Characterization of phosphofructokinase II and regulation of fructose 2,6-bisphosphate levels in *Trichoderma reesei*

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Phosphofructokinase II (PFK II) from *Trichoderma reesei* was partially purified (247-fold). The calculated $K_m$ values for fructose 6-phosphate and ATP were 0.7 mM and 40 μM, respectively. Upon incubation in the presence of $[γ-32P]ATP$, the enzyme formed a radioactive phosphoprotein with molecular mass of 67 kDa in autoradiography analysis after SDS-PAGE. Upon incubation in the presence of ATP-Mg and the catalytic subunit of cAMP-dependent protein kinase, its activity was not modified. The same result was obtained when a cell-free extract of *T. reesei* was incubated with ATP-Mg and CAMP. 2,4-Dinitrophenol caused a transient rise in cAMP levels in the fungal cell. These results provide evidence that the fructose 2,6-bisphosphate level in *T. reesei* is independent of cAMP concentrations and not related to a cAMP-dependent mechanism, but to the availability of substrate fructose 6-phosphate.

Introduction

Fructose 2,6-bisphosphate (Fru-2,6-P$_2$) controls glycolysis and gluconeogenesis by activating fructose 6-phosphate 1-kinase (PFK I; EC 2.7.1.11) and inhibiting fructose 1,6-bisphosphatase (EC 3.1.3.11). Fru-2,6-P$_2$ plays a central regulatory role in carbohydrate metabolism in eukaryotic organisms. Since its discovery (Van Schaftingen & Hers, 1980), Fru-2,6-P$_2$ has been found in plants (Larondelle et al., 1986), animals (Van Schaftingen, 1987) and fungi (Van Laere et al., 1990). An additional regulatory role of Fru-2,6-P$_2$ in regulation of the ‘futile cycle’ (fructose 6-phosphate/fructose 1,6-bisphosphate cycle) has been proposed (Leite et al., 1988).

Fru-2,6-P$_2$ is formed from ATP and Fru-6-P in a reaction catalysed by 6-phosphofructo-2-kinase (PFK II) (El-Maghrabi et al., 1981; Furuya & Uyeda, 1981; Hue et al., 1981; Van Schaftingen & Hers, 1981). The hydrolysis of Fru-2,6-P$_2$ to Fru-6-P and P$_i$ is catalysed, in liver and lower eukaryotes, by fructose-2,6-bisphosphatase (FBPase II; EC 3.1.3.46). In liver, muscle and higher plants, PFK II and FBPase II are associated in a bifunctional protein (Pilkis et al., 1984; Bazan et al., 1989). In yeast FBPase II and PFK II can be separated (Kretschmer et al., 1987; François et al., 1988).

Phosphorylation of the bifunctional enzyme from liver, by a cAMP-dependent protein kinase, causes PFK II inactivation and FBPase II activation (Van Schaftingen, 1987). In *Saccharomyces cerevisiae*, on the other hand, cAMP-dependent phosphorylation of PFK II activates the enzyme (François et al., 1984).

The filamentous fungus *Trichoderma reesei* secretes a very efficient cellulolytic system that has synergistic action on cellulose degradation to glucose (Montenecourt & Eveleigh, 1979; Béguin, 1990). This system is dependent on substrates that can induce the extracellular enzymes (El-Gogary et al., 1989).

The purpose of this work was to characterize PFK II of *T. reesei* and to investigate the control of its activity in relation to the production of Fru-2,6-P$_2$.

Methods

Materials. Blue trisacryl was from IBF Biotechnics; dithiothreitol, phenylmethylsulphonyl fluoride (PMSF), catalytic subunit of cyclic-AMP-dependent protein kinase, aldolase, triosephosphate isomerase...
and α-glycerophosphate dehydrogenase were from Sigma; SDS-PAGE molecular mass standards, Superose-6B, DEAE-Sepharose and Blue-Sepharose were from Pharmacia LKB Biotechnology. Other chemicals were from Merck and were of analytical grade. PFK II of Saccaromyces cerevisiae (Francois et al., 1988), potato tuber pyrophosphatase: fructose-6-phosphate 1-phosphate transferase (Van Schaftingen et al., 1982b), PFK phosphofructokinase of T. reesei (Leite et al., 1988), [2-32P]Fru-2,6-P2 (Van Schaftingen et al., 1982a) were prepared as described previously.

Inoculum and culture conditions. Cultures of T. reesei were maintained on potato dextrose agar slants at 4 °C. Inocula were prepared by harvesting spores from 7 d-old cultures in 0.9% NaCl and filtering through glass wool. Culture medium contained 0.08% glycerol, 0.2% Bacto-peptone (Difco), 0.21% (NH4)2SO4, 0.03% urea, 0.03% MgCl2, 1.3% NaCl, 0.05% CaCl2 and 0.1% trace metal solution (0.02% CoCl2, 0.5% FeSO4, 0.15% MnSO4, H2O and 0.17% ZnCl2) in 28 mM-HCl in 100 mM-potassium phosphate buffer (pH 7.6). Cultures were inoculated from spore inocula (final concentration, 10⁶ spores ml⁻¹) were incubated on a rotatory shaker for 14 h to yield a physiologically active inoculum.

Spores (5 ml) were added to the above culture medium containing the appropriate carbon source for study. All cultures were incubated on a rotary shaker (200 r.p.m.) at 28 °C.

Determination of enzyme activities. PFK II was assayed by determining the production of Fru-2,6-P2 in a mixture containing 50 mM-HEPES, pH 6.9, 10 mM-MgCl2, 100 mM-KCl, 1 mM-DTT, 1 mg bovine serum albumin ml⁻¹, 1 mM-Fru-6-P, 17.5 mM-Glu-6-P and 5 mM-ATP. At various times, samples (0.1 ml) were mixed with 1 vol. 0.1 M-NaOH, incubated for 10 min and used for determination of Fru-2,6-P2 (Van Schaftingen et al., 1982b).

PFK I activity was determined by following the rate of NADH oxidation at 340 nm in a reaction mixture containing 50 mM-HEPES, pH 7.6, 10 mM-NH4Cl, 5 mM-MgCl2, 2.5 mM-ATP, 5 mM-Fru-6-P, 2 mM-DTT, 0.15 mM-NADH, aldolase 100 μg and α-glycerophosphate dehydrogenase 0.5 μg.

All enzymic assays were performed at 30 °C. One unit is the amount of enzyme that catalyses the conversion of 1 μmol substrate in 1 min under the conditions of the assay.

Measurement of cyclic AMP and DNA. Samples of cultures were collected by filtration and the residue was scraped off and frozen in liquid nitrogen within less than 15 s after collection of the sample. The frozen pellet was weighed, mixed with 1 ml 20% (v/v) perchloric acid and freeze-thawed four times. The extract was centrifuged (3000 g for 2 min) and the supernatant divided into two samples. One sample was neutralized (2 M-KOH and 0.5 M-triethanolamine) and, after centrifugation, was used for determination of cAMP (kit supplied by Amersham). The other sample was used for determination of DNA as described by Abraham et al. (1972).

Purification of the PFK II of rat liver. Livers were isolated from fed rats killed by concussion and used immediately. The tissue was homogenized for 1 min in a Waring blender with 3 vols cold 25 mM-HEPES, pH 7.5, 1 mM-DTT, 1 mM-PMSE, 100 mM-KCl and 0.25 mM-sucrose. The purification was carried out essentially as described by Van Schaftingen & Hers (1986).

Purification of the PFK II of T. reesei. The cells, cultivated in 1% (w/v) glucose, were harvested in the stationary phase 10 h after glucose exhaustion. Cells (100 g) were homogenized for five 30 s periods in a cell homogenizer (Marconi T-120) in 300 ml ice-cold solution containing 20 mM-potassium phosphate, pH 7.5, 1 mM-DTT, 1 mM-PMSE, 5 mM-EDTA and 0.5 mM-KCl. The homogenate was centrifuged for 10 min at 35000 g and filtered through glass wool. The purification was performed as described by Francois et al. (1988).

Phosphorylation of PFK II of liver and T. reesei. The partially purified enzymes (20 μg) were incubated with the catalytic subunit of cAMP-dependent protein kinase (20 picomolar units ml⁻¹); 1 picomolar unit transfers 1 pmol phosphate from [γ-32P]ATP to hydrolysed, partially dephosphorylated casein per min at pH 6.5 and 30 °C at 30 °C in a final volume of 0.1 ml containing 10 mM-potassium phosphate, pH 7.5, 2 mM-ATP and 1 mM-DTT. After incubation for various times, samples were collected and mixed with 10 vols 10 mM-potassium phosphate, pH 7.5, 1 mM-DTT, 5 mM-EDTA and 20 mM-NaF. The PFK II activity was determined as described above.

Results

Purification of PFK II

In view of the differences in the catalytic and regulatory properties of PFK II in higher and lower eukaryotes, we undertook the simultaneous purification of the enzymes from T. reesei, S. cerevisiae and rat liver PFK II.

The purification of PFK II of T. reesei involved extraction, affinity chromatography on blue trisacryl and gel filtration on Superose-6B. The 247-fold purified PFK II was obtained with a yield of 13% (Table 1). The elution profile of this purification (Fig. 1) showed differences from previous results obtained with S. cerevisiae (Francois et al., 1988). The purification procedure for rat liver PFK II resulted in 6500-fold purified enzyme with a specific activity of 286 mU mg⁻¹ and a recovery of 24% (Table 2).

Effect of the catalytic subunit of cAMP-dependent protein kinase on the activity of purified PFK II of T. reesei and rat liver

Incubation of purified T. reesei PFK II with the catalytic subunit of cAMP-dependent protein kinase caused no change in the enzymic activity, whereas under the same conditions rat liver PFK II was inhibited (Table 3).

Experiment with a cell-free system

In order to verify a possible phosphorylation by a cell-specific cAMP-dependent protein kinase present in the homogenate, the extract of T. reesei was incubated with cAMP and ATP-Mg (Francois et al., 1984). A portion of extract (200 μl) was incubated (30 °C) with 5 mM-MgSO4, 2 mM-ATP and 10 μM-cAMP, and at various times samples were collected and mixed with 5 vols 10 mM-potassium phosphate, pH 7.5, 1 mM-DTT, 5 mM-EDTA and 20 mM-NaF. The PFK II activity was determined in
Table 1. Purification of PFK II from T. reesei

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Vol. (ml)</th>
<th>Protein (mg)</th>
<th>Total activity (mU)</th>
<th>Specific activity (mU (mg protein)-1)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
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<tbody>
<tr>
<td>Crude extract</td>
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<td>131</td>
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<td>100</td>
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<tr>
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<td>Superose-6B</td>
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<td>17</td>
<td>40.5</td>
<td>247</td>
<td>13</td>
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Table 2. Purification of PFK II from rat liver

<table>
<thead>
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<th>Purification step</th>
<th>Vol. (ml)</th>
<th>Protein (mg)</th>
<th>Total activity (mU)</th>
<th>Specific activity (mU (mg protein)-1)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
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<td>748</td>
<td>0.044</td>
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<td>100</td>
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<td>PEG 22%</td>
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<td>5625</td>
<td>394</td>
<td>0.070</td>
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<tr>
<td>DEAE-Sepharose</td>
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<td>264</td>
<td>3.1</td>
<td>70-4</td>
<td>35</td>
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<tr>
<td>Blue-Sepharose</td>
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<td>0.63</td>
<td>181</td>
<td>286</td>
<td>6500</td>
<td>24</td>
</tr>
</tbody>
</table>

Table 3. Phosphorylation of PFK II of T. reesei (A) and rat liver (B)

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Phosphorylated sample</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>1</td>
<td>34</td>
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<tr>
<td>20</td>
<td>34</td>
<td>100</td>
</tr>
<tr>
<td>30</td>
<td>33</td>
<td>100</td>
</tr>
</tbody>
</table>

The partially purified enzymes (20 µg) were incubated at 30 °C with 20 picomolar units (see Methods) of the catalytic subunit ml⁻¹ in 10 mM-potassium phosphate, pH 7.5, 2 mM-ATP and 1 mM-DTT. Samples were collected at the times indicated and mixed with 10 vols 10 mM-potassium phosphate, pH 7.5, 1 mM-DTT, 5 mM-EDTA and 20 mM-NaF. The PFK II activity was determined as described in Methods.

Complexation of PFK II of T. reesei with [γ-³²P]ATP

SDS-PAGE analysis of the fractions eluted from the Superose-6B purification of PFK II of T. reesei showed the presence of many bands. In order to identify the
Fig. 2. Complexation of PFK II of rat liver and T. reesei with \([\gamma-^3P]ATP\). The partially purified enzymes (10 µg) were incubated at 30 °C with 10 µM-[\(\gamma-^3P\)]ATP (12000 c.p.m. pmol\(^{-1}\)) in 20 mM-HEPES, pH 7.5, 0.1 mM-EDTA, 100 mM-KCl, 0.1 mM-EDTA, 5 mM-MgCl\(_2\), 1 mM-DTT, 1 mM-PP\(_i\), 5 mM-Fru-2,6-P\(_2\) and 1 mg bovine serum albumin ml\(^{-1}\). Samples (10 µl) were collected at the times indicated, mixed with 1 vol. loading buffer, heated in a boiling water bath for 7 min, and subjected to SDS-PAGE. For autoradiography the gel was dried and exposed at −80 °C to X-ray film with an intensifying screen.

Fig. 3. Saturation curve of PFK II of T. reesei for Fru-6-P. PFK II was assayed in the presence of 50 mM-HEPES, pH 6.9, 100 mM-KCl, 5 mM-MgCl\(_2\), 1 mM-DTT, 1 mM-Pi, 5 mM-ATP and 1 mg bovine serum albumin ml\(^{-1}\). The inset shows a plot of [Fru-6-P]/v against [Fru-6-P].

peptide band corresponding to the subunit of this enzyme, advantage was taken of experimental data suggesting that the mechanism of the (rat liver) PFK II catalysis involves the formation of a phosphoryl enzyme intermediate (E-P) from ATP and Fru-2,6-P\(_2\) (Pilkis et al., 1984). Incubation of purified PFK II of T. reesei and rat liver with 10 µM-[\(\gamma-^3P\)]ATP and 2 µM-[2,\(^32P\)]Fru-2,6-P\(_2\) allowed the identification of the protein band corresponding to PFK II of T. reesei (Fig. 2).

Kinetic properties of purified PFK II of T. reesei

The partially purified enzyme displayed typical Michaelis–Menten kinetics, with a \(K_m\) of about 0.7 mM for Fru-6-P (Fig. 3) and 40 µM for ATP (Fig. 4).

Stimulation of PFK I of T. reesei by Fru-2,6-P\(_2\)

The general rule that Fru-2,6-P\(_2\) is a positive effector also applies to T. reesei PFK I. Addition of Fru-2,6-P\(_2\) changed the saturation curve for Fru-6-P from sigmoidal to hyperbolic and decreased the \(K_m\) for Fru-6-P (Fig. 5).

Effect of glucose and 2,4-dinitrophenol (DNP) on the concentration of cAMP

In order to verify whether addition of glucose and DNP to a suspension of T. reesei in stationary phase caused a transient increase in concentration of cAMP, as described previously for the enzymes from S. cerevisiae (François et al., 1984) and Neurospora crassa (Ann Dumbrava & Pall, 1987), the culture of T. reesei was
As shown in Tables 1 and 2, the purification of PFK II of rat liver gave a purification factor much greater (6500-fold) than that of the enzyme of T. reesei. In order to separate PFK II of T. reesei from cAMP-dependent and cAMP-independent protein kinases, the suggestions of François et al. (1988) were followed. Thus we avoided procedures of purification involving, as a first step, fractionation with polyethylene glycol or chromatography on DEAE-trisacryl or blue-Sepharose which could case the possible activation of PFK II either during the purification or in the assay mixture. This activation, on the other hand, did not occur in the enzyme from liver, either in this work or in others (Van Schaftingen & Hers, 1986) (Table 2).

T. reesei PFK II was strongly retained on blue trisacryl and eluted only at a high salt concentration. In this respect the behaviour of the T. reesei enzyme is comparable to that of the yeast PFK II (François et al., 1988). The elution from blue trisacryl yields a purification of 100-fold. The active fractions (Fig. 1a) were pooled and concentrated by ultrafiltration and applied to the column of Superose-6B. This step was less efficient in terms of purification; however, it is worth noting that the elution profile of the enzyme was strikingly different from that of the preparation obtained with S. cerevisiae (François et al., 1988) (Fig. 1b).

A further, and more fundamental difference between the T. reesei enzyme and those of S. cerevisiae (François et al., 1984) and rat liver is the lack of cAMP-dependent regulation in the former enzyme (Table 3).

Catalysis by rat liver PFK II involves a double displacement with the transient formation of a phosphoryl enzyme intermediate (E-Pi) (Pilkis et al., 1988). Analysis of $[^{32}P]$phosphoprotein formed upon incubation of the purified enzymes with $[^{32}P]$ATP revealed a band with molecular mass of about 55 kDa for liver enzyme (El-Maghrabi et al., 1982; Furuya et al., 1982) and one with a molecular mass of about 67 kDa for the T. reesei enzyme (Fig. 2). On the other hand, it was not possible to obtain $[^{32}P]$phosphoprotein upon incubation of T. reesei enzyme with $[^{32}P]$Fru-2,6-P$_2$, indicating that this enzyme is not bifunctional. The purified enzyme (PFK II) of T. reesei did not catalyse the phosphorylation of histones, casein and albumin (not shown).

From the saturation curves of PFK II from T. reesei for Fru-6-P and ATP (Figs 3 and 4) $K_m$ values of about 0.7 mm and 40 μm, respectively, were obtained. The $K_m$ for Fru-6-P of PFK II from S. cerevisiae decreased from 1.3 mm to 0.6 mm after phosphorylation (François et al., 1984), values close to those obtained for the T. reesei enzyme. On the other hand, the $K_m$ of PFK II from S. cerevisiae for ATP (0.5 mm) is not affected by phosphorylation. Thus the enzyme of T. reesei is about tenfold more sensitive for ATP than its counterpart. In addition, the relatively high $K_m$ values for PFK II from S. cerevisiae and T. reesei indicate that these enzymes are highly dependent on the Fru-6-P concentration.

**Table 4. Effect of addition of glucose and 2,4-dinitrophenol (DNP) on the concentration of cAMP**

<table>
<thead>
<tr>
<th></th>
<th>cAMP concn [pmol (μg DNA)$^{-1}$]</th>
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<tbody>
<tr>
<td></td>
<td>S. cerevisiae</td>
</tr>
<tr>
<td>+ Glucose</td>
<td>+ DNP</td>
</tr>
<tr>
<td>Time (min)</td>
<td>(0-1 m)</td>
</tr>
<tr>
<td>0</td>
<td>3.0</td>
</tr>
<tr>
<td>1</td>
<td>3.5</td>
</tr>
<tr>
<td>2</td>
<td>4.0</td>
</tr>
<tr>
<td>3</td>
<td>4.5</td>
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<tr>
<td>5</td>
<td>4.5</td>
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</table>

*Fig. 5. Effect of Fru-2,6-P$_2$ on the saturation curve of T. reesei PFK I for Fru-6-P. PFK I was assayed in the presence of 50 mm-NH$_4$Cl, 5 mm-MgCl$_2$, 2.5 mm-ATP, 2 mm-DDT, 0.15 mm-NADH, 1 IU aldolase, 5 IU triosephosphate isomerase, 0.5 IU α-glycerophosphate dehydrogenase. The concentrations of Fru-2,6-P$_2$ are shown next to the curves.*

submitted to two concentrations of glucose and to 1 mm-DNP. The same procedure was also performed for S. cerevisiae in the presence of 0.1 m glucose alone (Table 4).

**Discussion**

As shown in Tables 1 and 2, the purification of PFK II of rat liver gave a purification factor much greater (6500-fold) than that of the enzyme of T. reesei. In order to separate PFK II of T. reesei from cAMP-dependent and
The general effect of Fru-2,6-P2 as a potent stimulator of glycolysis also holds for T. reesei, since the activity of PFK I in this fungus is greatly increased by this effector (Fig. 5).

The effect of Fru-2,6-P2 contrasts with that of cAMP in the regulation of carbohydrate metabolism. While Fru-2,6-P2 is known as the most positive effector of PFK I, it acts solely by increasing the glycolytic rate and decreasing gluconeogenesis by inhibition of fructose 1,6-bisphosphatase; cAMP shows a diverse action in eukaryotic cells, that may even be antagonistic. Thus, in liver, cAMP inhibits glycolysis, whereas it has the reverse effect in heart and muscle (FranGois et al., 1988; Kitamura et al., 1989, 1981; Van Schaftingen & Hers, 1986; Van Schaftingen, 1987).

FranGois et al (1984) added DNP to cultures of S. cerevisiae and obtained data that suggested a close relationship between levels of Fru-2,6-P2 and a transient rise of cAMP. This relation led to the proposal of a secondary activation of PFK II by a cAMP-dependent phosphorylation. It should be noted, however, that the transient rise of cAMP in lower eukaryotes treated with uncoupler agents, or even with antibiotics which induce pore formation, is a general response and should not be used for establishing specific correlations (Jaynes et al., 1982; Pall et al., 1981; TreviUian & Pall, 1979; Uno & Ishikawa, 1981). For example, the addition of glucose to a suspension of S. cerevisiae leads to a transient increase in the concentration of cyclic AMP, but addition to a culture of T. reesei, had the opposite effect. Furthermore, the addition of DNP to the T. reesei caused a transient rise of cAMP (Table 4) and a twofold increase in Fru-2,6-P2 after 30 min (not shown).

Results shown in this work indicate that, unlike S. cerevisiae, levels of Fru-2,6-P2 in T. reesei are independent of levels of cAMP, as in Blastocladiella emersonii (Vandercammen et al., 1990). Dictyostelium discoideum (Arag6n et al., 1986) and N. crassa (Ann Dumbrava & Pall, 1987). This may indicate that for these organisms the level of Fru-2,6-P2 is controlled by the availability of the Fru-6-P.

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