STABILIZATION OF DIFFERENT CONFORMATIONAL STATES OF L-TYPE PYRUVATE KINASE FROM RAT LIVER BY THE ALLOSTERIC INHIBITORS ALANINE AND ATP

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1. Introduction

The L-type pyruvate kinase (EC 2.7.1.40) from rat liver is an allosteric enzyme and its activity can be modulated to a great extent by various allosteric effectors [1]. The most important allosteric inhibitors are alanine and ATP, while Fru-1,6-P_2 is the most important activator. To describe the various allosteric interactions of the L-type, a model has been developed by Rozengurt et al. [2] which is based upon the allosteric model of Monod et al. [3]. This model predicts the existence of two unique conformational states. The R state is introduced by the activator Fru-1,6-P_2, H^+ ions, K^+ ions and high P-enolpyruvate concentrations, while the T state is stabilized by the inhibitors ATP and alanine. Recent evidence has shown that Fru-1,6-P_2 and K^+ ions induce a different conformational state rather than one unique R state [4]. From kinetic studies we concluded earlier [5] that alanine and ATP each introduce a different T conformation probably by binding to different sites. The here reported thermostability experiments support this interpretation and it can be concluded that sequential conformation changes [6] are involved in the allosteric transitions of the enzyme.

2. Materials and methods

L-type pyruvate kinase was isolated from rat liver according to the isolation procedure described earlier [5].

Stability experiments were performed at 53.0°C. The incubation mixture contained 0.25 M Tris-HCl pH 7.5, 5 mM reduced glutathione (GSH), 50 mM creatine-P and 0.1 mg creatine-P-kinase (EC 2.7.3.2). The total incubation volume was 1.0 ml containing precisely 6.5 mg protein. Samples were taken at the times indicated in the figures and immediately assayed for pyruvate kinase activity. The reaction mixture contained 1 mM ADP, 1 mM P-enolpyruvate, 0.5 mM Fru-1,6-P_2, 5 mM GSH, 225 mM KCl, 25 mM $MgCl_2$, 2 mM EDTA, 0.12 mM NADH, 0.1 mg lactate dehydrogenase and 25 mM Tris–HCl pH 7.5. To exclude effector changes during the incubation time at 53.0°C the reactions were stopped after 1 hr incubation with 5% perchloric acid. The effector concentrations were determined by standard procedures [7]. No significant changes were determined in the effector concentrations during the incubation procedure when compared to zero times.

3. Results

In fig.1 is plotted the effect of increasing alanine concentrations on the stability of the L-type pyruvate kinase at 53.0°C. It can be observed that alanine stabilizes the enzyme with a maximal effect at 10 mM alanine. A further increase in alanine concentration does not improve the thermostability of the enzyme. The semilogarithmic plot of the % activity vs time (fig.1B) shows biphasic curves, indicative for two pseudo first-order inactivation steps. Such a biphasic inactivation behaviour is also found for yeast pyruvate kinase [8,9].

Figs. 2A and 2B show that the effect of ATP on the L-type pyruvate kinase from rat liver is similar to the effect of alanine. Increasing concentrations of ATP stabilize the enzyme and a maximum stabilization

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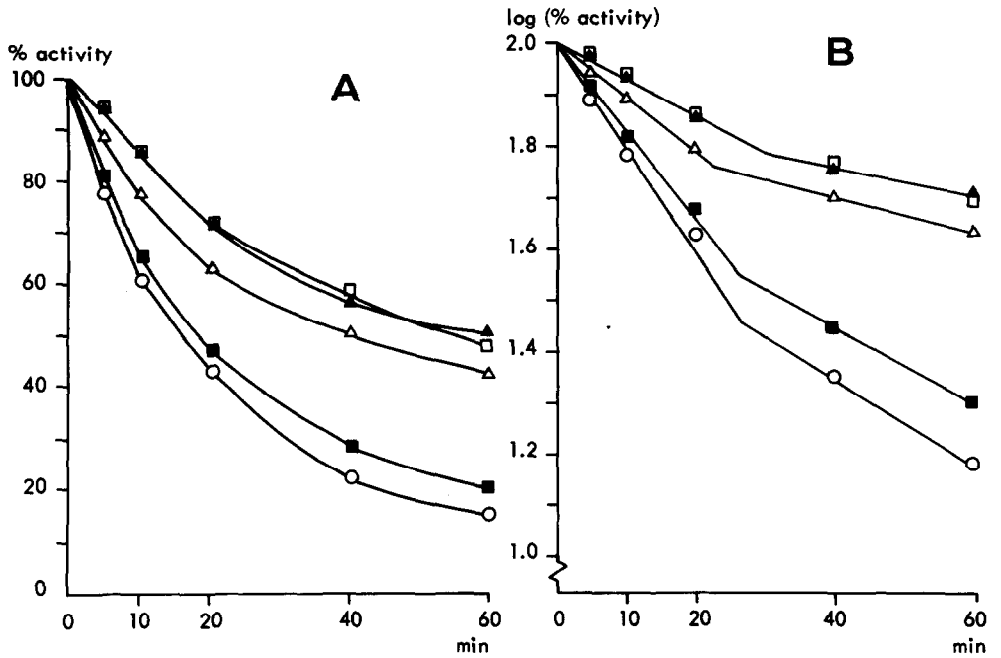

Fig.1A, B. Influence of increasing alanine concentrations on the stability of L-type pyruvate kinase at 53.0°C. (○—○) control. (■—■) 1 mM Ala. (△—△) 5 mM Ala. (▲—▲) 10 mM Ala. (□—□) 15 mM Ala.

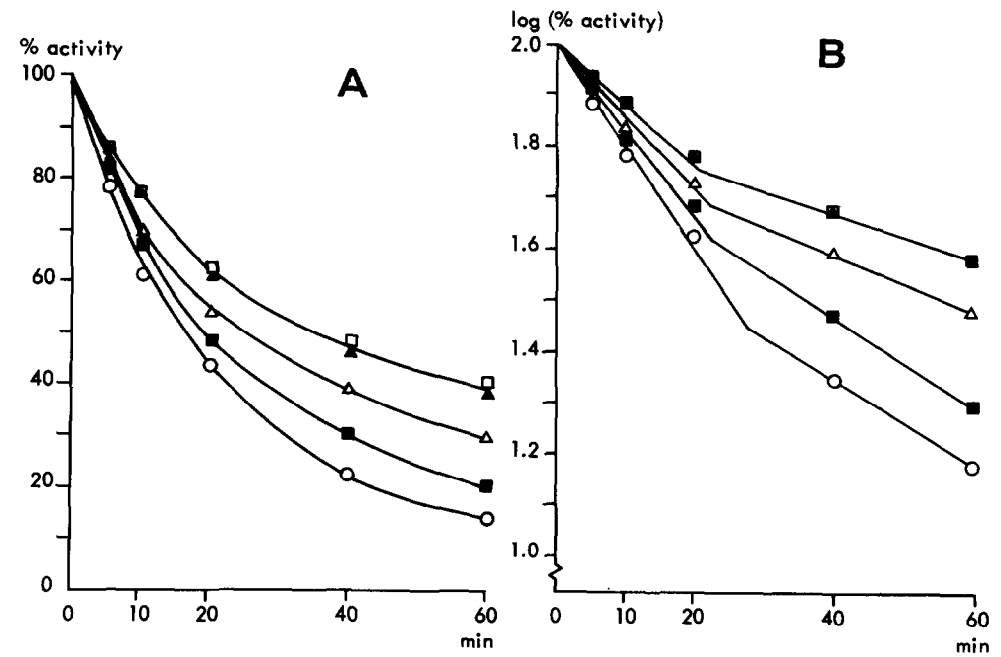

Fig.2A, B. Influence of increasing ATP concentrations on the stability of L-type pyruvate kinase at 53.0°C. (○—○) control. (■—■) 1 mM ATP. (△—△) 5 mM ATP. (▲—▲) 10 mM ATP. (□—□) 15 mM ATP.

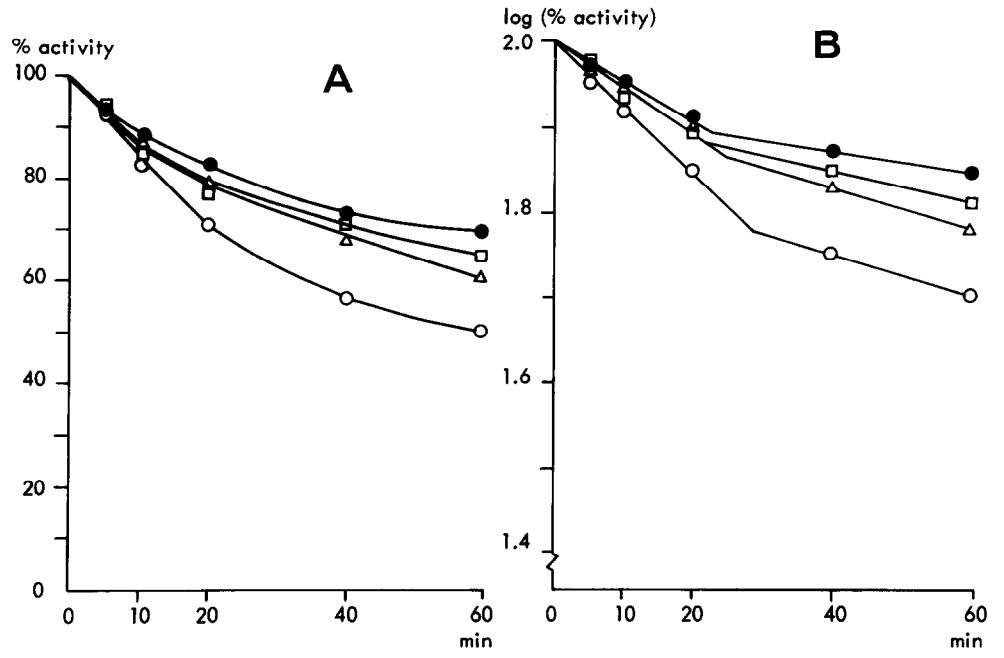

Fig.3A, B. Influence of increasing ATP concentrations on the stability of L-type pyruvate kinase at 53.0°C in the presence of 10 mM alanine. (○—○) 10 mM Ala. (Δ—Δ) 10 mM Ala + 1 mM ATP. (□—□) 10 mM Ala + 5 mM ATP. (●—●) 10 mM Ala + 10 mM ATP.

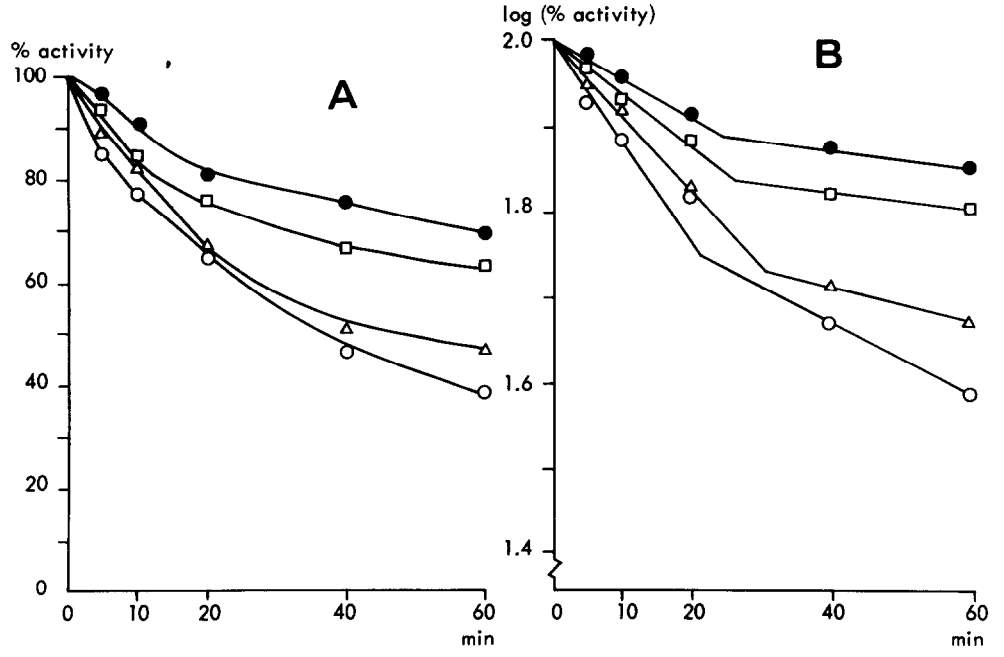

Fig.4A, B. Influence of increasing alanine concentrations on the stability of L-type pyruvate kinase at 53.0°C in the presence of 10 mM ATP. (○—○) 10 mM ATP. (Δ—Δ) 10 mM ATP + 1 mM Ala. (□—□) 10 mM ATP + 5 mM Ala. (●—●) 10 mM ATP + 10 mM Ala.

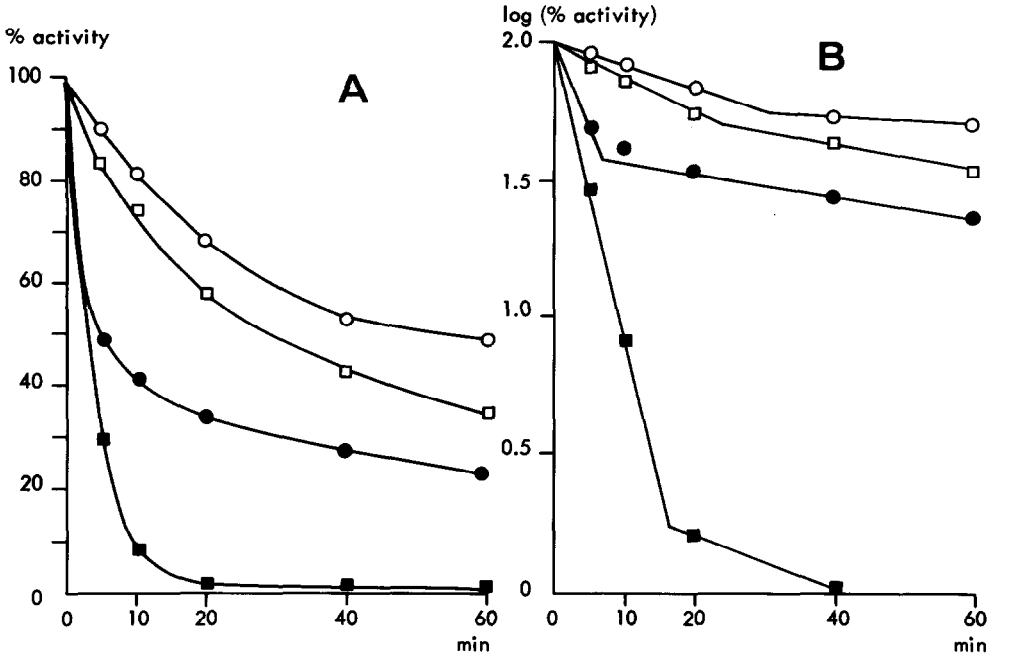

Fig.5A, B. Influence of ATP and alanine on the stability of L-type pyruvate kinase at 53.0°C in the absence and presence of 1 mM Fru-1,6-P_2. (□—□) 10 mM ATP. (■—■) 10 mM ATP + 1 mM Fru-1,6-P_2. (○—○) 10 mM Ala. (●—●) 10 mM Ala + 1 mM Fru-1,6-P_2.

occurs at 10 mM ATP. Also in the presence of ATP the semilogarithmic plot (fig.2B) is biphasic. However, the extent of maximal stabilization with ATP is lower than with alanine. With the five different preparations of L-type pyruvate kinase tested, it was found that the residual activity after incubation for 60 min with alanine (10 mM) was at least 10% higher than after incubation with ATP (10 mM). This difference in stability is indicative for different conformations, at least under the conditions used.

Figs. 3A and 3B show that addition of ATP to the maximally alanine (10 mM) – stabilized enzyme results in a further stabilization. The same phenomenon occurs when alanine is added to the enzyme which is maximally stabilized by ATP (figs. 4A and 4B). Also in the presence of both alanine and ATP the semilogarithmic plot is biphasic which indicates that the order of inactivation does not change. From figs. 3 and 4 we can conclude that the alanine-stabilized enzyme can be converted by ATP into a conformation (and vice versa) which is even more stable, suggesting that alanine and ATP do not stabilize one unique T conformation as predicted by the model of Monod et al. [3].

Figs. 5A and 5B show that also in the presence of the allosteric activator Fru-1,6-P_2 [10,11] the effects of ATP and alanine are not similar. Fru-1,6-P_2 labilizes the L-type pyruvate kinase in the same way (unpublished) as has been shown earlier for the yeast enzyme [8,9]. Fig.5 shows that also in the presence of alanine or ATP addition of Fru-1,6-P_2 results in a strong labilization of the enzyme. The loss of activity in the presence of ATP differs from that in the presence of alanine, indicating again that the effect of these compounds are not identical.

4. Discussion

By comparison of the effects of ATP and alanine on the apparent affinities of L-type pyruvate kinase for its allosteric activators Glc-1,6-P_2, Glc-6-P, Fru-1,6-P_2 and Fru-6-P, it was concluded earlier that ATP and alanine introduce a different conformational state,

probably by binding to different sites [5]. The results presented here support this conclusion. ATP and alanine both stabilize the L-type pyruvate kinase. However, the degree of maximal stabilization is greater for alanine than for ATP. This result is difficult to explain by the assumption that these compounds stabilize one unique T conformation as predicted by the Monod model [3] which has been applied to the L-type from liver [2]. Furthermore, figs. 3 and 4 show that a further stabilization occurs when ATP is added to the maximally alaninestabilized enzyme or vice versa, indicating additive effects of alanine and ATP on the stabilization. This indicates that ATP and alanine induce different conformations and bind to different sites on the enzyme. Fru-1,6-P_2 labilizes the enzyme, an effect which can be expected from an allosteric activator which normally decrease the interaction between the subunits. However, also in the presence of Fru-1,6-P_2 the effects of alanine and ATP are different. Rozengurt et al. [4] recently showed that the allosteric activators K^+ and Fru-1,6-P_2 each induce a different R conformation. Our data indicate that also different T conformations are possible. These results, together with the earlier obtained kinetic data [5] are difficult to explain with the two state model of Monod et al. [3] and indicate that sequential conformation changes [6] are involved in the allosteric transitions of this enzyme.

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