A NEW FORM OF PYRUVATE KINASE IN MYCELIUM OF *MUCOR ROUXII*

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1. Introduction

We have previously shown [1] that extracts of mycelium of the dimorphic fungus *Mucor rouxii* grown in glucose, contain two forms of pyruvate kinase (Type I and II), which can be separated by DEAE-cellulose chromatography and by electrophoresis in polyacrylamide gel. Extracts from ungerminated sporangiospores or yeast-like cells contain only the Type I form. Type II appears during aerobic germination of spores or during conversion of yeast-like cells to mycelium.

In the present communication we describe a third form of pyruvate kinase (Type III), which can be detected in extracts of mycelium grown on casaminoacids as sole carbon source. This form of the enzyme is labile to dilution and is much less responsive to activation by fructose-1,6-diphosphate (FDP) than Type I or II [2].

2. Materials and methods

Materials and methods were as described in the preceding paper [1] unless otherwise indicated.

Mucor rouxii (NRRL 1894) was grown from a spore inoculum (about 10^5 spores/ml) in a defined medium [3] supplemented with 0.4% Difco vitamin-free casaminoacids (AA-medium). When indicated, 2% glucose was added to the medium. The cultures were incubated aerobically for 18–20 hr in a rotatory shaker at 30°, harvested and stored as descibed elsewhere [1].

For preparation of mycelium extracts, the mycelial pads, frozen in liquid air, were ground in a porcelain mortar. The fine powder thus obtained was homogenized in a glass homogenizer with two volumes of 10 mM Tris-Cl buffer pH 7.4, containing 5 mM mercaptoethanol and 1 mM EDTA (buffer A), in 30% glycerol. The extract was centrifuged at 3000 *g* for 15 min, and the supernatant fraction was clarified by centrifugation at 30,000 *g* for 45 min. The supernatant fluid was used as source of enzyme (crude extract).

Initial attempts to purify pyruvate kinase Type III from AA-grown cells were hampered by the lability of the enzyme in fairly diluted solutions and under the conditions used for fractionation. We found subsequently that Mg^{2+} stabilized the enzyme activity. This allowed us to develop the following purification procedure: 12 g of AA-grown mycelium powder was suspended in two volumes of buffer A containing 5 mM $MgCl_2$, and one volume of glass beads. The mixture was homogenized in a blender for 15 min. The clear 30,000 *g* supernatant (200 mg of protein) was applied to a column (1.5 × 16 cm) of DEAE-cellulose previously equilibrated with the extracting buffer. The protein was eluted successively with the following solutions: 40 ml of 5 mM $MgCl_2$, 40 ml of 20 mM $MgCl_2$ and finally 60 ml of 40 mM $MgCl_2$, all made in buffer A. Two ml fractions were collected and tested for pyruvate kinase (PK) activity. PK Type II was eluted at 20 mM $MgCl_2$, well separated from Type III, which appeared in the first fractions of the 40 mM $MgCl_2$ step. Active fractions were pooled and the protein precipitated by the addition of solid ammonium sulfate to 80% saturation. The suspension was centrifuged at 12,000 rpm for 30 min, and the pellet was resuspended in a small volume of buffer A containing 10 mM $MgCl_2$ and 30% glycerol. Undissolved material was removed by centrifugation. The purification ranged from 5- to 10-fold. The recovery was about 60% of the initial activity.

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The enzymatic pattern of crude extracts could not be determined by the stepwise procedure because it does not separate Type I and II pyruvate kinases.

3. Results and discussion

The specific activity of pyruvate kinase in extracts from AA-grown mycelium (3.5–6 units/mg protein) was lower than that of glucose-grown mycelium (10–15 units/mg protein). Extracts from glucose and AA-grown cells, containing 15–30 mg of protein were chromatographed on DEAE-cellulose columns.

As shown in fig. 1, the pyruvate kinase activity from glucose-grown mycelium was resolved in two peaks (Type I and II) [1]. Extracts from AA-grown mycelium yielded a small amount of Type II (less than 15% of the total activity recovered after chromatography) and a new peak of PK activity (Type III), eluted by approximately 0.15 M KCl. No pyruvate kinase activity was detected in the zone of the chromatogram corresponding to Type I, even after increasing the time of incubation or the amount of sample. Fractions corresponding to PK activity (Type III) were concentrated, filtered through Sephadex G-25 and rechromatographed in a DEAE-cellulose column. The chromatographic mobility was the same as that observed in the crude extract. Pyruvate kinase Type III, was absent in the extracts of the organism grown in 2% glucose.

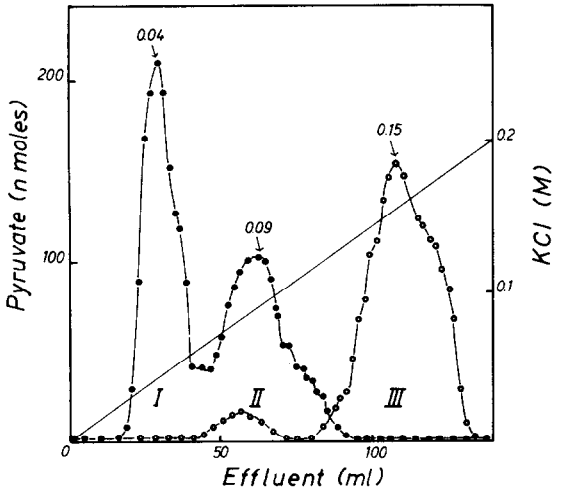

Fig. 1. Chromatography on DEAE-cellulose column (1 × 9 cm) of the pyruvate kinase activity of extracts of *M. rouxii* mycelium grown on 2% glucose (●—●) and casaminoacids (○—○) as carbon source; containing 17 and 26 mg of protein respectively. The protein was eluted with a linear gradient of 0–0.2 M KCl in buffer A containing 30% glycerol. 1 ml fractions were collected and tested for pyruvate kinase activity.

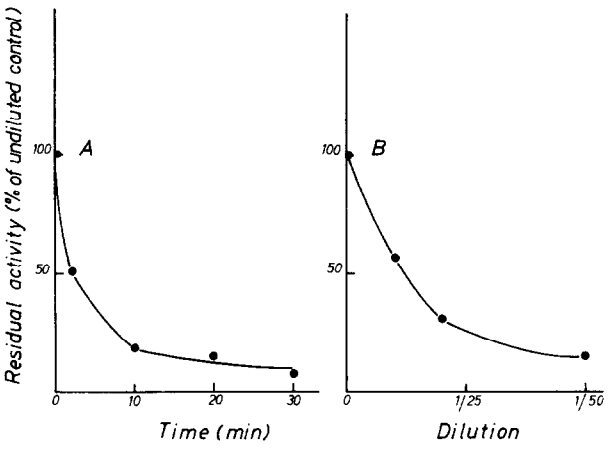

Fig. 2. (A) Time dependent inactivation of pyruvate kinase in crude extracts of AA-grown mycelium. A crude extract (20 mg of protein per ml) was prepared as described in the text. An aliquot was diluted 10-fold in buffer A at 0°. At different intervals of time samples were assayed for PK activity. (B) Sensitivity of pyruvate kinase to dilution. Aliquots of the extract were diluted to varying extents in buffer A at 0°. After 2 min samples of each dilution were tested for PK activity. The amount of protein was the same in all assays.

As can be seen in fig. 2A, a rapid loss of pyruvate kinase activity was observed when crude extracts from AA-grown mycelium were diluted in buffer A at 0°. The data of fig. 2B indicate an increasing loss of activity with dilution.

Several chemicals were examined for a possible protecting effect against inactivation of PK by dilution. As can be seen in table 1, the addition of Mg^{2+}, glycerol or ADP to the dilution mixture led to a substantial stabilization of pyruvate kinase activity. The presence of both Mg^{2+} and glycerol prevented almost completely inactivation promoted by dilution. The addition of 1 mg/ml of albumin, 2 mM FDP, 3 mM PEP, or 20 mM KCl was ineffective in preventing inactivation of pyruvate kinase. The loss of enzymatic activity upon dilution did not occur in crude extracts from glucose-grown mycelium, even 200-fold diluted.

It was shown in the preceding paper [1], that PK_s

Table 1
Effect of several chemicals on inactivation by dilution of pyruvate kinase activity in crude extracts.

Conditions of dilution	Residual activity (% of undiluted control)
Buffer only	13
+5 mM Mg^{2+}	64
+30% glycerol	62
+5 mM Mg^{2+} + 30% glucerol	82
+10 mM Mg^{2+}	65
+10 mM Mg^{2+} + 30% glycerol	89
+3 mM PEP	26
+3 mM ADP	71
+2 mM FDP	24
+1 mg per ml albumin	27

Equal quantities of crude extract from AA-grown cells, containing 22 mg of protein per ml were diluted 50-fold in buffer A containing various additives. After 30 min at 0°, samples were taken for assay of PK activity. The control activity was that present in an undiluted extract stored at 0°.

Type I and Type II have similar kinetic properties. Both isoenzymes are allosterically activated by one of the substrates, phosphoenolpyruvate (PEP), with an $S_{0.5}$ of about 2.5 mM. At low concentrations of PEP, the activity of both enzymes is greatly stimulated by FDP.

In order to search for differences among the isoenzymes, some kinetic measurements were performed with pyruvate kinases Type I and Type III, both partially purified by DEAE-cellulose chromatography. Some interesting differences were observed between them. Although Type III exhibits sigmoid kinetics with respect to PEP, the $S_{0.5}$ is lower (1 mM) than that of Type I (2.5 mM). Moreover, although FDP also stimulates PK activity Type III at low concentrations of PEP, the stimulatory effect is several times lower than that observed with Type I. The possibility that FDP bound to the enzyme could be responsible for this diminished response to FDP seems unlikely, since preincubation of the enzyme with excess aldolase for 5 min did not change its activity measured at 0.5 and 1 mM PEP.

Preliminary experiments indicated that the activation of pyruvate kinase Type II is half-way between the other two isoenzymes.

Incubation of mixtures of crude extracts, both from glucose and AA-grown cells, with ATP-Mg, Mn^{2+}, Mg^{2+} plus mercaptoethanol, or FDP did not change the elution profile of the three types of pyruvate kinase. When extracts were preincubated with FDP, this metabolite, at a concentration of 1 mM was present in the eluent of the column and also in the buffer of extraction.

These results argue against the interconvertibility of the three forms of the enzyme, as has been reported for the pyruvate kinases of rat epididymal adipose tissue [4] and red blood cells [5].

In summary, glucose and AA-grown mycelium are quantitatively characterized by different total PK activity. Qualitatively, a mjaor difference between them is the enzymatic pattern of both extracts. DEAE-

Table 2
Activity of pyruvate kinase Type I and Type III in the absence (a) and in the presence (b) of 2 mM FDP, at different levels of PEP.

PEP (mM)	Type I[a]			Type III[a]		
	Activity[b]		Activation ratio (b)/(a)	Activity[b]		Activation ratio (b)/(a)
	−FDP (a)	+FDP 2 mM (b)		−FDP (a)	+FDP 2 mM (b)	
0.5	0	66	–	24	95	3.95
1	2.6	154	59.4	62	110	1.78
2	12.5	160	12.8	89	112	1.26
3	33	155	4.5	–	–	–
7.5	150	155	1.0	126	120	0.95

[a] Pyruvate kinases Type I [1] and Type III both partially purified by DEAE-cellulose chromatography were used as enzyme.
[b] nmoles of pyruvate/min.

cellulose chromatography showed that, in AA-grown cells, pyruvate kinase Type I completely disappeared, Type II activity was greatly diminished and a new form of pyruvate kinase, Type III, absent in glucose-grown cells, was detected. This form of the enzyme is rapidly inactivated by dilution of crude extracts and is quite insensitive to FDP activation.

It has been reported that rat liver [6] and *Escherichia coli* K12 [7] contain two forms of pyruvate kinase; one of these is activated by FDP and is inducible, the other is not affected by FDP and appears to be formed constitutively.

M. rouxii pyruvate kinases seem to be controlled in a different way, in the sense that none of the isoenzymes seems to be constitutive; the level of each depends of the conditions of growth.

A detailed study of the nutritional factors that determine the enzymatic pattern of pyruvate kinases in mycelium of *M. rouxii* will be described in a following paper.

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