INTERCONVERTIBLE FORMS OF CLASS A PYRUVATE KINASE FROM EHRLICH ASCITES TUMOUR CELLS

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1. Introduction

Three major types of pyruvate kinase have been identified in mammalian tissues by electrophoretic [1,2] and immunological techniques [3]. Recently, Carbonell et al. [4] have achieved by kinetic studies on pyruvate kinases of rat and human tissues, the identification of three classes* of isozymes with qualitative differences in regulatory properties: the well known classes L (liver) and M (muscle), and a regulable new one which was designated as class A because it was first observed in adipose tissue [5,6]. This class A appears to be the same that the M_2-PK [3], PK-III [7] and PK-K [8] identified in other laboratories. It is the most widely extended class of pyruvate kinase in mammalian tissues including different tumoral cells [3,9,10].

The occurrence of interconvertible forms of pyruvate kinase has been previously reported. Pogson [5] found two interconvertible forms in pyruvate kinase from rat adipose tissue, and recently Walker and Potter [7] in pyruvate kinase from cultured hepatic cells.

We report here the kinetic characterization of the pyruvate kinase from Ehrlich ascites tumour cells as class A and evidence of two interconvertible forms of the enzyme with different regulatory properties. The form present in fresh extracts depends on the metabolic conditions of the cells prior to the homogenization process.

2. Materials and methods

2.1. *Biological material*

A hypertriploid strain of Ehrlich ascites carcinoma cells was grown in 2-month-old Swiss mice, and harvested about the seventh day after inoculation. The cells were withdrawn from the sacrificed animal, washed in an isotonic solution (140 mM NaCl and 5 mM Tris–HCl pH 7.4), and centrifuged at low speed at 2–4°C.

2.2. *Preparation of extracts*

Washed cells were suspended in 10 mM Tris–HCl pH 7.4 and 0.5 mM dithioerythritol, placed in a propilene centrifuge tube and frozen and thawed twice in liquid nitrogen. The resulting homogenate was centrifuged at 30 000 *g* for 15 min at 2–4°C. Samples of the supernatant were used for kinetic studies, generally within 1 or 2 hr after its prepara-

* This grouping of the isozymes in classes does not interfere with the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature [11] for the designation of individual isozymes.

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tion and kept on ice from this moment until it was added to the assay mixture.

2.3. *Enzyme assay*

Pyruvate kinase was assayed by the method of Bücher and Pfleiderer [12] with a Gilford model 2.400 spectrophotometer at 25°C. The final vol of the reaction mixture was 1 ml containing, unless indicated otherwise, 50 mM Tris–HCl pH 7.4, 100 mM KCl, 5 mM $MgCl_2$, 0.15 mM NADH, 2 mM MgADP, 2.5 mM phosphoenolpyruvate and 1 unit lactate dehydrogenase. Protein concentration was estimated by the method of Lowry et al. [13].

Phosphoenolpyruvate (monosodium salt), fructose 1,6-bisphosphate (trisodium salt), nucleotides and auxiliary enzymes were from Boehringer. Amino acids were of the L-series, unless indicated otherwise, and obtained from Mann and Sigma.

2.4. *Preparation of starved cells*

Starved cells were prepared by incubating washed cells 4–6 hr in a phosphate saline solution in a shaking bath at 30°C, with aeration. The phosphate saline solution contained: 144 mM NaCl, 17 mM Na_2HPO_4 and 3 mM NaH_2PO_4, at final pH 7.4. The percentage of viable cells was controlled at the beginning and at the end of the incubation, by Tripan Blue staining [14]. Initially the amount of stained cells was of 2–3 per cent, and it did not increase after the starvation period.

In two experiments the glycolytic flux, as indicated by the lactate production, of the tumour cells was measured by continuous recording of pH changes as described previously [15]. In recently harvested cells the endogenous glycolysis was high, but in starved cells no lactate production was detected. However, after glucose addition the lactate production became normal.

3. Results

3.1. *Kinetic characterization*

We have characterized the pyruvate kinase from extracts of recently harvested Ehrlich ascites tumour cells as class A, by the criteria developed by Carbonell et al. [4], since as shown in table 1, it is strongly inhibited by phenylalanine and alanine and essentially unaffected by 2 mM MgATP, in contrast with the marked inhibition produced by the same concentration of MgATP on class L pyruvate kinase [3,16].

Table 1
Regulatory properties of pyruvate kinase from extracts of recently harvested Ehrlich ascites tumour cells

Effectors	Activity (units/mg protein)	Inhibition (%)
None	0.55	–
Alanine	0.2	64
Alanine + Fru-P_2	0.55	–
Phenylalanine	0.08	86
Phenylalanine + Fru-P_2	0.53	4
MgATP	0.53	4

The assay mixture was as indicated in Materials and methods, with 0.25 mM phosphoenolpyruvate. The final concentration of alanine, phenylalanine and MgATP was 2 mM. Fructose 1,6-bisphosphate (Fru-P_2) was assayed at 0.02 mM.

3.2. *Pyruvate kinase from recently harvested, non-starved, tumour cells*

Extracts from recently harvested ascites cells were prepared as indicated above, and immediately assayed for pyruvate kinase activity. In these conditions the substrate–velocity curve, in the absence of effectors, was hyperbolic with an apparent K_m for phosphoenolpyruvate of 0.065 mM, but in the presence of 2 mM alanine or phenylalanine it displayed a sigmoidal shape with n_H values of 1.4 and 2 respectively.

Fig.1 shows the effect of alanine and phenylalanine at different concentrations on the pyruvate kinase activity. The apparent $I_{0.5}$ values (inhibitor concentration producing half-maximal inhibition), for alanine and phenylalanine were 0.1 and 0.4 mM respectively. As shown in the insert of this figure, the enzyme displays homotropic cooperativity for the amino acid inhibitions, with n' values calculated by the method of Jensen and Nester [17] of 1.3 for alanine and 1.5 for phenylalanine.

Fructose 1,6-bisphosphate did not activate the enzyme in the absence of inhibitory amino acids, but reversed the inhibition produced by alanine and phenylalanine with an apparent K_a of 0.003 mM (not shown). Similar results have been obtained by Sparmann et al. [18].

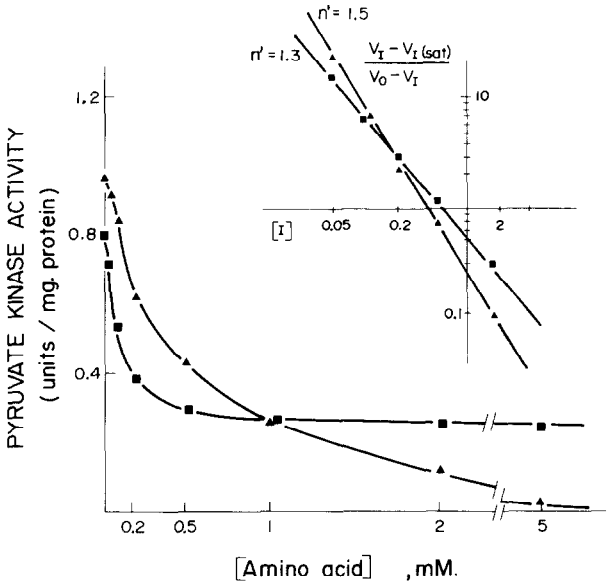

Fig.1. Effects of alanine and phenylalanine on the activity of pyruvate kinase from recently harvested tumour cells. (■) Alanine and (▲) phenylalanine. Assay as indicated in Materials and methods, with 0.25 mM phosphoenolpyruvate. In the insert, plots by the method of Jensen and Nester [17].

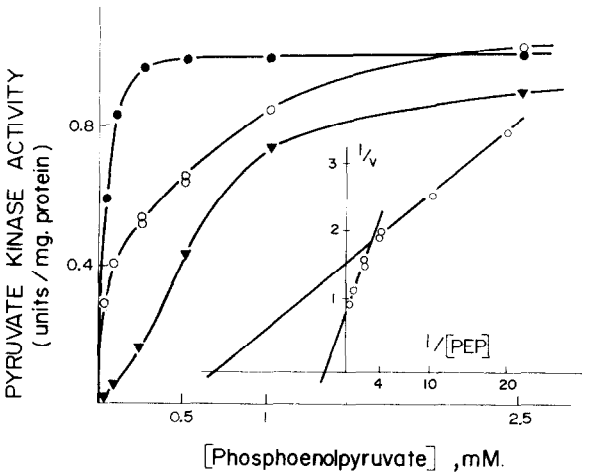

Fig.2. Activity of pyruvate kinase from aged extracts of recently harvested tumour cells, at various concentrations of phosphoenolpyruvate. (○) Without preincubation; preincubated with 0.02 mM fructose 1,6-bis-phosphate (●) or 1 mM ATP (▼), at 25°C for 20 min. Assay as indicated in Materials and methods. In the insert a double-reciprocal plot of the kinetics in the absence of preincubation.

3.3. *Effect of the extract ageing on the substrate–velocity curve for phosphoenolpyruvate*

The outlined kinetic study, as indicated, was carried out in fresh extracts of recently harvested cells. However when extracts kept in ice or at room temperature for hours or extracts from cells with delayed homogenization were used, the substrate–velocity curve for phosphoenolpyruvate displayed a biphasic shape (fig.2). By the Lineweaver Burk plot two enzymatic activities became evident with different apparent affinities for this substrate, one of them with a K_m of 0.06 mM and the other, showing smaller affinity, with a K_m of 0.5 mM. The incubation of the extracts displaying this anomalous kinetics with 0.02 mM fructose 1,6-bisphosphate led to a Michaelis–Menton type, with high affinity for the phosphoenolpyruvate. In contrast, the incubation of the same extract with 1 mM ATP resulted in a pyruvate kinase form with sigmoidal substrate–velocity curve for phosphoenolpyruvate, with a $S_{0.5}$ of 0.5 mM and a n_H value of 1.4 (fig.2).

The effectors used in the preincubation experiments of the extracts, fructose 1,6-bisphosphate and ATP, were diluted 200 times when the extract sample was added to the reaction mixture. At the resulting concentrations, there was no apparent effect on the kinetics, as tested separately.

3.4. *Pyruvate kinase from starved tumour cells*

Pyruvate kinase from extracts of cells kept in starvation conditions (see Materials and methods) for 4–6 hr, showed a sigmoidal substrate–velocity curve for phosphoenolpyruvate with an apparent $S_{0.5}$ of 0.5 mM and a n_H value of 1.4, similar to the kinetics displayed by pyruvate kinase from aged extracts incubated with ATP, as reported above. The addition of 5 mM glucose to the starved cells carrying pyruvate kinase with sigmoidal kinetics against phosphoenolpyruvate, converted, in vivo, within 5 min, the enzyme to the form with hyperbolic kinetics and higher affinity for this substrate.

Fig.3 shows the effect of alanine and phenylalanine at various concentrations on the activity of pyruvate kinase from starved tumour cells. The $I_{0.5}$ values of the amino acids for this form of the enzyme are 0.02 mM for alanine and 0.05 mM for phenylalanine. The

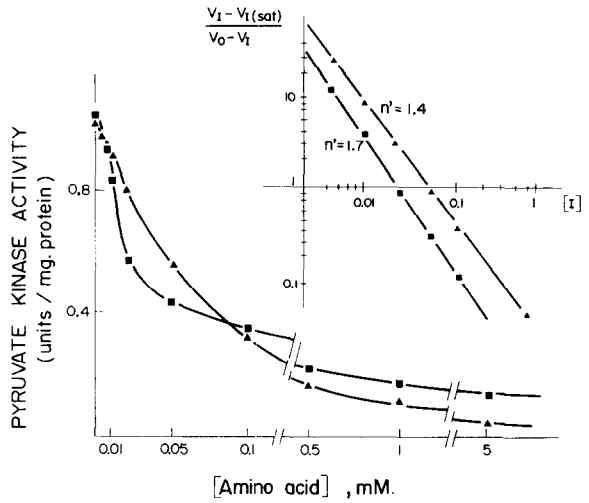

Fig.3. Effects of alanine and phenylalanine on the activity of pyruvate kinase from extracts of starved cells. (■) Alanine and (▲) phenylalanine. Assay as indicated in Materials and methods, with 0.25 mM phosphoenolpyruvate. In the insert, plots by the method of Jensen and Nester [17].

enzyme shows also homotropic cooperativity for the amino acid inhibitions with n' values of 1.4 and 1.3 for alanine and phenylalanine respectively. Cysteine also inhibits the enzyme, but to a lesser extent. The $I_{0.5}$ value for cysteine was 0.02 mM with 0.25 mM phosphoenolpyruvate. The corresponding D-amino acids were completely ineffective.

4. Discussion

We report here that pyruvate kinase from Ehrlich ascites tumour cells belongs to class A, according to its regulatory properties, bringing new evidence on the presence of two interconvertible forms in pyruvate kinase class A, as reported for this class of pyruvate kinase from other sources [5,7,18], and showing regulatory differences between these two interconvertible forms. One of these forms, present in extracts from cells with high glycolytic flux, shows a Michaelis–Menton kinetics against phosphoenolpyruvate with high affinity for this substrate. It is the physiologically active form of the enzyme, since at the phosphoenolpyruvate and fructose 1,6-bisphosphate concentrations in glycolysing ascites cells [19] there is no inhibition by alanine and phenylalanine. The second form, present in extracts from cells undergoing little or no glycolysis, displays sigmoidal kinetics against phosphoenolpyruvate with low affinity for this substrate, and high sensitivity to alanine and phenylalanine inhibition. It should be practically inactive at in vivo concentrations of substrates and effectors [19].

Aged extracts or extracts from cells with delayed homogenization, showed a biphasic substrate–velocity curve for the phosphoenolpyruvate that seems to correspond to a mixture of the two interconvertible forms of the enzyme, as indicated by the incubation of these extracts with ATP and fructose 1,6-bisphosphate. In vitro, fructose 1,6-bisphosphate shifts the equilibrium between the two interconvertible forms, to the hyperbolic one, and ATP to the sigmoidal form of the enzyme.

In vivo, the conversion of the sigmoidal (physiologically inactive) form, present in starved cells, to the hyperbolic (highly active) form of the enzyme, following glucose addition to the suspension of starved cells, could be mediated by the rising of the intracellular concentration of fructose 1,6-bisphosphate in the first minute after the glucose addition [20,21].

The physiological significance of these two interconvertible forms of pyruvate kinase could be related to the coordination of the overall functioning of the glycolytic pathway. Besides phosphofructokinase, pyruvate kinase seems to be a key enzyme in the regulation of the lower part of glycolysis [6,15]. The fructose 1,6-bisphosphate level should be the metabolic indicator of the activity of the higher part of this pathway. When the level of this indicator drops because of slow glycolysis, the pyruvate kinase should be converted to the inactive form, braking the glycolytic flux in the lower part of the pathway, buffering in this manner the changes in the levels of the intermediates between phosphofructokinase and pyruvate kinase.

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