ALANINE-MEDIATED REVERSIBLE INACTIVATION OF TUMOUR PYRUVATE KINASE CAUSED BY A TETRAMER-DIMER TRANSITION

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1. Introduction

Pyruvate kinase (ATP: pyruvate phosphotransferase, EC 2.7.1.40) from Ehrlich ascites tumour cells resembles the M_2-type of mammalian pyruvate kinases both in its electrophoretic mobility and in its basic kinetic properties [1]. This enzyme undergoes a slow dimer-tetramer transition being oppositely influenced by negative and positive allosteric effectors [2].

In this paper experiments will be presented showing that the effect of the allosteric inhibitor L-alanine on the activity of ascites tumour pyruvate kinase is directly related to a temperature and time dependent, as well as ligand influenced reversible dissociation of the tetrameric enzyme into dimers.

2. Materials and methods

The ascites tumour cell pyruvate kinase used in this study was obtained after step 4 of the purification procedure [3]. Before use, the enzyme preparation was subjected to gel filtration on a Sephadex G-25 column in the following medium: 40 mM triethanol–HCl, 50 mM KCl, and 5 mM $MgSO_4$, pH 7.2. The protein concentration was determined spectrophotometrically at 280 nm using an absorption coefficient of $\epsilon = 0.54$ ml/mg · cm [4]. Thereafter the enzyme was diluted using the same medium to a definite final protein concentration and kept at fixed temperatures. To this incubation mixture the respective ligand has been added as indicated in the text.

At different times, the activity was measured in small aliquots (10–50 μl) of the incubation mixture by immediately adding to the complete reaction mixture (0.5 or 1.0 ml respectively) in the cuvette containing 2.5 mM ADP, 2.5 mM phosphoenolpyruvate, 100 mM KCl, 25 mM $MgSO_4$, 0.3 mM NADH, 200 mM triethanolamine pH 7.2, and 1.5 or 3 U lactate dehydrogenase. All assays were carried out at 25°C in an Eppendorf Photometer at 334 or 366 nm. No reactivation of the cold induced alanine-inactivated enzyme was observed in the course of the short period of measuring enzyme activity at 25°C.

The sedimentation coefficients of the incubated enzyme were determined by means of sucrose density gradient centrifugation as described previously [2] except that the centrifugation was performed for 4 hr at 22°C. To assure full enzyme activity the fractions were brought to 1 mM fructose 1,6-bisphosphate before the enzymatic assay.

3. Results and discussion

It was shown in a previous paper, that pyruvate kinase from Ehrlich ascites tumour cells undergoes a partial inactivation when it is incubated with L-alanine at 4°C. A complete reactivation was achieved by fructose 1,6-bisphosphate [2].

Fig.1 demonstrates that in presence of alanine an inactivation–reactivation cycle can be produced by alternating changes of the incubation temperature. Table 1 shows the activity of pyruvate kinase at 25°C after incubation of the enzyme at different temperatures in presence and absence of alanine. From fig.1 and table 1 it becomes evident that alanine induces a reversible cold inactivation of tumour pyruvate kinase.

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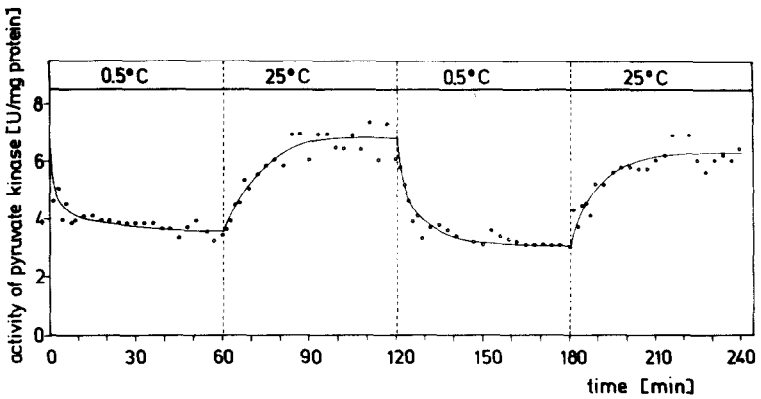

Fig.1. Activity pattern of ascites tumour pyruvate kinase during alternative incubation at 0.5°C and 25°C in presence of alanine. 0.25 mg enzyme per ml were incubated in presence of 5 mM L-alanine. For medium composition and activity measurements see in 'Materials and methods'. About 3 min after temperature change the incubation mixture had reached the desired temperature indicated on the top of the figure. In absence of alanine (control) no changes in the activity during the alternative incubation is detectable.

Only fructose 1,6-bisphosphate, but none of the two substrates, phosphoenolpyruvate or ADP, appear to be able to counteract the alanine-induced cold inactivation of the enzyme.

In fig.2 cold inactivation and thermal reactivation of pyruvate kinase in dependence on the protein concentration is shown. Immediately after dilution no change of specific activity is observed (curve a), indicating that the rate of enzyme inactivation by alanine is low.

Table 1
Activity of pyruvate kinase at 25°C in dependence of the temperature of preincubation in presence and absence of alanine

Incubation	with 5 mM L-alanine	without alanine
Temperature (°C)	Activity of pyruvate kinase (U/mg prot.)	
6	14.3	24.0
16	16.2	25.9
25	24.4	23.0
35	24.6	13.8

The enzyme has been preincubated at the respective temperature for 70 min and initial activity has then been measured at 25°C. At 35°C a protection against heat inactivation by alanine can be observed during the preincubation period.

With increasing protein concentrations the degree of cold inactivation is diminished (curve b) whereas thermal reactivation is significantly promoted (curve c). These results provide evidence that reversible inactivation of the enzyme could be due to a temperature dependent reversible dissociation. Indeed, in previous experiments at 4°C a tetramer-dimer transition of the enzyme could be demonstrated by means of sucrose density gradient centrifugation [2].

As it is shown in fig.3A, at 22°C in absence of any ligand the dimer and tetramer are coexisting indicating an equilibrium between these two oligomeric forms of the enzyme. Because at 4°C only the dimer is detectable one may conclude, that lower temperature favours dissociation of the tetramer to the dimer. In presence of alanine, only dimers are occuring both at 4°C and at 22°C.

By taking into account that low protein concentrations have been used in the sucrose density gradient centrifugation experiments, one may conclude that the reason of the inactivating effect of alanine is the dissociation of the tetrameric enzyme into dimers which might be subactive or even inactive. From fig.2 it may be derived that the alanine caused dissociation is diminished by increasing temperatures as well as increasing enzyme concentrations. Fructose 1,6-bisphosphate under all conditions has been found to associate the dimers by forming tetramers in presence as well as in absence of alanine.

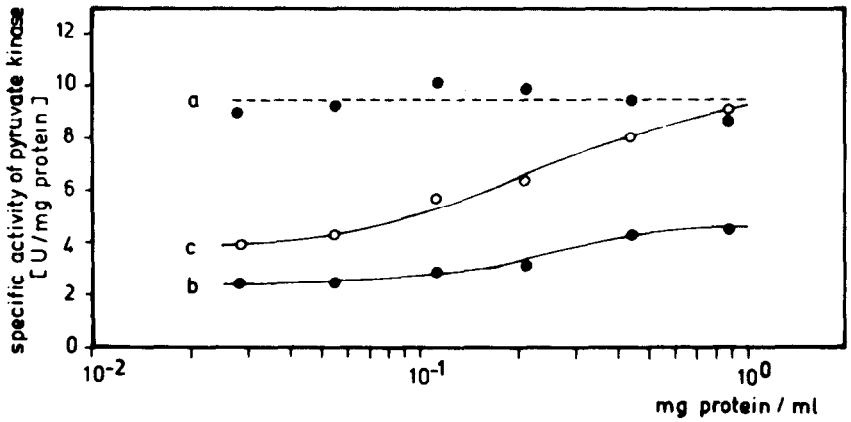

Fig.2. Effect of the protein concentration on the alanine-mediated cold inactivation of pyruvate kinase and its thermal reactivation. The enzyme was diluted to the respective protein concentrations at 22°C in presence of 5 mM L-alanine. Then the mixture was kept at 1°C for 42 min, followed by incubation at 22°C for 38 min. Specific activity of pyruvate kinase; (a) immediately after dilution of the enzyme at 22°C (control), (b) after reaching a constant level of activity at 1°C, and (c) after maximum reactivation at 22°C.

The effects of the two substrates, phosphoenolpyruvate and ADP, on the dimer-tetramer transitions are significantly different from that of fructose 1,6-bisphosphate. In absence of alanine the dimers promptly associate to tetramers at low concentrations of either of the two substrates both at 4°C [2] and 22°C (fig.3A).

In presence of alanine at 4°C neither ADP nor phosphoenolpyruvate have an associating effect on the dimers in a concentration range from 0.1 up to 3 mM of each substrate [2]. The actual effects of the three ligands on the alanine stabilized dimeric form of the tumour pyruvate kinase are directly related to their influence on the alanine mediated inactivation of the enzyme. Only fructose 1,6-bisphosphate is able to reactivate and to reassociate the cold treated enzyme in presence of alanine, whereas ADP or phosphoenolpyruvate at the concentrations tested are at 4°C without any detectable effect.

At 22°C and low enzyme concentrations however,

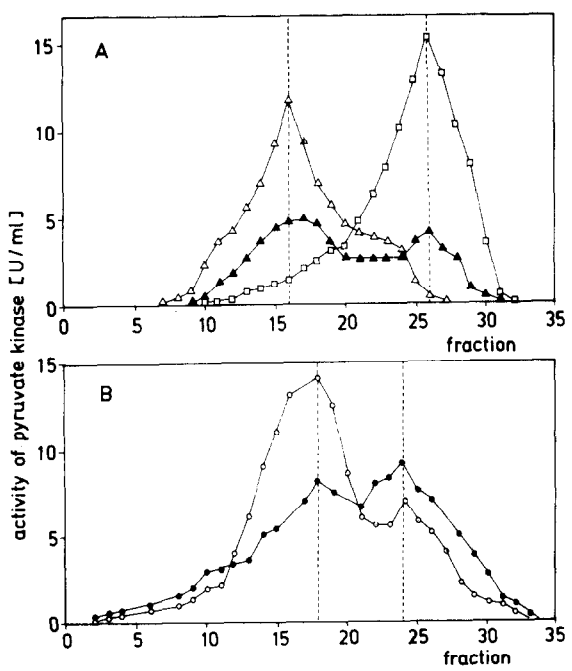

Fig.3. Sucrose density gradient centrifugation of pyruvate kinase in absence and presence of alanine and phosphoenolpyruvate. At 22°C the diluted enzyme was incubated with the respective ligand and then centrifuged as described in 'Materials and methods'. Ligand concentrations: 0.25 mM phosphoenolpyruvate or 0.25 mM ADP (□–□–□); 1 mM L-alanine (△–△–△); without any ligand (▲–▲–▲); 0.25 mM phosphoenolpyruvate plus 2 mM L-alanine (○–○–○); 2.5 mM phosphoenolpyruvate plus 2 mM L-alanine (●–●–●). Fig. A and B represent independent experiments. The vertical dotted lines indicate the peak fractions of the dimer (left) and of the tetramer (right).

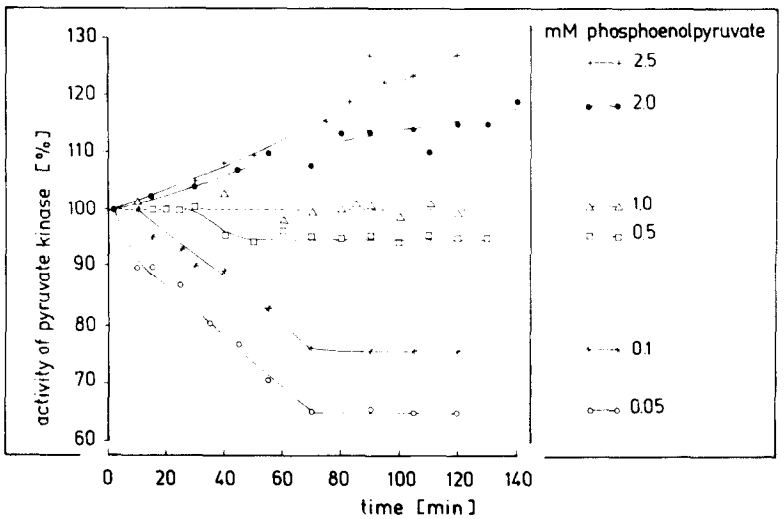

Fig.4. Influence of phosphoenolpyruvate on the alanine induced temporal changes of pyruvate kinase activity. At zero the enzyme was diluted to a final concentration of 1 μg protein per ml and then incubated at 22°C in 40 mM triethanolamine buffer pH 7.2, 50 mM KCl, 5 mM $MgSO_4$, 0.1 mM NADH, 3 U lactate dehydrogenase per ml, 2 mM L-alanine, and phosphoenolpyruvate as indicated. At various time intervals the catalytic reaction was started by addition of ADP to a final concentration of 0.5 mM. Activity of pyruvate kinase is expressed as percentage of the activity at zero. The absolute values of enzyme activity depend on the phosphoenolpyruvate concentration.

the alanine promoted dissociation is reversed by phosphoenolpyruvate, but not by ADP.

Clearly a concentration dependence of phosphoenolpyruvate on reassociation is detectable (fig.3B).

Therefore one could expect that phosphoenolpyruvate should also influence the inactivation of pyruvate kinase by alanine at 22°C. A respective experiment is shown in fig.4. During incubation of the enzyme at low concentration and at 22°C with 2 mM alanine and varying concentrations of phosphoenolpyruvate time-dependent changes of the activity are observed. At phosphoenolpyruvate concentrations less than 1 mM the activity decreases before reaching a plateau, while at concentrations higher than 1 mM the activity increases. Both processes are very slow in respect to the catalytic reaction, and hence, they might reflect a hysteretic response of the enzyme [5]. The velocity of the reactivation by fructose 1,6-bisphosphate [2] or upon rewarming of the cold treated alanine containing enzyme as discussed previously has also been found to be relatively slow.

By connecting the temperature dependence of activity (table 1) with that of the respective sedimentation pattern of the enzyme, it seems that in absence of alanine the addition of substrates (as well as fructose 1,6-bisphosphate) to the enzyme causes a rapid reassociation of the dimer to the full active tetramer. In fact, the activity of the enzyme incubated at 5°C equals that at 25°C in absence of alanine, although the free enzyme occurs at 5°C only as dimers. Because alanine strongly favours the dimer, one may assume that in presence of this effector the dimer is

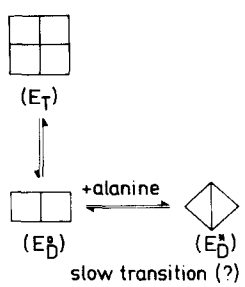

Fig.5. Model of the tetramer-dimer transition of tumour pyruvate kinase. E_T = full active tetramer, E_D^o = dimer able to associate, E_D^* = dimer unable to associate by being bound to L-alanine.

trapped by an isomerisation reaction between a free and an alanine bound dimer fraction which might be the rate limiting step of the slow inactivation and reactivation of the enzyme (fig.5).

A paper dealing with the kinetic consequences of the three-state model of tumour pyruvate kinase is in preparation.

References

[1] Hofmann, E., Kurganov, B. I., Schellenberger, W., Schulz, J., Sparmann, G., Wenzel, K.-W. and Zimmermann, G. (1975) in: Advances in Enzyme Regulation (G. Weber, ed.) 13 (in press), Pergamon Press.
[2] Sparmann, G., Schulz, J. and Hofmann, E. (1973) FEBS-Lett. 36, 305–308.
[3] Schulz, J., Wilhelm, G., Lorenz, G. and Hofmann, E. Eur. J. Biochem., submitted for publication.
[4] Bücher, T. and Pfleiderer, G. (1955) in: Methods in Enzymology (S. P. Colowick and N. O. Kaplan, eds.) Vol. 1, Academic Press, New York, p. 435.
[5] Frieden, C. (1970) J. Biol. Chem. 245, 5788–5799.