ACTIVATION OF PYRUVATE KINASE OF *MUCOR ROUXII* BY MANGANESE IONS

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1. Introduction

Mucor rouxii, a filamentous fungus, is able to grow either anaerobically, exhibiting a yeast-like morphology, or aerobically as a filamentous mycelium. It was also demonstrated that yeast-like cells will develop in a culture under any conditions favoring a high level of fermentation. [1].

In order to gain insight into the process of morphogenesis a general study of one of the key glycolytic enzymes (pyruvate kinase) was undertaken.

In the present communication some kinetic properties of the *Mucor* pyruvate kinase (ATP: pyruvate phosphotransferase, E.C. 2.7.1.40) are described. Evidence is presented that in addition to fructose-1,6-diphosphate (FDP), Mn^{2+} is also an allosteric activator of the *Mucor* pyruvate kinase at physiological concentrations of substrate (phosphoenol pyruvate, PEP).

2. Materials and methods

All reagents and chemicals used were of analytical grade.

Mucor rouxii (NRRL 1894) was grown from a spore inoculum (about 10^5 spores/ml) in a complex medium (YPG) [2] containing glucose as the main carbon source. The cultures were incubated aerobically or anaerobically as described elsewhere [1] and harvested by filtration and rinsed with distilled water. Pads of mycelium or yeast-like cells were blotted with filter paper and kept at -80°C until use.

Cell extracts were prepared by suspending the cells in three volumes of 50 mM tris-HCl buffer, pH 7.4, containing 5 mM mercaptoethanol, and two volumes of glass beads. The mixture was disrupted for 2 to 3 min in a Nossal disintegrator. The extracts were centrifuged at 1500 *g* for 15 min, and the supernatant fraction was spun again at 30,000 *g* for 40 min. The clear supernatant was passed through a column of Sephadex G-25 equilibrated with the same buffer, and the eluate was used without further purification.

The effectiveness of the Sephadex filtration in eliminating small molecules was tested in preliminary experiments; FDP intentionally added to the extract was completely removed by Sephadex filtration.

2.1. *Enzymatic assay*

Pyruvate kinase activity was measured by the spectrophotometric method described by Bücher and Pfleiderer [3]. NADH oxidation was followed in a Gilford model 2000 automatic spectrophotometer. Each assay was done with 40–60 μg of protein.

The effect of Mn^{2+} on the lactate dehydrogenase reaction was checked over a large range of pyruvate and NADH concentrations. No interference was observed.

3. Results

3.1. *Comparison of pyruvate kinase activity in mycelium and yeast-like cell extracts*

Crude extracts obtained from yeast-like cells consistently exhibited 3 to 5 times more total specific activity than those obtained from mycelium. The ratio of activation by FDP (activity with FDP/activity without FDP) was also higher in yeast-like cell extracts. This resulted from a comparatively higher

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Table 1
Total specific activity (*b*) and ratio of activation by FDP (*b*/*a*) of pyruvate kinase measured in extracts from mycelium and yeast-like cells of *Mucor rouxii*.

Enzyme source	1 mM PEP (*a*) mμmoles NADH/ min/mg protein	1 mM PEP, 2 mM FDP (*b*) mμmoles NADH/ min/mg protein	Ratio of activation
Mycelin	64.5	1,160	18.4
Yeast-like cells	61.0	3,060	50.0

activity of the mycelium enzyme at low PEP concentrations in the absence of FDP (table 1). On the other hand, the kinetic behaviour of pyruvate kinase from mycelium and yeast-like cells toward the substrate (PEP) and effectors was closely similar. In view of these results, and the higher specific activity of pyruvate kinase of the yeast-like cell extracts, this material was used for most of the kinetic measurements.

3.2. *Interaction with PEP and FDP*

Stimulation of pyruvate kinase by FDP depends not only on the FDP concentration but also on PEP concentration. The interrelationships between FDP and PEP are shown in fig. 1A. In the absence of the effector the curve for initial velocity against PEP concentration is sigmoidal. The Hill plot gives an exponent (n_H) of 3.6 and half-maximal activity is attained at 3.3 mM PEP. In the presence of 2 mM FDP the response curve is transformed into a hyperbola, with an n_H of approximately 1 and the $S_{0.5}$ for PEP is lowered to 0.25 mM, 13 times less than in the absence of FDP. The saturation curve for FDP shows a sigmoidicity and the Hill plot gives an exponent (n_H) of 1.9 and a $[FDP]_{0.5}$ value of 0.2 mM (fig. 2A).

3.3. *Interaction with bivalent cations*

Mn^{2+} activates *Mucor* pyruvate kinase at physiological concentrations of PEP in the presence or in the absence of Mg^{2+} (figs. 2B and 3). As shown in fig. 3 at a concentration of metal of 3 mM the velocity of the reaction in the presence of Mn^{2+} is at least 160 times higher than in the presence of Mg^{2+}; moreover, even 20 mM Mg^{2+} is unable to activate pyruvate kinase appreciably. The Mn^{2+}-induced activation is not competed by the addition of 5 mM Mg^{2+}; on the contrary, Mg^{2+} enhances the activity of the enzyme at low levels of Mn^{2+}. These results suggest that free Mn^{2+} is responsible for the activation of pyruvate kinase, rather than a complex ADP-Mn^{2+}, since the addition of Mg^{2+} should decrease the concentration of such a complex by displacing the equilibrium towards the liberation of free Mn^{2+}.

At low concentrations of PEP (0.25 mM) and in the presence of 6 mM Mg^{2+}, the Mn^{2+} activation curve is sigmoidal with an n_H value of 3.25 and a $Mn^{2+}{}_{0.5}$ of 1.7 mM (fig. 2B).

As can be seen in fig. 1B, 3 mM Mn^{2+} transforms the response curve toward PEP concentration, as does FDP, into a Michaelis hyperbola, with an n_H value of 1.25 and the $S_{0.5}$ for PEP is lowered from 3.3. to 0.2 mM. The maximal velocity attained in the presence of Mn^{2+} is 20% lower than that reached at saturating concentrations of PEP in the absence of the metal.

Filtration of the Mn^{2+}-activated pyruvate kinase through Sephadex G-25 in order to remove the Mn^{2+} resulted in a full recovery of both the original activity and kinetic characteristics, indicating that the effect of Mn^{2+} on the enzyme is freely reversible.

4. Discussion

The kinetic characteristics of *M. rouxii* pyruvate kinase demonstrate that this enzyme belongs to the allosteric type, similar to that described by Haeckel et al. [4] for the baker's yeast enzyme. Preliminary experiments indicate that two pyruvate kinases are present in the extracts of mycelium and only one in those of the yeast form, but no important kinetic differences were detected in the crude extract.

A major property of *M. rouxii* pyruvate kinase is the strong cooperative activation induced by Mn^{2+}, which abolishes, as FDP does, substrate interaction and decreases 15-fold the apparent Km for PEP. These observations support the view that Mn^{2+} can act as an allosteric positive effector of *Mucor* pyruvate kinase.

Although cooperative effects were also observed with Mg^{2+} by Haeckel et al. [4] for the baker's yeast enzyme, the activation produced by Mn^{2+} in our system occurred at a much lower concentration of metal and at a physiological range of substrate con-

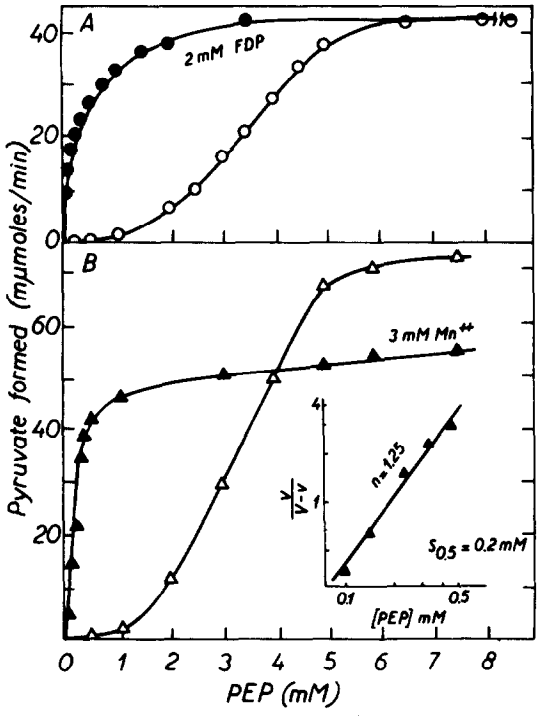

Fig. 1. Activity of *M. rouxii* pyruvate kinase as a function of PEP concentration, in the presence (•) and absence (○) of 2 mM FDP (fig. 1*A*), and in the presence (▲) and absence (△) of 3 mM manganous sulfate (fig. 1*B*). The assay mixture contained: Tris-HCl buffer, pH 7.4, 0.1 M; ADP, 4 mM; $MgCl_2$, 6 mM; KCl, 40 mM, NADH, 0.2 mM, lactate dehydrogenase, PEP as indicated and enzyme in a final volume of 0.5 ml. The reaction was done at 30°C. Preincubation of the extract for 5 min with all the concentrations of PEP used (in the absence of effectors) did not change the velocity of NADH oxidation in the complete system.

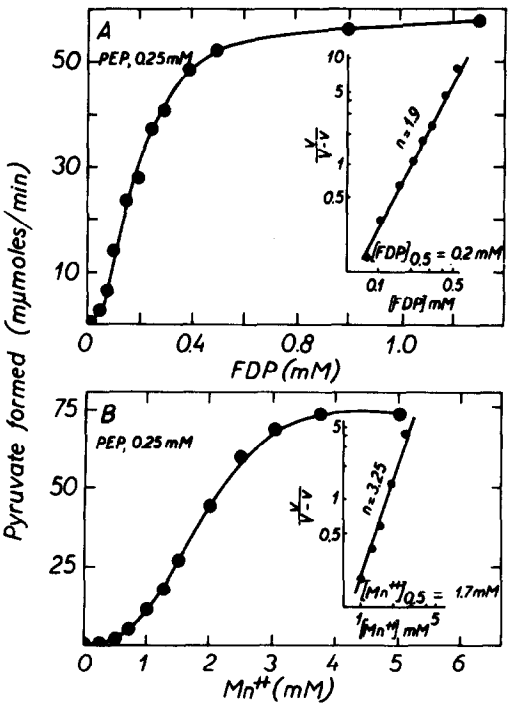

Fig. 2. Activity of pyruvate kinase measured at 0.25 mM PEP, as a function of FDP concentration (2*A*) and Mn^{2+} concentration (2*B*). Mg^{2+} (6 mM) was present in the assays. Other conditions as in fig. 1. Preincubation of extracts for 5 min with FDP (up to 0.4 mM) did not alter the velocity of NADH oxidation in the complete system.

centration. Moreover, the Mn^{2+} concentration at which these effects were observed is far below the total concentration of this metal reported to occur in several kinds of yeasts [5]. The maximal activity observed for the Mn^{2+}-activated enzyme was about 60% less than that obtained in the presence of Mg^{2+} and FDP. Furthermore, the Mn^{2+}-activated enzyme was not only insensitive to FDP, but was slightly inhibited by this effector (data not shown).

A detailed interpretation of the multiple possible interactions between substrates, cofactors and modifiers is beyond the scope of this paper and should await the availability of a more purified enzyme.

The role that metals might play in metabolic

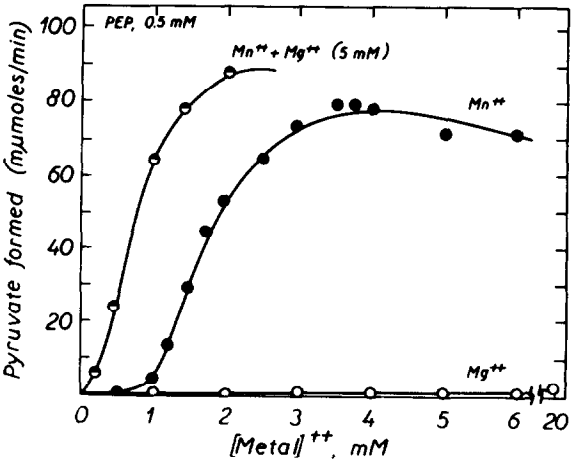

Fig. 3. Effect of Mg^{2+} (○), Mn^{2+} (•) and Mn^{2+} plus 5 mM Mg^{2+} (⊖) on the activity of pyruvate kinase measured at 0.5 mM PEP. Other assay conditions as in fig. 1, except that metal was varied as indicated.

regulations has been stressed by Bygrave [6]. Kingdon and Stadtman [7] recently observed an activation of glutamine synthetase of *E. coli* produced by its binding with Mn^{2+} which induced conformational changes on the enzyme. Also Gentner and Preiss [8], working with the *E. coli* B ADP-glucose pyrophosphorylase reported a number of differences in the kinetic properties of the enzyme, depending on whether Mg^{2+} or Mn^{2+} served to fulfil the bivalent cation requirement.

Our results do not permit so far to equate the effect of Mn^{2+} with the regulatory behaviour of *Mucor* pyruvate kinase inside the cell. It is however tempting to speculate on the possibility that these changes may occur *in vivo* in response to intracellular variations in the concentration of metals, like variations in the level of metabolites affect enzyme activity. Thus, Mn^{2+} ions could serve to modulate the activity of *Mucor rouxii* pyruvate kinase in aerobic conditions, when the level of FDP is too low to insure the provision of the pyruvate required for cell growth.

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