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TWO INTERCONVERTIBLE FORMS OF L-TYPE PYRUVATE KINASE FROM RAT LIVER

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SUMMARY

1. Reduced L-type pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40) from rat liver can be converted into an oxidized form by incubation with oxidized mercaptoethanol and oxidized glutathione. This interconversion can be completely reversed by incubation with reduced mercaptoethanol.

2. The kinetic and allosteric properties of the reduced and oxidized forms are described.

3. The results are discussed in view of a possible regulation of the enzyme.

INTRODUCTION

Pyruvate kinase (ATP: pyruvate phosphotransferase, EC 2.7.1.40) catalyses the last step of glycolysis. Its activity is important in the regulation of the dynamic balance between gluconeogenesis and glycolysis in the liver. In the liver two types of pyruvate kinase are present[1]. The L-type pyruvate kinase shows an allosteric response to one of its substrates phosphoenolpyruvate (PEP). Its activity is influenced by Fru-1,6-P_2, Glc-1,6-P_2 and other phosphorylated hexoses[2–5]. These compounds increase the apparent affinity for the substrate PEP. On the other hand ATP and alanine act as allosteric inhibitors[3,6]. It has been generally accepted that the L-type pyruvate kinase, localized in the rat liver hepatocytes[7], must be inhibited during gluconeogenesis in order to increase the [PEP] necessary for gluconeogenesis. In a previous paper[4] we pointed out that the L-type pyruvate kinase cannot be regulated by a fluctuating [Fru-1,6-P_2] in the liver. The ATP concentration *in vivo* only fluctuates between 2 and 3 mM (refs 8 and 9) and, therefore, some authors[6,10] assumed that during gluconeogenesis alanine is the most important inhibitor of pyruvate kinase activity. However, during gluconeogenesis the [alanine] in the liver is lowered (65%) due to an increased flux into the gluconeogenic pathway[11]. These properties would lead to an uninhibited enzyme under gluconeogenic conditions, which is rather

Abbreviation: PEP, phosphoenolpyruvate.

unlikely. Another possibility is that the reduction state of the enzyme plays an important role in its regulation. We investigated the influence of sulfhydryl compounds on the activity of the enzyme, after preliminary studies with coenzyme A (containing glutathione) indicated an influence of thiol groups on the allosteric behaviour of pyruvate kinase.

MATERIALS AND METHODS

Pyruvate kinase type L was isolated from rat liver according to the isolation procedure described earlier[4], except that during this procedure 1 mM mercaptoethanol was omitted and the final preparation was dissolved in 0.25 M Tris–HCl pH 8.0. Pyruvate kinase activity was assayed by following the decrease in absorbance at 340 nm in a coupled reaction with lactate dehydrogenase at 23 °C according to Valentine and Tanaka[12]. The triethanol–HCl buffer (0.4 M, pH 7.5) was replaced by Tris–HCl buffer (0.25 M, pH 8.0). Oxidized mercaptoethanol was prepared by bubbling O_2 through a 100-mM solution of reduced mercaptoethanol for 24 h. Oxidized L-type pyruvate kinase was prepared by incubating the enzyme for 6 h at 5 °C with 1 mM oxidized mercaptoethanol or 2.5 mM oxidized glutathione.

ADP, PEP, NADH, Fru-1,6-P_2, Glc-1,6-P_2 and oxidized glutathione were obtained from Boehringer (Mannheim, Germany). Glc-1,6-P_2 was free of Fru-1,6-P_2, when analysed by the method of Bücher and Hohorst[13]. Reduced glutathione was obtained from Sigma. All other reagents were of analytical grade purity.

RESULTS

Fig. 1 shows the *v vs* [PEP] plot at [ADP] = 0.5 mM for the freshly prepared pyruvate kinase (L-type) and the oxidized enzyme (preincubated with oxidized mercaptoethanol; compare Materials and Methods). In the presence of Fru-1,6-P_2

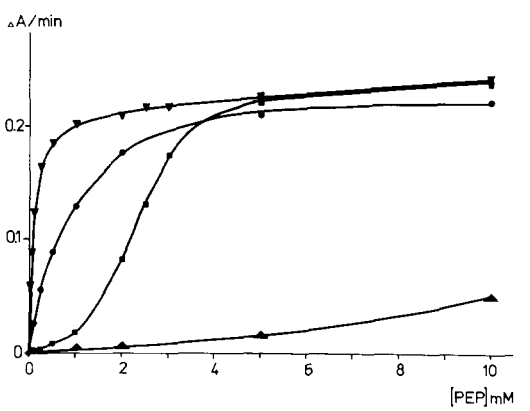

Fig. 1. The *v vs* [PEP] plot of the freshly isolated and oxidized L-type pyruvate kinase at [ADP] = 0.5 mM measured in the presence and absence of Fru-1,6-P_2 (0.5 mM). ■—■, the activity of the freshly isolated L-type pyruvate kinase; ▼—▼, the activity of the freshly isolated L-type pyruvate kinase measured in the presence of Fru-1,6-P_2; ▲—▲, the activity of the oxidized L-type pyruvate kinase (by oxidized mercaptoethanol); ●—●, the activity of the oxidized L-type pyruvate kinase (by oxidized mercaptoethanol) measured in the presence of Fru-1,6-P_2.

the freshly prepared enzyme gives an apparent K_m value of 0.1 mM for PEP, whereas in the absence of Fru-1,6-P_2 a $K_{0,5}$ value of 2.5 mM was obtained. Oxidation of the enzyme leads to a marked change in these activity curves. In the presence of Fru-1,6-P_2 the K_m value for PEP is increased to 0.7 mM and the $K_{0,5}$ value in the absence of Fru-1,6-P_2 was not measurable. This means that at least under our test conditions the oxidized enzyme cannot reach its maximal activity in the absence of Fru-1,6-P_2. The addition of Fru-1,6-P_2 stimulates the enzymatic activity to the same value as obtained with the freshly prepared enzyme, indicating that no loss of maximal activity occurred after oxidation of the enzyme.

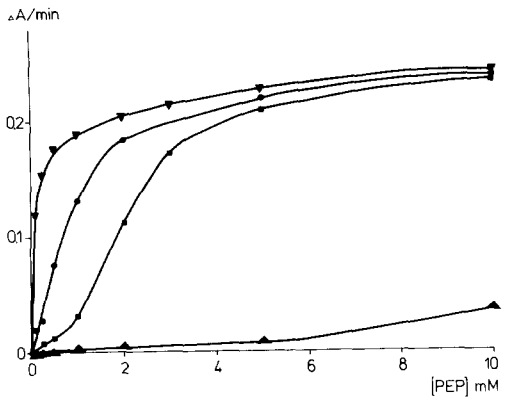

Fig. 2. The *v vs* [PEP] plot of the oxidized L-type pyruvate kinase (by oxidized glutathione) and the oxidized L-type pyruvate kinase after incubation for 1 h at 10 °C with 1 mM mercaptoethanol (reduced enzyme) measured in the presence and absence of Fru-1,6-P_2; ▲—▲, the activity of the oxidized L-type pyruvate kinase (by oxidized glutathione); ●—●, the activity of the oxidized L-type pyruvate kinase (by oxidized glutathione) measured in the presence of Fru-1,6-P_2; ■—■, the activity of the reduced L-type pyruvate kinase; ▼—▼, the activity of the reduced L-type pyruvate kinase measured in the presence of Fru-1,6-P_2.

Fig. 2 shows that the L-type pyruvate kinase can also be oxidized by incubation with oxidized glutathione In the absence and presence of Fru-1,6-P_2 the same pattern was obtained as in Fig. 1. Fig. 2 also shows that oxidation of the enzyme is a reversible process. By incubating the oxidized enzyme with 1 mM reduced mercaptoethanol for 1 h at 10 °C (reduced enzyme) the same kinetic data were obtained as with the freshly isolated enzyme. The $K_{0,5}$ value in the absence of Fru-1,6-P_2 becomes again 2 mM and the apparent K_m for PEP in the presence of Fru-1,6-P_2 is again lowered to 0.1 mM. The same curves were obtained when the enzyme was oxidized by using oxidized mercaptoethanol and incubated again with reduced mercaptoethanol. When the oxidized enzyme was incubated with reduced glutathione (5 mM), intermediate curves were obtained (Fig. 3).

From Figs 1, 2 and 3 we can conclude that there exist at least two forms of pyruvate kinase with different kinetic properties, which can be interconverted, probably by oxidation and reduction of the –SH groups of the enzyme.

The increase in K_m value for PEP in the presence of Fru-1,6-P_2, obtained with the oxidized enzyme as compared with the reduced enzyme, might be of physiological importance. Therefore the enzymatic activity of the oxidized and reduced

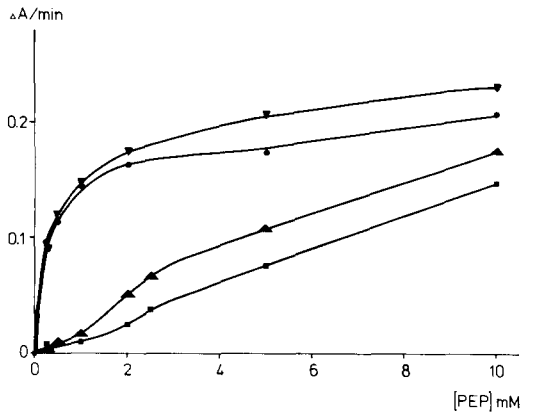

Fig. 3. The *v vs* [PEP] plot of the oxidized L-type pyruvate kinase after incubation for 1 h at 10 °C with 5 mM reduced glutathione measured in the presence and absence of Fru-1,6-P_2. ■—■, the activity of the oxidized L-type (by oxidized mercaptoethanol) incubated with reduced glutathione; ●—●, the activity of the oxidized L-type (by oxidized mercaptoethanol) which has been incubated with reduced glutathione measured in the presence of Fru-1,6-P_2; ▲—▲, the activity of the oxidized L-type (by oxidized glutathione) incubated with reduced glutathione; ▼—▼, the activity of the oxidized L-type (by oxidized glutathione) incubated with reduced glutathione measured in the presence of Fru-1,6-P_2.

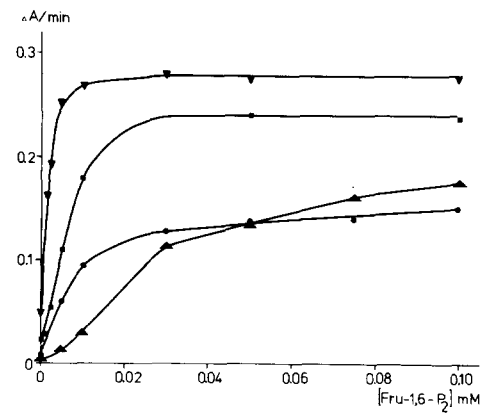

Fig. 4. The *v vs* [Fru-1,6-P_2] plot of the oxidized L-type pyruvate kinase and the oxidized L-type after incubation for 1 h at 10 °C with 5 mM reduced glutathione or 1 mM mercaptoethanol at [PEP] = 1 mM. ●—●, oxidized L-type pyruvate kinase (by oxidized mercaptoethanol); ▲—▲, oxidized L-type pyruvate kinase (by oxidized glutathione); ■—■, oxidized L-type pyruvate kinase (by oxidized glutathione) after incubation with reduced glutathione; ▼—▼, oxidized L-type pyruvate kinase (by oxidized mercaptoethanol) after incubation with reduced mercaptoethanol.

enzymes as a function of the [Fru-1,6-P_2] was studied. Fig. 4 shows that the reduced enzyme is already fully activated at 5 μM Fru-1,6-P_2, while the oxidized enzyme reaches this maximal activity at a concentration of about 30 μM Fru-1,6-P_2. Fig. 4 also shows the difference in maximal activities of the reduced and oxidized enzymes at [PEP] = 1 mM and [ADP] = 0.5 mM. When the oxidized enzyme is incubated with reduced mercaptoethanol, the same curve is obtained as with the freshly prepared enzyme. Incubation of the oxidized enzyme with reduced glutathione gives

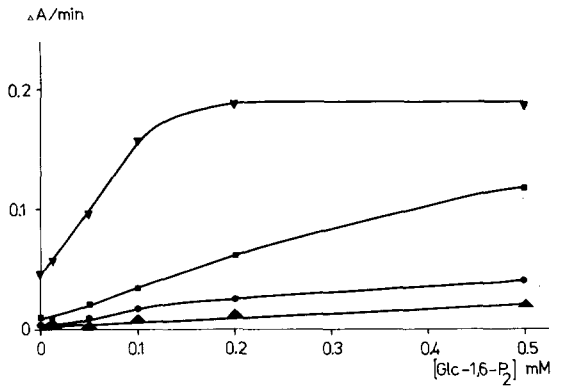

Fig. 5. The *v vs* [Glc-1,6-P_2] plot of the oxidized L-type pyruvate kinase and the oxidized L-type after incubation for 1 h at 10 °C with 5 mM reduced glutathione or 1 mM mercaptoethanol at [PEP] = 1 mM. ●—●, oxidized L-type pyruvate kinase (by oxidized mercaptoethanol); ▲—▲, oxidized L-type pyruvate kinase (by oxidized glutathione); ■—■, oxidized L-type pyruvate kinase (by oxidized glutathione) after incubation with reduced glutathione; ▼—▼, oxidized L-type pyruvate kinase (by oxidized mercaptoethanol) after incubation with reduced mercaptoethanol.

an intermediate curve; when this enzyme is further incubated with reduced mercaptoethanol, the same curve is obtained as with the freshly prepared enzyme.

Fig. 5 shows that the oxidized enzyme is rather insensitive to Glc-1,6-P_2 stimulation. When the oxidized enzyme was incubated with reduced glutathione for 1 h, a more marked stimulation by Glc-1,6-P_2 is found. Incubation with (reduced) mercaptoethanol for 1 h gives the same stimulation with Glc-1,6-P_2 as it is found with the freshly isolated enzyme. From Figs 4 and 5 the conclusion can be drawn that the oxidized enzyme is rather insensitive to Glc-1,6-P_2, whereas Fru-1,6-P_2 is still able to stimulate the enzyme, although the concentration necessary for maximal stimulation has increased from 5 μM with the reduced enzyme to 30 μM for the oxidized enzyme.

DISCUSSION

From the data presented it is clear that the L-type pyruvate kinase can exist in two forms with different kinetic properties. The two forms can be interconverted by incubation with oxidized or reduced mercaptoethanol or glutathione. Since these compounds affect the –SH groups of proteins, it seems likely that thiol groups are involved in the interconversion. When glutathione (5 mM) was used to reduce the oxidized enzyme, the results obtained were not identical with those obtained with mercaptoethanol (1 mM) as a reducing agent. This might be due to incomplete reduction of the enzyme with glutathione, suggesting at least two different types of –SH groups in the enzyme, involved in the interconversion.

For pyruvate formiate lyase from *Escherichia coli*[14,15] and also for xanthine oxidase (EC 1.2.3.2) from rat liver[16–18] an enzyme regulation by the oxidation and reduction of thiol groups has been shown and it is also likely that these interconversions are enzyme-catalysed. Also the fructose-1,6-diphosphatase (EC 3.1.3.11) activity from rabbit liver seems to be influenced by SH reagents[19–21]. For plants the re-

dox state of the SH groups of a number of enzymes is a general kind of control[22]. The existence of such an enzyme-catalysed interconversion for rat liver pyruvate kinase cannot be concluded from the presented data. The *in vitro* oxidation is a rather slow process (for full oxidation 6 h are required, data not shown) and subsequent reduction takes 1 h. These data are obtained with the partially purified enzyme. By analogy with other enzymes, it is tempting to speculate the existence of an *in vivo* enzyme-catalysed interconversion. One condition for physiological regulation is fullfilled in that the interconversion is reversible.

The difference in kinetic properties between the oxidized and reduced enzymes makes regulation of the enzymatic activity possible. In the oxidized enzyme the interaction of the PEP binding sites is raised (Figs 1 and 2) and Fru-1,6-P_2 is still able to stimulate the reaction. However the [Fru-1,6-P_2] needed for full activity is approximately 6 times higher than for the reduced enzyme and exceeds the liver [Fru-1,6-P_2] (ref. 23). This decrease in affinity for PEP and Fru-1,6-P_2 can be important during fasting, when PEP is needed for gluconeogenesis. In this view the markedly decreased influence of Glc-1,6-P_2 (of which the concentration in the liver remains constant during fasting and feeding, at least in the mouse[24]) on the oxidized enzyme is of great importance. These properties can make the interconversion of the two forms of pyruvate kinase (L-type) of physiological importance, if a system exists which can catalyse these interconversions more rapidly than under our *in vitro* conditions.

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