EFFECTS OF L-ALANINE AND FRUCTOSE (1, 6-DIPHOSPHATE) ON PYRUVATE KINASE FROM EHRLICH ASCITES TUMOUR CELLS

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1. Introduction

In mammalian tissues multiple forms of pyruvate kinase (ATP, pyruvate phosphotransferase, EC 2.7.1.40) have been found with different kinetic, physicochemical and immunological properties [1–3]. In particular, significant differences exist in the regulatory properties of the various types of this enzyme, especially in their sensitivity against allosteric effectors, like certain amino acids [3] and fructose 1, 6-diphosphate [2] which are considered as being important for the coordination of glycolysis and gluconeogenesis [4–6]. In the course of an investigation of the regulation of glycolysis in Ehrlich ascites tumour cells it became necessary to study the pyruvate kinase of these cells [7] in more detail. In this paper, evidence will be presented that the negative allosteric effector L-alanine, causes a dissociation of the tumour pyruvate kinase into two half molecules. Evidently, this dissociation may be reversed by the positive effector fructose 1, 6-diphosphate.

2. Materials and methods

Ascites tumour pyruvate kinase was brought to a specific activity of 30 units per mg of protein by ultrasonic disintegration of the cells, ammonium sulphate fractionation in the range of 40–80% saturation and gel filtration on Sephadex G-200. The enzyme preparation contained less than 0.03% of enolase, adenylate kinase and alanine aminotransferase and no detectable activities of NADH oxidases, fructose 1, 6-diphosphatase, aldolase, and proteases, respectively. Initial velocity was measured by coupling the pyruvate kinase with lactate dehydrogenase in an Eppendorf Photometer at 334 nm. All assays were carried out at pH 7.2 and 25°C with final concentrations of 50 mM Tris–maleate buffer, 100 mM KCl, 25 mM $MgSO_4$, 0.3 mM NADH and 2 units lactate dehydrogenase per ml reaction mixture.

The sedimentation coefficients were determined by sucrose density gradient centrifugation according to Martin and Ames [8], and the molecular weights were calculated assuming that the relationship between the sedimentation coefficient and the molecular weight of pyruvate kinase is the same as for the globular proteins used as references: ox heart lactate dehydrogenase (6.5 S, mol. wt = 135 000), rabbit muscle aldolase (7.82 S, mol. wt = 149 000), pig heart fumarase (9.09 S, mol. wt = 220 000), rabbit skeletal muscle pyruvate kinase (10.04 S, mol. wt = 237 000) and yeast phosphofructokinase (17.8 S, mol. wt = 570 000). The centrifugation was carried out at 53 000 rpm (5 hr, 4°C) in a 5–20% linear sucrose concentration gradient by using a Beckmann centrifuge type L2-65 B and the rotor SW 65 L-TI.

Before its application to the gradient the enzyme was dialysed for 1 hr and diluted in the following buffer: 40 mM triethanolamine–HCl (pH 7.2), 50 mM KCl and 5 mM $MgSO_4$, in addition to the respective effector(s), as indicated in fig. 4. After dividing the gradient into 45 fractions, the activity was determined by applying conditions, where maximum activity is assured (see fig. 4).

3. Results and discussion

L-alanine acts as negative allosteric effector on the

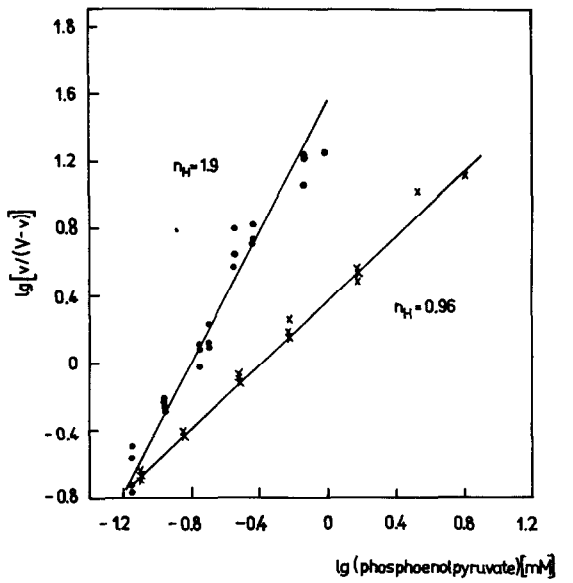

Fig. 1. Hill plot of pyruvate kinase for phosphoenolpyruvate in absence and presence of L-alanine. Experimental: ADP concentration was 0.3 mM (•) in absence of alanine; (×) in presence of 0.8 mM alanine.

enzyme from ascites tumour cells. The inhibition curve by alanine is sigmoidal with a Hill-coefficient of 1.8 (at 2.5 mM phosphoenol pyruvate and 0.3 mM ADP). The heterotropic effect of alanine gives rise to apparent cooperative interactions of phosphoenolpyruvate (fig. 1). Without alanine the Hill-coefficient for this substrate is about one, in its presence the respective value is 1.9.

Fig. 2 shows a Dixon plot of the dependence of pyruvate kinase activity on the alanine concentration at different concentrations of ADP. The enzyme is apparently non-competitively inhibited by alanine in respect to ADP. One may conclude from the results of figs. 1 and 2, that the binding sites for alanine are different from those of the two substrates. The inhibition of the enzyme by alanine is very effectively counteracted by fructose 1, 6-diphosphate. At 2 mM alanine the half-saturation concentration is about 2 μM (at 0.3 mM phosphoenolpyruvate and 0.3 mM ADP).

In the absence of alanine no effect of fructose 1, 6-diphosphate on the enzyme activity at saturating concentrations of phosphoenolpyruvate and ADP could be detected.

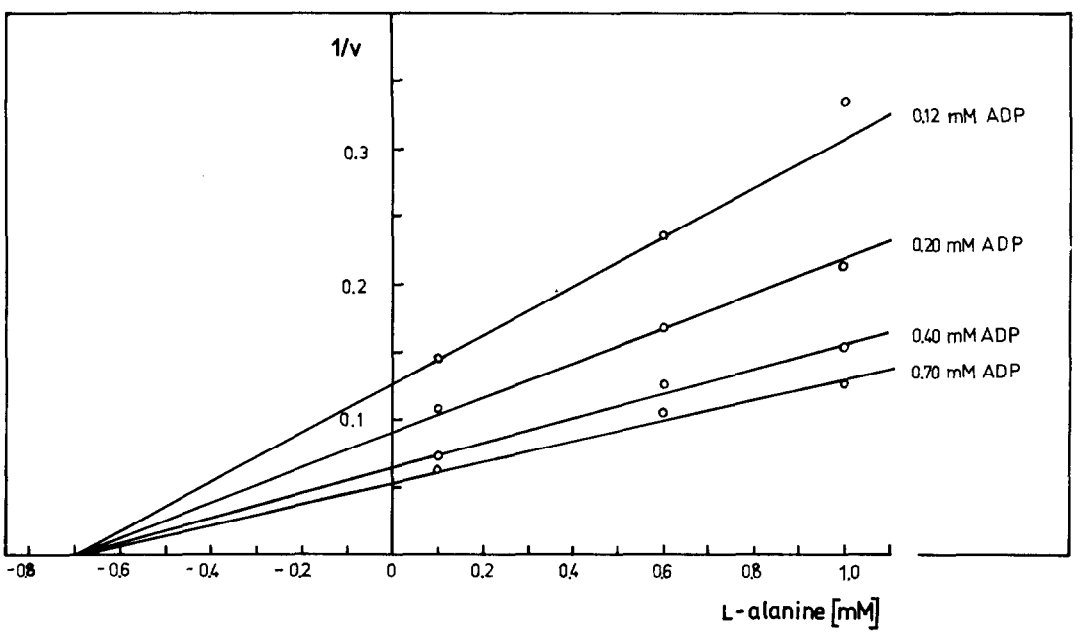

Fig. 2. Dependence of pyruvate kinase activity on L-alanine at various concentrations of ADP (Dixon plot) 3.0 mM phosphoenolpyruvate.

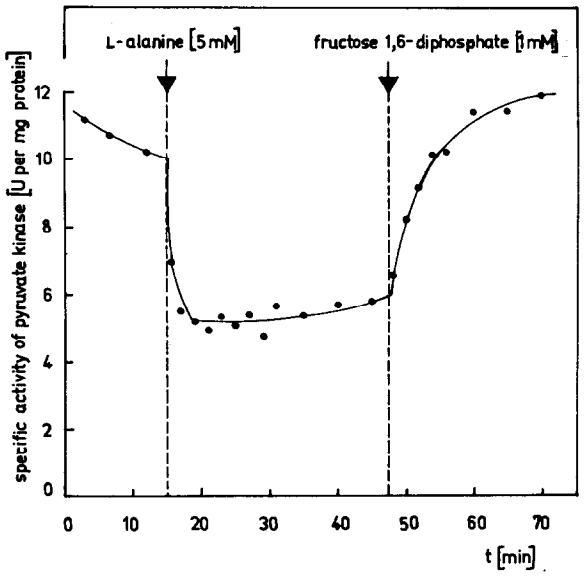

Fig. 3. Inhibition and reactivation of pyruvate kinase by L-alanine and fructose 1,6-diphosphate. Experimental: The enzyme was diluted in 40 mM Tris buffer, pH 7.2; 0.1 mM EDTA; 50 mM KCl; 5 mM $MgSO_4$; to a final protein concentration of 0.25 mg per ml incubation medium. Thereafter the enzyme was kept at 4°C and activity was measured at 25°C in aliquots taken from the mixture. The low temperature of incubation is necessary owing to a progressive inactivation of the diluted enzyme at room temperature in absence of stabilizing effectors like phosphoenolpyruvate, ADP or fructose 1,6-diphosphate, respectively.

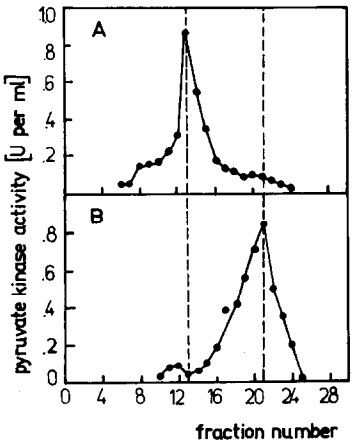

Fig. 4. Sucrose density gradient centrifugation of ascites tumour cell pyruvate kinase. Conditions as described in 'Materials and methods'. Enzyme activity was measured in aliquots after fractionation of the gradient under the following optimum conditions: 2.5 mM phosphoenolpyruvate; 2.5 mM ADP and 1.0 mM fructose 1,6-diphosphate, pH 7.2. *Gradient A.* This type of enzyme distribution is obtained under the following conditions: (1) without any ligand or; (2) 2 mM L-alanine or; (3) 2 mM L-alanine plus 0.5 mM phosphoenolpyruvate or; (4) 2 mM L-alanine plus 0.5 mM ADP, respectively. Fraction No. 13 corresponds to 5.3 S mol. wt about 100 000).*Gradient B.* This type of enzyme distribution is obtained under the following conditions: (1) 0.1 mM phosphoenolpyruvate or; (2) 0.2 mM ADP or; (3) 0.02 mM fructose 1,6-diphosphate or; (4) 2 mM L-alanine plus 0.05 mM fructose 1,6-diphosphate. Fraction No. 21 corresponds to 10 S mol. wt about 220 000).

Further information about the effects of alanine and fructose 1,6-diphosphate has been obtained by adding both effectors successively to the enzyme with an interval of about 30 min (fig. 3). The inhibition of the enzyme by alanine as well as its reactivation by fructose 1,6-diphosphate are time-dependent reactions requiring several minutes before attaining their respective steady state values. This result suggests that both effectors might induce relatively slow structural changes in the enzyme.

In fig. 4 the sedimentation pattern of pyruvate kinase after sucrose density gradient centrifugation under different conditions is presented. Both effectors influence the dissociation—reassociation equalibrium of the enzyme. In the presence of alanine the enzyme sediments with about 5.3 S, which corresponds to a molecular weight of about 100 000. Addition of fructose 1,6-diphosphate to the alanine -containing gradient causes an association of the enzyme sedimenting with about 10 S. Referring to experiments described above, one may suppose that the 5.3 S form is less active then the 10 S molecule.

The calculated molecular weight of the latter form is about 220 000, which is in fair agreement with the respective values of the pyruvate kinase from other biological sources [9]. Neither phosphoenolpyruvate nor ADP at the reported concentrations are able to reassociate the enzyme in the presence of alanine, underlining the specificity of the effect of fructose 1,6-diphosphate. When the enzyme is centrifuged without any effector it sediments with 5.3 S too, as is the case in the presence of alanine. However, in this case not only fructose

1, 6-diphosphate but also phosphoenolpyruvate or ADP, respectively, are effective in the reassociation of the enzyme to the 10 S molecule. Evidently, alanine stabilizes the 5.3 S form of tumour pyruvate kinase in such a way that it only may be reassociated by fructose 1, 6-diphosphate but not by one of its two substrates. In view of the molecular weight of the enzyme (about 220 000) and the molecular weight of its subunits (around 50–60 000, unpublished experiments) , one may conclude that the effector-mediated control of ascites tumour pyruvate kinase activity is caused by a dimer–tetramer equilibrium.

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