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COMPARATIVE KINETIC STUDIES ON THE L-TYPE PYRUVATE KINASE FROM RAT LIVER AND THE ENZYME PHOSPHORYLATED BY CYCLIC 3′, 5′-AMP-STIMULATED PROTEIN KINASE

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Summary

The kinetics of rat liver L-type pyruvate kinase (EC 2.7.1.40), phosphorylated with cyclic AMP-stimulated protein kinase from the same source, and the unphosphorylated enzyme have been compared. The effects of pH and various concentrations of substrates, Mg^{2+}, K^+ and modifiers were studied.

In the absence of fructose 1,6-diphosphate at pH 7.3, the phosphorylated pyruvate kinase appeared to have a lower affinity for phosphoenolpyruvate ($K_{0.5}$ = 0.8 mM) than the unphosphorylated enzyme ($K_{0.5}$ = 0.3 mM). The enzyme activity vs. phosphoenolpyruvate concentration curve was more sigmoidal for the phosphorylated enzyme with a Hill coefficient of 2.6 compared to 1.6 for the unphosphorylated enzyme. Fructose 1,6-diphosphate increased the apparent affinity of both enzyme forms for phosphoenolpyruvate. At saturating concentrations of this activator, the kinetics of both enzyme forms were transformed to approximately the same hyperbolic curve, with a Hill coefficient of 1.0 and $K_{0.5}$ of about 0.04 mM for phosphoenolpyruvate. The apparent affinity of the enzyme for fructose 1,6-diphosphate was high at 0.2 mM phosphoenolpyruvate with a $K_{0.5}$ = 0.06 μM for the unphosphorylated pyruvate kinase and 0.13 μM for the phosphorylated enzyme. However, in the presence of 0.5 mM alanine plus 1.5 mM ATP, a higher fructose 1,6-diphosphate concentration was needed for activation, with a $K_{0.5}$ of 0.4 μM for the unphosphorylated enzyme and of 1.4 μM for the phosphorylated enzyme.

The results obtained strongly indicate that phosphorylation of pyruvate kinase may also inhibit the enzyme in vivo. Such an inhibition should be important during gluconeogenesis.

Introduction

The regulation of the activity of liver pyruvate kinase type L (ATP : phosphotransferase; EC 2.7.1.40) is important for the regulation of glycolysis and

gluconeogenesis in the liver cell [1—3]. It has been shown that metabolites can affect the activity of pyruvate kinase [1,4—7]. Thus, ATP and alanine, at physiological concentrations, inhibit the activity of the enzyme. Fru-1,6-P_2 partially reverses this inhibition or even activates the enzyme. The synthesis of the L-form of the enzyme can be induced or repressed by different diets [2,7,8]. Nevertheless it is disputable whether modifiers and dietary factors alone can account for the total demand of regulation that would be needed when switching over from glycolysis to gluconeogenesis or vice versa [7,9,10].

It has been shown that glucagon administration gives a rapid decrease in the pyruvate kinase activity in the liver. These conditions also result in an increase in the cyclic AMP level in the cell [11,12] thereby leading to an activation of the protein kinase [13]. The phosphorylation of pyruvate kinase by cyclic AMP-stimulated protein kinase has been demonstrated in vitro [14,15] and the work cited above suggests that this could be relevant even in vivo.

At physiological concentrations of phosphoenolpyruvate, i.e. 0.02—0.5 mM [16], phosphorylation leads to decreased activity of pyruvate kinase [14,15]. In the presence of 10 μM Fru-1,6-P_2 the difference in activity between phosphorylated and unphosphorylated pyruvate kinase is abolished. However, since several other compounds affect the activity of pyruvate kinase, it was of interest to investigate the difference between the unphosphorylated and phosphorylated enzyme with special attention to physiological concentrations of substrates, ions and modifiers to establish whether the phosphorylation of pyruvate kinase may influence its activity in vivo.

Materials and Methods

Rabbit lactate dehydrogenase (EC 1.1.1.27) was a Boehringer-Mannheim product. Bovine serum albumin, Fru-1,6-P_2, NADH, ATP, ADP, cyclic AMP, phosphoenolpyruvate and dithiothreitol were purchased from Sigma. The L-alanine used was bought from S.A.F., Hoffman-La Roche and Co. Ltd. Ce., Basle, Suisse. Sephadex G-50 was from Pharmacia, Uppsala, Sweden and hydroxyapatite from Bio-Rad Laboratories, Richmond, Calif., U.S.A. All other chemicals were of reagent grade.

Protein kinase and pyruvate kinase were prepared by the methods used by Titanji, V., Zetterqvist, O. and Engstrom, L. [22] as far as to the hydroxyapatite step. The specific activity of the pooled material was 800 units/mg for protein kinase and 366 units/mg for pyruvate kinase.

Protein concentrations were determined from absorbance measurements at 280 nm by using the value of $A^{1\%}_{1cm} = 10.0$. [γ-^{32}P] ATP was prepared according to Engström [17]. Radioactivity was measured as Čerenkov radiation as described by Mårdh [18].

Enzyme assays. The methods of Kimberg and Yielding [19] as modified in this laboratory [14] were used for determination of pyruvate kinase during purification and phosphorylation of the enzyme. One unit of enzyme is defined as the amount of enzyme that transforms 1 μmol of substrate per min.

The assay medium, adjusted to pH 7.30—7.35 with HCl, contained 0.020 unit of unphosphorylated or phosphorylated pyruvate kinase, 5 mM K_2HPO_4, 0.1 mM dithiothreitol, 0.1% albumin, 0.15 mM NADH and 1.5 units /ml of lactate dehydrogenase. The latter had been dialysed overnight against 20 mM po-

tassium phosphate (pH 7.5) and 1 mM dithiothreitol. It was shown that this coupling enzyme was not rate limiting. The concentrations of KCl and ADP were, unless otherwise stated, 100 and 1 mM, repectively. The concentration of free Mg^{2+} was 5 mM when calculated according to O'Sullivan and Perrin [20]. The concentration of phosphoenolpyruvate was varied as described under Results. The reactions were carried out at 30° C and started by the addition of phosphoenolpyruvate after preincubation at the same temperature of the enzyme with all the other components for 3 min. The activity of the enzyme forms was stable during preincubation times of up to 20 min. The rate of the reaction was calculated by drawing the tangent to the initial part of the curve (first 2 min). The pH remained constant over the assay period. Addition of albumin, hexokinase and glucose did not have any effect on the pyruvate kinase activity. $K_{0.5}$ values were calculated from Hill plots by the methods of least squares. The V used was estimated according to Lineweaver and Burk unless otherwise stated.

In all experiments, both unphosphorylated and phosphorylated enzyme were diluted to the same activity, 0.020 unit per incubation, when assayed with 2.5 mM phosphoenolpyruvate and 25 μM Fru-1,6-P_2. Under these conditions the two enzyme forms had the same V. The activity of the enzyme was proportional to the concentration of enzyme in the range of 0.005—0.040 unit per incubation irrespective of phosphoenolpyruvate concentration used (0.2—5 mM).

Protein kinase was assayed by the method used by Titanji et al. [22]. One unit of protein kinase was defined as the amount of enzyme that catalyses the incorporation of 1 pmol of phosphate into histone per min.

Phosphorylation of pyruvate kinase. Pyruvate kinase (63 units /ml) was incubated with protein kinase (100 units/ml) for 45 min at 30° C in a medium containing 10 mM magnesium acetate, 0.1 mM [^{32}P] ATP (spec. act. 20 000 cpm/nmol) and 0.01 mM cyclic AMP in a 50 mM potassium phosphate buffer (pH 7.2) with 0.1 mM dithiothreitol and 15% (v/v) glycerol added. The total volume was 5 ml. Thereafter all manipulations were carried out at 4° C. Excess ATP was removed by chromatography on a 1.2 × 45 cm Sephadex G-50 column equilibrated and eluted with 1 mM potassium phosphate buffer (pH 7.0), 30% glycerol, 0.1 mM dithiothreitol and 10^{-6} M Fru-1,6-P_2. In order to separate pyruvate kinase from protein kinase, the radioactive protein peak from the Sephadex chromatography was applied to a hydroxyapatite column (1 × 2 cm) equilibrated with the previous buffer. The radioactive pyruvate kinase was eluted with the same buffer except that the concentration of potassium phosphate was increased to 10 mM. The degree of [^{32}P] ATP incorporation was estimated to be 3.6 mol/mol of enzyme using a specific activity of 450 units/mg for the pure enzyme and a molecular weight of 250 000 (Titanji et al., [22].

Both enzyme forms were dialysed against 20 mM potassium phosphate buffer (pH 7.0), 30% glycerol, 0.1 mM dithiothreitol and 10^{-6} M Fru-1,6-P_2 overnight. They were maintained in this form at -18° C.

Results

Effect of pH. As seen in Fig. 1, there was no great difference in pH optima for unphosphorylated and phosphorylated enzyme in the pH region tested. For both enzyme forms, the maximum activity was reached around pH 6.5. At 0.2

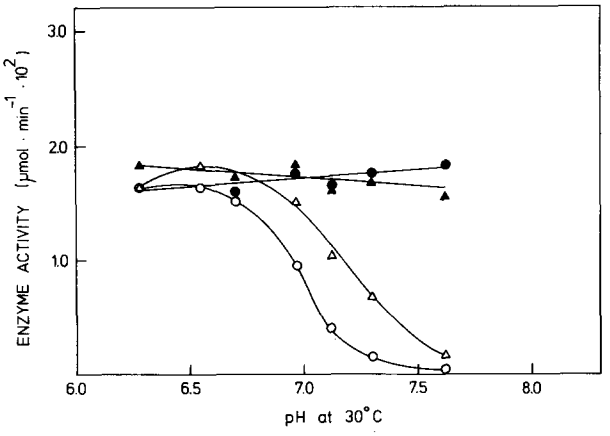

Fig. 1. Activity of pyruvate kinase as a function of pH. The various pH values were obtained by adjusting the assay medium with NaOH or HCl. The standard reaction mixture was used. The phosphoenolpyruvate concentration was 0.2 mM. Open symbols represent pyruvate kinase activity in the absence of Fru-1,6-P_2. Filled symbols represent the activity of pyruvate kinase in the presence of 5 μM Fru-1,6-P_2. △——△, ▲——▲, unphosphorylated enzyme; ○——○, ●——●, phosphorylated enzyme.

mM phosphoenolpyruvate, which was the concentration used in these experiments, the activity of the phosphorylated enzyme was lower than that of the unphosphorylated enzyme at all pH values tested except for the most acid point where they had the same activity. The difference became more pronounced in the alkaline range. For example, the apparent activity ratio of unphosphorylated to phosphorylated pyruvate kinase was 1.2 at pH 6.7 increasing to 4.7 at pH 7.3. However, in the presence of 5 μM Fru-1,6-P_2 the ratio was about 1 irrespective of the pH tested.

Dependence on concentration of phosphoenolpyruvate. The activity of pyruvate kinase as a function of the concentration of phosphoenolpyruvate is described in Fig. 2. Both enzyme forms described a sigmoidal curve (Fig. 2A). The Hill coefficient for the unphosphorylated enzyme was 1.6 and for the phosphorylated enzyme 2.6 (Fig. 2B). $K_{0.5}$ for the unphosphorylated pyruvate kinase was about 0.3 mM phosphoenolpyruvate and this constant increased to about 0.8 mM phosphoenolpyruvate for the phosphorylated enzyme. With 5 or 25 μM Fru-1,6-P_2 there was no significant difference between the two pyruvate kinase forms and the value of $K_{0.5}$ was about 0.04 mM phosphoenolpyruvate for both. The curves were hyperbolic with a Hill coefficient of 1.0. The same maximal activities of unphosphorylated and phosphorylated enzyme could also be obtained with 5 mM phosphoenolpyruvate in the absence of Fru-1,6-P_2.

Pyruvate kinase activity as a function of ADP concentration. Fig. 3 demonstrates that using 0.2 mM phosphoenolpyruvate, the phosphorylated pyruvate kinase attains an activity maximum at about 0.2 mM ADP. The maximum appeared to be somewhat higher (0.3 mM ADP) for the unphosphorylated pyruvate kinase at the same phosphoenolpyruvate concentration. When 2.5 mM phosphoenolpyruvate was used, both unphosphorylated and phosphorylated enzyme reached maximal activity at 1.0 mM ADP. At saturating concentrations of phosphoenolpyruvate, K_m for both enzyme forms was 0.25 mM with respect

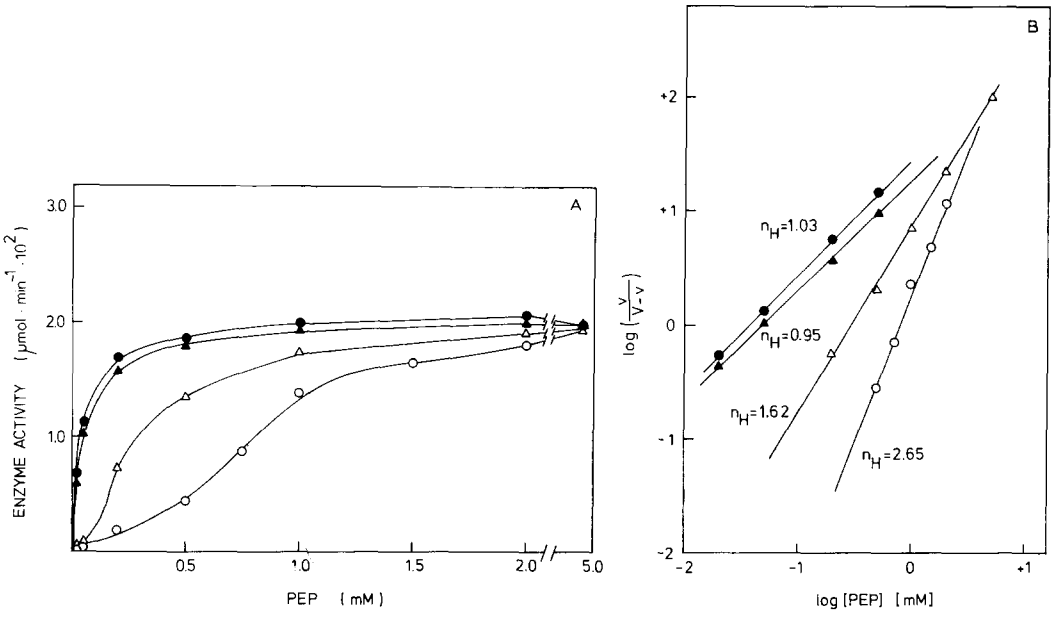

Fig. 2 (A) Dependence of pyruvate kinase activity on phosphoenolpyruvate concentration. The assay conditions were as described in Materials and Methods. Open and filled symbols represent pyruvate kinase activity in the absence and presence of Fru-1,6-P_2, respectively. When Fru-1,6-P_2 was used the concentration was 5 μM. △——△, ▲——▲, unphosphorylated pyruvate kinase; ○——○, ●——●, phosphorylated pyruvate kinase. (B) Re-plot of data from A for calculations of the Hill coefficients.

to ADP as calculated from Lineweaver-Burk plots. In all cases tested, there was a slight inhibition with ADP concentrations above that giving maximal activity. The concentration of free Mg^{2+} was adjusted to 5 mM in each of these experiments.

Requirements of K^+ and Mg^{2+}. At 0.2 and 2.5 mM phosphoenolpyruvate, a potassium concentration of 25 mM gave maximal activity for both enzyme forms (Fig. 4A).

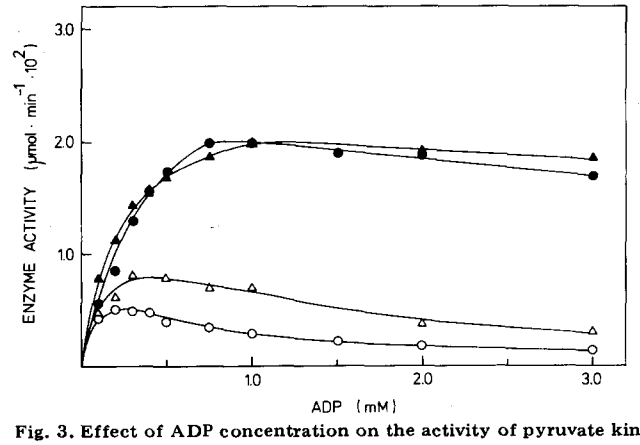

Fig. 3. Effect of ADP concentration on the activity of pyruvate kinase. The standard reaction mixture was used. Open and filled symbols represent activity with 0.2 and 2.5 mM phosphoenolpyruvate, respectively. △——△, ▲——▲, unphosphorylated pyruvate kinase; ○——○, ●——●, phosphorylated pyruvate kinase.

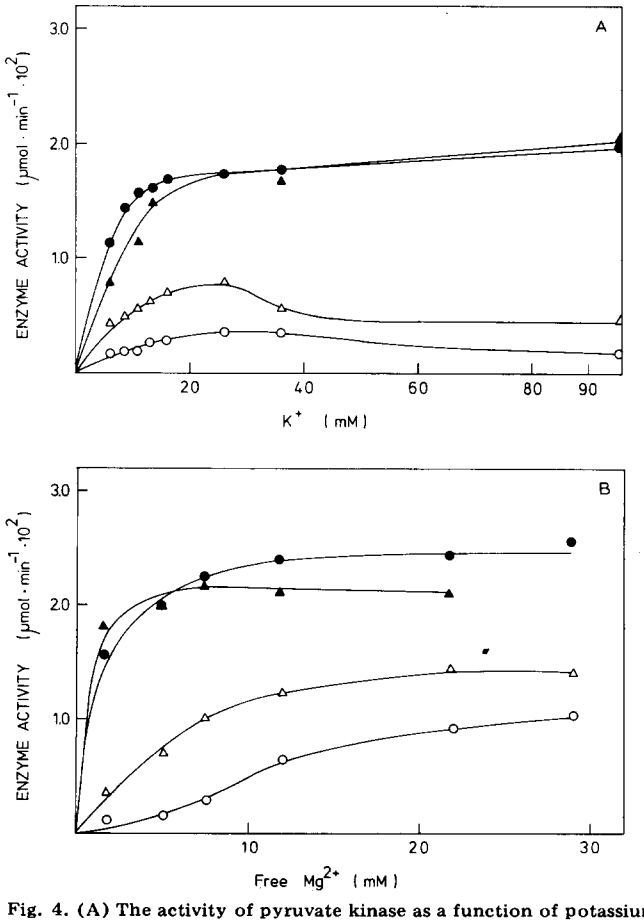

Fig. 4. (A) The activity of pyruvate kinase as a function of potassium concentration. The assay conditions were as described in Materials and Methods except that at lower potassium concentrations than 25 mM the chloride content was adjusted to 25 mM by the addition of NaCl. Open symbols and closed symbols represent activity with 0.2 and 2.5 mM phosphoenolpyruvate, respectively. △——△, ▲——▲, unphosphorylated enzyme; ○——○, ●——●, phosphorylated enzyme. (B) Stimulation of the activity of pyruvate kinase by magnesium concentration. The routine assay mixture was used. Open symbols and closed symbols represent activity in the presence of 0.2 and 2.5 mM phosphoenolpyruvate, respectively. △——△, ▲——▲, unphosphorylated pyruvate kinase; ○——○, ●——●, phosphorylated pyruvate kinase.

Both unphosphorylated and phosphorylated pyruvate kinase were activated by the divalent Mg^{2+} (Fig. 4B). Half maximal activity of both pyruvate kinase forms was attained with about 0.7 mM free Mg^{2+} at 2.5 mM phosphoenolpyruvate. This parameter was 6.5 and 23 mM for the unphosphorylated and the phosphorylated enzyme, respectively, when assayed with 0.2 mM phosphoenolpyruvate.

Inhibition by ATP and alanine. As can be seen in Fig. 5 the phosphorylated enzyme was more sensitive to inhibition by ATP and alanine than the unphosphorylated enzyme. This inhibition was more accentuated when tested with 0.5 mM phosphoenolpyruvate than with 2.5 mM phosphoenolpyruvate.

Alanine had a more pronounced inhibitory effect than ATP at the same concentrations except in the case of unphosphorylated enzyme at 2.5 mM phosphoenolpyruvate where the inhibition was about equal for ATP and alanine.

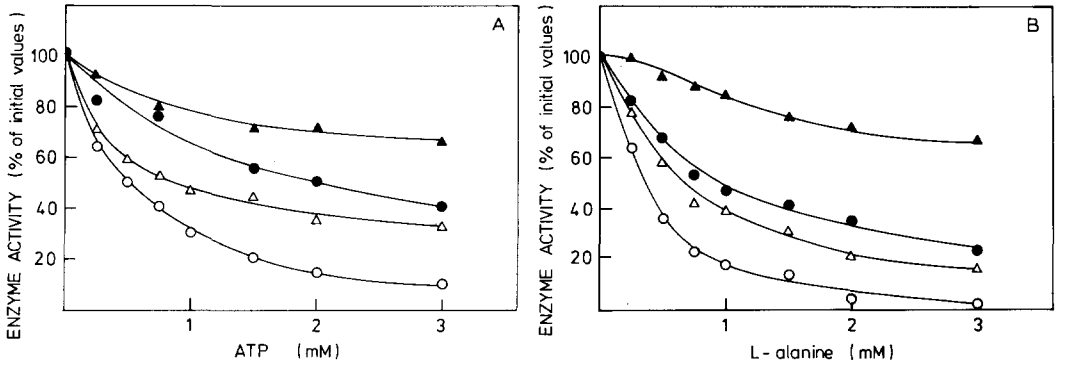

Fig. 5. (A) ATP inhibition of the activity of pyruvate kinase. The assay conditions were as described in Materials and Methods. Open symbols and filled symbols represent activity in the presence of 0.5 and 2.5 mM phosphoenolpyruvate, respectively. △——△, ▲——▲, unphosphorylated enzyme; ○——○, ●——●, phosphorylated enzyme. (B) The inhibition of unphosphorylated and phosphorylated pyruvate kinase by alanine. The standard reaction mixture was used. Open symbols and filled symbols represent activity in the presence of 0.5 and 2.5 mM phosphoenolpyruvate, respectively. △——△, ▲——▲, unphosphorylated enzyme; ○——○, ●——●, phosphorylated enzyme.

Effect of Fru-1,6-P_2. In order to investigate whether a difference in activity between the unphosphorylated and the phosphorylated pyruvate kinase could be relevant in the cell their activities were tested in the presence of the presumed physiological concentrations of substrates, and the inhibitors ATP and alanine [16]. For comparison, the activities of the unphosphorylated and the phosphorylated enzyme were tested in the absence of inhibitors and under these conditions a difference was observed (see Fig. 6). $K_{0.5}$ for the unphosphorylated and the phosphorylated pyruvate kinase were 0.06 and 0.13 μM, respectively, for Fru-1,6-P_2. In the presence of 0.5 mM alanine and 1.5 mM ATP the activity was totally abolished for both enzyme forms in the absence of Fru-1,6-P_2. When present, Fru-1,6-P_2 activated the unphosphorylated pyruvate kinase at lower concentrations than was the case with the phosphorylated enzyme. Neither the activity of the unphosphorylated nor the phosphorylated enzyme, when inhibited, could be restored completely by the addition of 25 μM

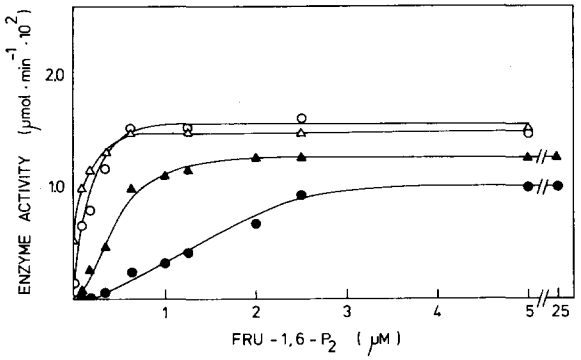

Fig. 6. The activity of pyruvate kinase as a function of Fru-1,6-P_2 concentration in the presence (filled symbols) and absence (open symbols) of ATP and alanine. 0.2 mM phosphoenolpyruvate was used. When ATP and alanine were added the concentrations were 1.5 mM of ATP and 0.5 mM of alanine. △——△, ▲——▲, unphosphorylated pyruvate kinase; ○——○, ●——●, phosphorylated pyruvate kinase.

Fru-1,6-P_2. When alanine and ATP were present, the $K_{0.5}$ for Fru-1,6-P_2 was 0.4 μM for the unphosphorylated pyruvate kinase and 1.4 μM for the phosphorylated enzyme. The apparent Hill coefficients for the uninhibited enzyme forms were about 1 and for the inhibited enzyme forms about 2.

Discussion

The kinetic parameters for the unphosphorylated pyruvate kinase given in this report were in good agreement with most previous works [1,4,6—8,10,14, 16,21,22].

The inhibitory effect of the phosphorylation of pyruvate kinase was reversed by two effectors: H^+ and Fru-1,6-P_2. As the pH was reduced the activity of the unphosphorylated and the phosphorylated pyruvate kinase became similar (Fig. 1).

In the absence of the inhibitors ATP and alanine, a very low concentration of Fru-1,6-P_2 cancelled the inhibition of pyruvate kinase due to phosphorylation. When ATP and alanine were present in the physiological range of concentration, there was a considerable inhibition of the phosphorylated enzyme around 1—2 μM Fru-1,6-P_2 (Fig. 6). In the liver the gluconeogenesis takes place in the hepatocytes [9,21]. The content of free Fru-1,6-P_2 is probably very low in these cells under gluconeogenetic conditions [10]. Therefore, the phosphorylation of the L-type of pyruvate kinase may well be of importance for the regulation of its activity in vivo in rat liver. This hypothesis is further supported by the work of Taunton et al. [11,12].

Results similar to the present ones have been obtained with the L-type pyruvate kinase from pig liver (Ljungström, O., Berglund, L. and Engström, L., unpublished).

In conclusion it is highly probable that the activity of the L-type of rat liver pyruvate kinase in vivo is not only regulated by allosteric effectors and changes in enzyme concentration but also by phosphorylation of the enzyme by cyclic AMP-stimulated protein kinase.

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