Tris–maleate buffer was used. Routinely about 1.5 mg enzyme protein was added to start the reaction and the absorbance decrease was registered within 10 s after the addition of enzyme. The plotted values are the initial velocities and the activity was corrected for activity present in the absence of *P*-enolpyruvate or ADP. When effectors were added, a corresponding blank was measured for all the effector concentrations used.

The Mg^{2+}_{free} concentrations as indicated in the legends and in the figures were

* Originally no distinction was made between M_1- and M_2-type and both have been described as M-type, while the tissue of origin was indicated. However, as it appears that the M-type can be differentiated in two isoenzymes, we follow the classification as given by Imamura and Tanaka [1].

calculated by a method based on that of Morrison et al. [17–19] in which the following equation was used:

[total Mg^{2+}] = [Mg^{2+}_{free}] + [$MgADP^-$] + [MgPEP] + [$MgATP^{2-}$] where PEPmeans *P*-enolpyruvate.

The [$MgADP^-$] was calculated from the equation [$MgADP^-$]/[Mg^{2+}] [ADP^{3-}] = *K* in which for *K* a value of 4000 M^{-1} was used [18].
[MgPEP] can be calculated from [MgPEP]/[Mg^{2+}] [PEP^{2-}] = *K*. For *K* a value of 180 M^{-1} [20] was used, and [$MgATP^{2-}$] was calculated from [$MgATP^{2-}$]/[Mg^{2+}] [ATP^{4-}] = *K* in which a *K* of 20 000 M^{-1} [21, 22] was used. Each addition of *P*-enolpyruvate, ADP or ATP was accompanied by the calculated amount of Mg^{2+} to be bound by these compounds to assure the presence of the free Mg^{2+} concentrations as indicated in the legends to the figures. No calculations were performed for the presence of $HATP^{3-}$ or $HADP^{2-}$ at pH 8.0 because under the applied condition the concentrations of these compounds are less than 1 % of the total nucleotide content [19]. For the assay at pH 5.9 corrections were made according to Morrison and Heyde [19]. The *K* values selected from literature are the values valid in the presence of the applied salt concentration.

ADP, ATP, *P*-enolpyruvate, NADH and Fru-1,6-P_2 were obtained from Boehringer (Mannheim, Germany), L-alanine was obtained from Merck (Darmstadt, Germany). All other reagents were of analytical grade purity.

RESULTS

Influence of the Mg^{2+}_{free} concentration on the affinity for the substrates

Fig. 1 shows the influence of the Mg^{2+}_{free} concentration on the enzymatic activity of M_2-type pyruvate kinase as a function of the total *P*-enolpyruvate concentration at pH 8.0. It can be seen that the Mg^{2+}_{free} concentration not only affects the *V* but also influences the affinity of the enzyme for the substrate *P*-enolpyruvate. In the presence of 10 mM Mg^{2+}_{free} a curve is obtained which is characterized by two *n* values, calculated

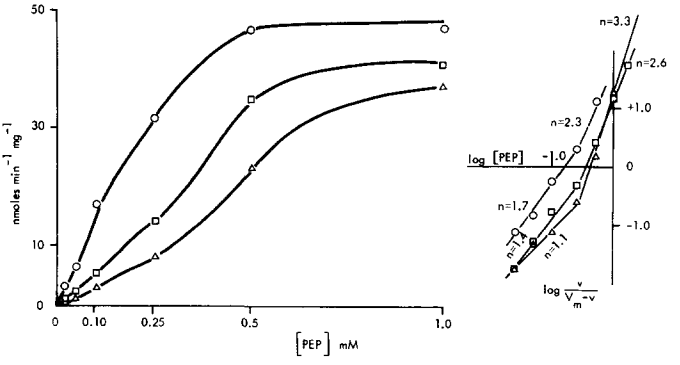


Fig. 1. Influence of the Mg^{2+}_{free} concentration on the *P*-enolpyruvate saturation curve of the M_2-type pyruvate kinase at pH 8.0. ○—○, 10 mM Mg^{2+}_{free}; □—□, 1 mM Mg^{2+}_{free}; △—△, 0.5 mM Mg^{2+}_{free}. The insert is the Hill plot of the values obtained. The calculated Hill coefficients (*n*) are indicated.

from the Hill plot (see insert Fig. 1). The obtained $K_{0.5}$ at 10 mM Mg^{2+}_{free} is 0.16 mM while in previous experiments with 23 mM total Mg^{2+} a $K_{0.5}$ value for *P*-enolpyruvate of 0.14 mM was obtained. Lowering of the Mg^{2+}_{free} concentration to 1 mM results in an increase of the $K_{0.5}$ value for *P*-enolpyruvate to 0.34 mM while the V is only slightly lowered. A further decrease in the Mg^{2+}_{free} concentration to 0.5 mM potentiates these effects and the $K_{0.5}$ value for *P*-enolpyruvate becomes 0.45 mM. From the Hill plot (insert Fig. 1) it can be observed that the breakpoints of the lines drop to lower log $v/V-v$ values when the free Mg^{2+} concentration is lowered. This indicates that at the lower free Mg^{2+} concentration the relative contribution of the enzymatic activity with the lower n value to the overall activity is less. For reason that the lowering of the free Mg^{2+} concentration is accompanied by a lowering of the $MgADP^-$ concentration it might be possible that the $MgADP^-$ concentration is responsible for the reported effects. Therefore, we increased the total ADP and total Mg^{2+} concentrations at 0.5 mM free Mg^{2+} in such a way that the same $MgADP^-$ concentrations were present both with 1 and 10 mM free Mg^{2+}. Although the V was raised, the $K_{0.5}$ for *P*-enolpyruvate in both cases was 0.45 mM (not shown) which indicates that the shift in the $K_{0.5}$ for *P*-enolpyruvate is indeed due to the decrease in the Mg^{2+}_{free} concentration. Furthermore we plotted the values obtained in Fig. 1 as a function of the free *P*-enolpyruvate and MgPEP concentrations (not shown). When the activity is plotted against the MgPEP concentration the curves are abnormal, especially in the presence of Fru-1,6-P_2, which makes it unlikely that MgPEP is the true substrate. Activity plots against the free *P*-enolpyruvate concentration yields curves qualitatively the same to those in Fig. 1, although the differences in $K_{0.5}$ for free *P*-enolpyruvate at different Mg^{2+}_{free} concentrations are larger. However, to allow comparison with data reported in literature, we decided to plot the activity against the total *P*-enolpyruvate concentration.

In Fig. 2 is plotted the pyruvate kinase activity under the same circumstances as for Fig. 1 except that 0.5 mM Fru-1,6-P_2 is present. This figure shows that in the presence of Fru-1,6-P_2 the curves have a biphasic pattern. To investigate this pheno-

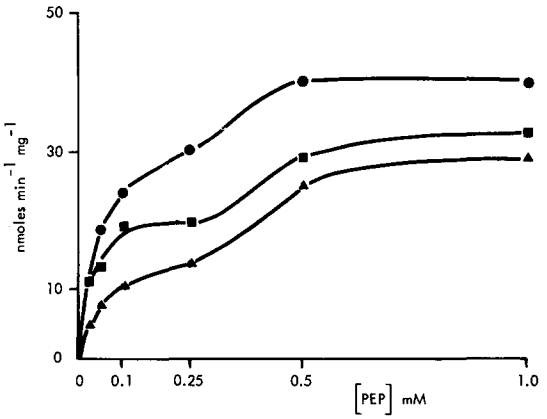


Fig. 2. Influence of the Mg^{2+}_{free} concentration on the *P*-enolpyruvate saturation curve of the M_2-type pyruvate kinase at pH 8.0 in the presence of 0.5 mM Fru-1,6-P_2. ●—●, 10 mM Mg^{2+}_{free}; ■—■, 1 mM Mg^{2+}_{free}; ▲—▲, 0.5 mM Mg^{2+}_{free}.

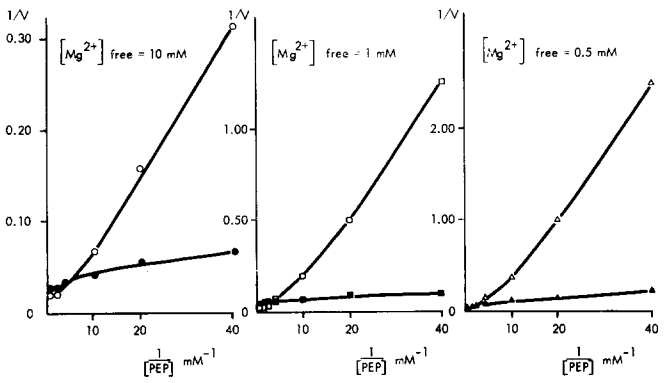


Fig. 3. The $1/v$ vs 1/[PEP] plot of the M_2-type pyruvate kinase activity at pH 8.0 tested in the absence and presence of Fru-1,6-P_2 (0.5 mM) at different Mg^{2+}_{free} concentrations. ○—○, 10 mM Mg^{2+}_{free} present; ●—●, 10 mM Mg^{2+}_{free} and 0.5 mM Fru-1,6-P_2 present; □—□, 1 mM Mg^{2+}_{free} present; ■—■, 1 mM Mg^{2+}_{free} and 0.5 mM Fru-1,6-P_2 present; △—△, 0.5 mM Mg^{2+}_{free} present; ▲—▲, 0.5 mM Mg^{2+}_{free} and 0.5 mM Fru-1,6-P_2 present.

menon more closely the $1/v$ vs 1/[PEP] plot was made (Fig. 3) from the values shown in Figs 1 and 2. In the presence of Fru-1,6-P_2 (0.5 mM) the $1/v$ vs 1/[PEP] curve has two components: a straight part at low *P*-enolpyruvate concentrations and a concave upward part at high *P*-enolpyruvate concentrations. This indicates that at low *P*-enolpyruvate concentrations in the presence of Fru-1,6-P_2 the enzyme follows Michaelis–Menten kinetics whereas the concave upward part is indicative for an allosteric response. By extrapolation of the straight part an apparent K_m for the enzyme at low *P*-enolpyruvate concentrations of 0.05 mM can be calculated irrespective of the Mg^{2+}_{free} concentration used. In the absence of Fru-1,6-P_2 the upward concave curves are characterized by two different n values (see Hill plot Fig. 1). This figure shows clearly that only the curve obtained at low *P*-enolpyruvate concentrations, and characterized by a low n value, is stimulated by Fru-1,6-P_2. The latter addition converts the upward concave curve into a straight line.

It seems likely that these results reflect the presence of two forms of the enzyme with different affinities for *P*-enolpyruvate. From results [13] reported earlier it was concluded that we could not discriminate between this possibility and the presence of negative cooperativity [13, 23]. The results obtained at different Mg^{2+}_{free} concentrations allow the conclusion that the Mg^{2+}_{free} concentration influences the ratio between two different forms of the enzyme. The Hill plot (insert Fig. 1) shows that the n values for the form active at low *P*-enolpyruvate concentrations decrease by lowering the Mg^{2+}_{free} concentration. However, when the data are replotted by applying the V belonging to this form this decrease disappears and both at 1 and 0.5 mM Mg^{2+}_{free} a n value of 1.7 is obtained. This result indicates that the Mg^{2+}_{free} concentration regulates the relative amounts of the two forms without influencing the allosteric behaviour of the form active at low *P*-enolpyruvate concentrations.

Fig. 4 shows that the Mg^{2+}_{free} concentration does not change the apparent K_m for ADP. Both at 10 and 1 mM Mg^{2+}_{free} the enzyme follows Michaelis–Menten kinetics with an apparent K_m for ADP of 0.31 mM at 10 mM Mg^{2+}_{free} and 0.33 mM at 1 mM Mg^{2+}_{free}. Determination of the apparent K_m values from the plot $1/v$ vs 1/[MgADP]

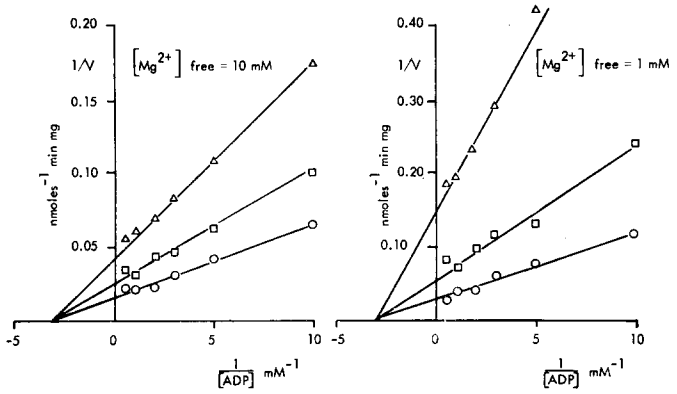


Fig. 4. The $1/v$ vs 1/ADP plot of the M_2-type pyruvate kinase activity at pH 8.0 tested at 10 and 1 mM Mg^{2+}_{free} at different *P*-enolpyruvate concentrations. △—△, 0.1 mM *P*-enolpyruvate; □—□, 0.25 mM *P*-enolpyruvate; ○—○, 0.5 mM *P*-enolpyruvate. The applied Mg^{2+}_{free} concentrations are indicated in the figure.

yields values of 0.30 and 0.27 mM, respectively. These values are in good agreement with the values reported earlier for M_2-type from liver [24] and other tissues [3, 10, 14].

The afore-mentioned experiments were performed at pH 8.0 to exclude the presence of MgHADP and $HADP^{2-}$. At pH 7.5 the effect of the Mg^{2+}_{free} concentration is qualitatively similar to that obtained at pH 8.0 (Table I). At 10 mM Mg^{2+}_{free} a slight sigmoidal response towards increasing *P*-enolpyruvate concentrations is obtained with a n value of 1.4 (not shown). At 1 mM Mg^{2+}_{free} the response is a curve characterized by two n values of 1.1 and 2.2, while the same phenomenon occurs at 0.25 mM Mg^{2+}_{free}. Also at this pH a replotting of the activities correlating with the lower n value, by applying the corresponding V, indicates that the n value observed at 10 mM Mg^{2+}_{free} remains constant by lowering the Mg^{2+}_{free} concentration. Table I summarizes the $K_{0.5}$ values observed both in the absence and presence of Fru-1,6-P_2. It can be seen

TABLE I

EFFECT OF THE Mg^{2+}_{free} CONCENTRATION ON THE $K_{0.5}$ VALUE FOR *P*-ENOLPYRUVATE IN THE ABSENCE AND PRESENCE OF FRU-1,6-P_2 (0.5 mM)

Assay conditions are indicated in the legends to Figs 1 and 2. The $K_{0.5}$ in the presence of Fru-1,6-P_2 is calculated from the straight part of the $1/v^{-1}$/[PEP] curve (see Fig. 3).

Free Mg^{2+} concentrations	pH	$K_{0.5}$ (mM)	$K_{0.5}$ (mM) of the high affinity form measured in the presence of Fru-1,6-P_2
10	5.9	0.05	0.08
1	5.9	0.05	0.07
10	7.5	0.06	0.05
1	7.5	0.28	0.06
0.5	7.5	0.32	0.06
0.25	7.5	0.35	0.06
10	8.0	0.16	0.04
1	8.0	0.34	0.04
0.5	8.0	0.45	0.06

that at pH 5.9 the same $K_{0.5}$ value is obtained for 1 and 10 mM Mg^{2+}_{free}. At increasing pH values the effect of Mg^{2+} on the $K_{0.5}$ is enforced. Further investigations however are needed to see if the effect of the pH on the affinity for *P*-enolpyruvate is due to a proton effect on the equilibrium of the two interconvertible forms or on the affinity of the low affinity form for *P*-enolpyruvate.

Although the observed activity of pyruvate kinase was linear in time during the assay it was stated [1, 10, 14] that slow transitions may be involved in the B⇌A equilibrium. Imamura and Tanaka [1] showed that pre-incubation with Fru-1,6-P_2 is able to change the biphasic *P*-enolpyruvate dependency in the presence of Fru-1,6-P_2 into normal curves. We have shown earlier that by incubation of M_2-type pyruvate kinase with oxidized glutathione the ability of Fru-1,6-P_2 to relieve the alanine inhibition is lost [13] and higher $K_{0.5}$ values for *P*-enolpyruvate are obtained also at high Mg^{2+} concentrations. The same result is obtained after prolonged dialysis (3 h) of the enzyme in the absence of Mg^{2+}. If the dialysed enzyme was incubated with Mg^{2+} (10 mM) + Fru-1,6-P_2 (0.5 M) for 30 min the original B form is restored. These data indicate that after a prolonged stay of the enzyme in the A form the conversion to the B form is a slow transition. Under these conditions also at high Mg^{2+}_{free} concentrations the properties of the A form are obtained.

Inhibitory action of alanine and ATP

The inhibitory actions of alanine and ATP on the M_2-type pyruvate kinase have been noticed by many authors [1–3, 12–14, 18]. Earlier we reported that alanine acts as an allosteric inhibitor [12, 13] and that the ATP inhibition differs from the amino acid inhibition. Imamura and Tanaka [1] reported that the ATP inhibition can be partly reversed by Mg^{2+}. However, the nature of the ATP inhibition has not been studied in detail. For reason, that this inhibition can be of regulatory importance we studied the effect of ATP at low free Mg^{2+} concentrations. Furthermore we compared this effect with the inhibition by alanine. Fig. 5 shows the pyruvate kinase activity at increasing *P*-enolpyruvate concentrations at 1 mM free Mg^{2+} in the presence of 2 mM

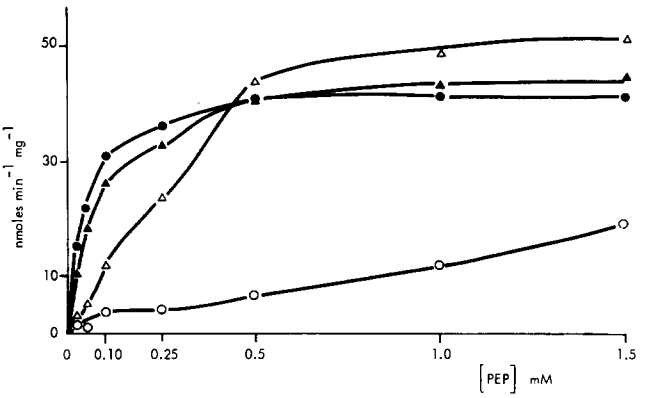


Fig. 5. M_2-type pyruvate kinase activity vs *P*-enolpyruvate concentration in the presence of 2 mM MgATP or 1 mM alanine at 1 mM Mg^{2+}_{free}. △—△, 2 mM MgATP present; ▲—▲, 2 mM MgATP and 0.5 mM Fru-1,6-P_2 present; ○—○, 1 mM alanine; ●—●, 1 mM alanine and 0.5 mM Fru-1,6-P_2 present.

MgATP or 1 mM alanine. In the presence of 2 mM MgATP the obtained $K_{0.5}$ for *P*-enolpyruvate of 0.30 mM indicates that MgATP in the physological concentration does not change the affinity for *P*-enolpyruvate. This is in contrast to the effect of 1 mM alanine, which is a strong inhibitor under the applied conditions. Under both conditions, Fru-1,6-P_2 (0.5 mM) is able to activate the enzyme and the obtained $K_{0.5}$ values in the presence of Fru-1,6-P_2 are simular whether ATP or alanine were present or not.

To differentiate between the effect of ATP on the Mg^{2+} concentration and a direct inhibitory action on the enzyme we plotted in Fig. 6 the influence of ATP at different Mg^{2+} concentrations while the effect of increasing EDTA concentrations is plotted in the same figure. It can be seen that the inhibitory action of ATP is mainly

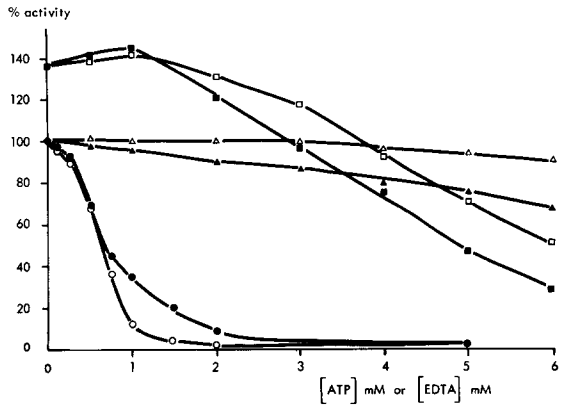


Fig. 6. The influence of ATP and EDTA on the activity of M_2-type pyruvate kinase at 0.5 mM P-enolpyruvate and pH 8.0. Open symbols are the added EDTA concentrations and the closed symbols the added ATP concentrations. ○—○, EDTA and 1 mM total Mg^{2+}; ●—●, ATP and 1 mM total Mg^{2+}; □—□, EDTA and 5 mM total Mg^{2+}; ■—■, ATP and 5 mM total Mg^{2+}; △—△, EDTA and 1 mM Mg^{2+}_{free}; ▲—▲, ATP and 1 mM Mg^{2+}_{free}. The 100% value is the activity at 0.5 mM *P*-enolpyruvate and total 1 mM Mg^{2+}. For 1 mM Mg^{2+}_{free} the 100% value is the activity at 0.5 mM *P*-enolpyruvate and 1 mM Mg^{2+}_{free}.

the consequence of Mg^{2+} binding. At 1 mM total Mg^{2+} the inhibitory action of ATP can be completely explained by Mg^{2+} binding. EDTA used in the same concentration is under the applied condition even more inhibitory, which is probably due to its higher affinity for Mg^{2+}, as compared with ATP. At 5 mM total Mg^{2+}, inhibition of the enzymatic activity is found at higher ATP concentrations while the data obtained at 1 mM Mg^{2+}_{free} allow the conclusion that under this probably physiological condition [15] the ATP inhibition is very small and does not seem to be of regulatory importance.

Affinity for Fru-1,6-P_2 and effect of other phosphorylated hexoses

It is well-known that Fru-1,6-P_2 activates the L-type pyruvate kinase from liver [8], erythrocytes [25] and yeast [26] in the micromolar range. For the M_2-type pyruvate kinase from liver Carbonell et al. [2] showed that 10 μM Fru-1,6-P_2 can overcome the alanine inhibition completely. Fig. 7 shows that the enzymatic form present at high Mg^{2+} concentrations shows a high affinity for Fru-1,6-P_2. At 0.1 mM

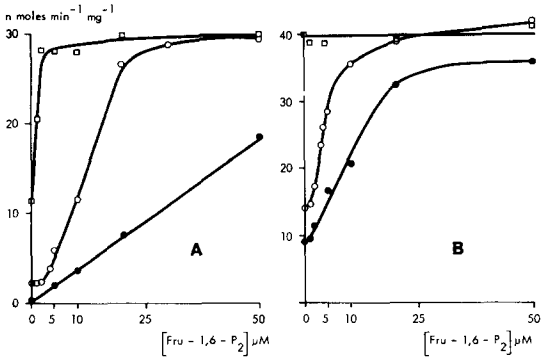


Fig. 7. (A and B) The affinity of M_2-type pyruvate kinase for Fru-1,6-P_2 in the absence and presence of alanine at: A, 0.1 mM *P*-enolpyruvate and B, 0.5 mM *P*-enolpyruvate, pH 8.0, and total Mg^{2+} concentration of 23 mM. □—□, control; ○—○; 1 mM alanine present; ●—●, 5 mM alanine present.

P-enolpyruvate full activation of the enzyme occurs already at 2 μM Fru-1,6-P_2. Addition of 1 mM alanine lowers the affinity of the enzyme for Fru-1,6-P_2 and for half maximal activation a concentration of 13 μM Fru-1,6-P_2 is needed. In the presence of 5 mM alanine the affinity for Fru-1,6-P_2 is even lower. Fig. 7B shows that at a higher *P*-enolpyruvate concentration (0.5 mM) the effect of alanine on the affinity of the enzyme for Fru-1,6-P_2 is less. This effect of alanine and *P*-enolpyruvate on the affinity of M_2-type pyruvate kinase for Fru-1,6-P_2 is very similar to that obtained with the L-type. This indicates that at high Mg^{2+} concentrations the experimental data can be explained by the earlier proposed R⇌T model [13] for M_2-type pyruvate kinase.

Fig. 8 shows that a lowering of the Mg^{2+}_{free} concentration to its physiological level of 1 mM does not change the affinity of the enzyme for Fru-1,6-P_2 at 0.1 mM *P*-enolpyruvate. The concentrations of Fru-1,6-P_2 necessary for half maximal activation measured at high free Mg^{2+} (10 mM) and low free Mg^{2+} (1 mM) are comparable and this is also the case in the presence of 1 mM alanine.

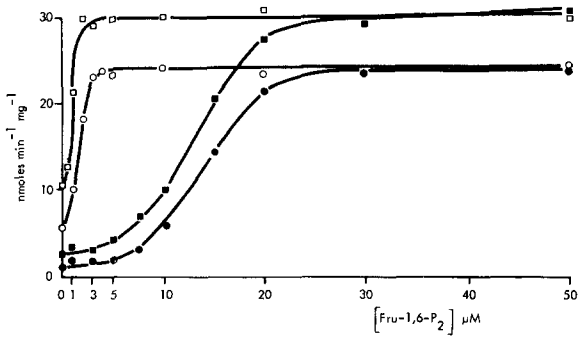


Fig. 8. The influence of the Mg^{2+}_{free} concentration on the affinity of M_2-type pyruvate kinase for Fru-1,6-P_2 in the absence and presence of 1 mM alanine at pH 8.0 and 0.1 mM P-enolpyruvate. □—□, 10mM Mg^{2+}_{free}; ○—○, 1 mM Mg^{2+}_{free}; ■—■, 10 mM Mg^{2+}_{free} and 1 mM alanine present; ●—●, 1 mM Mg^{2+}_{free} and 1 mM alanine present.

TABLE II

EFFECT OF P_i AND THE PHOSPHORYLATED HEXOSES ON THE ACTIVITY OF M_2-TYPE PYRUVATE KINASE

Addition (0.5 mM)	Relative activity* (%)	Relative activity in the presence of 1 mM alanine (%)
None	28	12
Glc-1-*P*	24	10
Glc-6-*P*	28	12
Glc-1,6-P_2	61	14
Gal-1-*P*	24	10
Fru-1-*P*	27	15
Fru-6-*P*	26	10
Fru-1,6-P_2	100	111
P_i (15 mM)	25	11

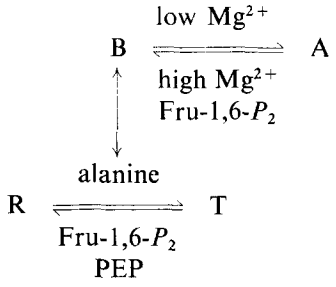
* The 100% value is the activity at 0.1 mM *P*-enolpyruvate in the presence of Fru-1,6-P_2 (0.5 mM) pH 8.0, and 1 mM Mg^{2+}_{free}.

The L-type pyruvate kinase from rat liver is not only activated by Fru-1,6-P_2 but also by P_i and phosphorylated hexoses [6, 7]. Since these latter compounds may also contribute to the "in vivo" regulation of the enzyme, we investigated the effect of P_i and phosphorylated hexoses on the M_2-type (Table II). The only effective compound besides Fru-1,6-P_2 is Glc-1,6-P_2. However, in the presence of alanine (1 mM) (a physiological concentration for liver [33]) the effect of Glc-1,6-P_2 has disappeared, which allows the conclusion that these compounds besides Fru-1,6-P_2 do not play a regulatory role in vivo.

DISCUSSION

The kinetic behaviour of M_2-type pyruvate kinase of rat liver towards *P*-enolpyruvate at low free Mg^{2+} concentrations can be completely explained by the presence of two interconvertible forms with different affinities for the substrate *P*-enolpyruvate. Pogson [10] has shown with the pyruvate kinase from adipose tissue that the presence of EDTA in the extraction buffer modifies the enzyme, resulting in an enzyme with a completely different kinetic behaviour (called by Pogson Pyk-A). The B form of the enzyme, isolated in the absence of EDTA, shows a high affinity for *P*-enolpyruvate (K_m for *P*-enolpyruvate, 0.06 mM) whereas with the A form a $K_{0.5}$ of 0.6 mM has been obtained. Recently Walker and Potter [14] have shown that rat liver cells, cultured in the absence of glucose, contain this A form whereas in the presence of glucose the B form of M_2-type pyruvate kinase was obtained. The data presented make it clear that isolation of M_2-type pyruvate from normal liver in the absence of an unphysiological metal ion chelator yields an enzyme which exists in interconvertible forms at physiological Mg^{2+} concentrations. From the data presented (Fig. 1) it can be concluded that the relative contribution of the two forms to the overall pyruvate kinase activity is regulated by the free Mg^{2+} concentration. The B form of M_2-type pyruvate kinase which is present at high Mg^{2+} concentrations shows a high affinity for *P*-enolpyruvate and it was shown earlier [13] that its properties can be explained on the basis of the R⇌T model of Monod et al. [27]. In this model Fru-

1,6-P_2 and *P*-enolpyruvate should favour the R state whereas alanine and the other inhibitory amino acids possess a higher affinity for the T state. This paper shows that the B form of M_2-type pyruvate kinase has a high affinity for Fru-1,6-P_2. The effect of alanine on the affinity for Fru-1,6-P_2 at different *P*-enolpyruvate concentrations (Figs 7A and 7B) is in complete agreement with this earlier proposed R⇌T model of Monod et al. [27]. Lowering of the Mg^{2+}_{free} concentration introduces the A form, which has less affinity for *P*-enolpyruvate. The following scheme might help to explain the observed kinetic data



The A form of the enzyme is characterized by a lower affinity for the substrate *P*-enolpyruvate while this form is not sensitive to Fru-1,6-P_2. However, Fru-1,6-P_2 can convert the A form into the B form which is potentiated by high Mg^{2+} concentrations. A prolonged stay of the enzyme in the A form makes the A to B conversion more difficult and longer incubation times are needed. The occurrence of the interconvertible forms of M_2-type pyruvate kinase might also explain the variations in kinetic behaviour described in the literature. Imamura and Tanaka [1] described for the highly purified enzyme that Fru-1,6-P_2 was not able to restore the alanine inhibition, a property which we found after a prolonged incubation of the enzyme in the A form. Furthermore Costa et al. [28] recently described a new isoenzyme of pyruvate kinase in rat kidney cortex. The described kinetic properties of this enzyme are very similar to the properties of the A form of M_2-type pyruvate kinase. Since the authors purified this enzyme in the presence of 5 mM EDTA, it seems likely that the A form of M_2-type pyruvate kinase is responsible for their results.

The given scheme also explains the difference in behaviour of Fru-1,6-P_2 and Mg^{2+}. Besides an effect of Fru-1,6-P_2 on the A⇌B transition, this phosphorylated hexose is an allosteric activator of the B form, which explains the stimulation also at higher Mg^{2+} concentrations. Recently a similar kind of enzyme regulation has been shown for phosphofructokinase [29, 30] and it will be interesting to see if this kind of regulation can also account for other enzymes.

Whether Mg^{2+} itself can act as a regulatory modulator of the M_2-type pyruvate kinase in liver seems not likely, as Veloso et al. [15] have shown that the free Mg^{2+} concentration in liver is nearly constant. From the kinetic data obtained in vitro it is rather speculative to draw conclusions about the regulation of the enzyme in vivo. This remark may account for every enzyme, but is of special importance for the M_2-type from liver as this type is not uniformly localized in this tissue [31, 32]. However, applying the physiological concentrations of Mg^{2+}, *P*-enolpyruvate and alanine (for these compounds there exists no large differences between various tissues [15, 33] the

activity of the M_2-type pyruvate kinase would be low, unless the enzyme is activated by Fru-1,6-P_2 (Figs 5 and 8). An distinction between the L-type pyruvate kinase and the M_2-type is the difference in ATP inhibition. With the L-type the influence of ATP is comparable with that of analine [8], whereas with the M_2-type ATP inhibition under physiological conditions seems negliglible (Figs 5 and 6). Whether this difference in ATP inhibition is related with the regulation of gluconeogenesis, for which the presence of L-type seems obligatory, needs further investigation.

The physiological meaning of the B⇌A equilibrium is not completely clear at the moment. As the Mg^{2+}_{free} concentration for liver, brain and kidney [15] is rather constant the B⇌A equilibrium will be dependent upon the Fru-1,6-P_2 concentration. This can explain the A form found in cells cultured in the absence of glucose. Under these conditions the Fru-1,6-P_2 will be low. However, for liver and also for leucocytes this will imply that the equilibrium will be on the B side because these cells have easily available glucose present. Preliminary studies with M_2-type from different cell types indicate that this is also the case with M_2-type from thrombocytes, whereas for rat small intestine the results are indicative for the fact that this enzyme occurs in vivo mainly in the A form [34]. The M_2-type pyruvate kinase is widely distributed in glycolytic tissues and the B⇌A equilibrium may provide an additional property of the enzyme to accommodate its properties to the specific requirements of the several tissues.

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