Fructose-2,6-bisphosphatase and 6-phosphofructo-2-kinase are separable in yeast

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Fructose-2,6-bisphosphatase was purified from yeast and separated from 6-phosphofructo-2-kinase and alkaline phosphatase. The enzyme released Pi from the 2-position of fructose 2,6-bisphosphate and formed fructose 6-phosphate in stoichiometric amounts. The enzyme displays hyperbolic kinetics towards fructose 2,6-bisphosphate, with a $K_m$ value of 0.3 $\mu$M. It is strongly inhibited by fructose 6-phosphate. The inhibition is counteracted by L-glycerol 3-phosphate. Phosphorylation of the enzyme by cyclic-AMP-dependent protein kinase causes inactivation, which is reversible by the action of protein phosphatase 2A.

INTRODUCTION

PFK-2 was prepared from yeast and, in contrast with the liver enzyme, shown to be activated by cyclic-AMP-dependent phosphorylation (François et al., 1984; Yamashoji & Hess, 1984a,b). The increase in cellular Fru-2,6-P$_2$ on addition of glucose to yeast was attributed to increases in the concentrations of cyclic AMP and Fru-6-P (François et al., 1984).

The degradation of Fru-2,6-P$_2$ in yeast is still unresolved. François et al. (1986) were unable to find a Fru-2,6-P$_2$-degrading enzyme activity in yeast extracts, but more recently Purwin et al. (1987) reported the purification of a phosphatase that, in contrast with the FBPase-2 from liver and from other tissues, releases P$_i$ preferentially from the 6-position of Fru-2,6-P$_2$.

In the present paper, the partial purification of an enzyme from yeast is reported, which specifically acts on the phosphate in the 2-position of Fru-2,6-P$_2$ and which was found to be separable from PFK-2 and alkaline phosphatase. Evidence is given for its regulation by phosphorylation/dephosphorylation and by glycolytic intermediates.

EXPERIMENTAL

The C-subunit of cyclic-AMP-dependent protein kinase type II from bovine heart was kindly provided by Professor F. Hofmann (Homburg, Federal Republic of Germany). The catalytic subunit of protein phosphatase 2A from rabbit skeletal muscle (Alemany et al., 1984) was a gift by Professor P. Cohen (Dundee, Scotland, U.K.). All substrates and auxiliary enzymes were purchased from Boehringer (Mannheim, Germany). DEAE-Sephadex, CM-Sephadex C-50 and Sephacryl S-300 were from Pharmacia (Uppsala, Sweden). Cibacron Blue F3G-A was coupled to Sephacryl S-300 by a modification of the method described by Böhme & Kopperschläger (1972). Fru-2,6-P$_2$ was prepared as described by Van Schaftingen & Hers (1981), Fru-2,6-[2-32P]P$_2$ as described by Van Schaftingen et al. (1982), and Fru-2,6-[6-32P]P$_2$ with hexokinase (Khym & Cohn, 1953) and PFK-2. All enzymic assays were performed at 30 °C. One unit of enzyme is the amount of protein that transforms 1 $\mu$mol of substrate/min. Protein was determined, with human serum albumin as standard, as described by Bradford (1976).

Assay of PFK-2

PFK-2 was measured in a medium containing 50 mM-Tris/acetate, pH 7.6, 6 mM-ATP, 2 mM-Fru-6-P, 6.5 mM-Glc-6-P, 20 mM-magnesium acetate, 2 mM-potassium phosphate and 5 mM-mercaptoethanol. Samples of the alkali-treated reaction mixture were assayed for Fru-2,6-P$_2$ as described by Van Schaftingen & Hers (1983).

Assay of FBPase-2

The assay mixture contained 50 mM-Tris/acetate, pH 7.6, 3 $\mu$M-Fru-2,6-P$_2$, 2 mM-magnesium acetate, 2 mM-potassium phosphate and 5 mM-mercaptoethanol. Samples from this solution were removed, and the disappearance of Fru-2,6-P$_2$ was monitored. The release of [32P]P$_i$ from Fru-2,6-[2-32P]P$_2$ was measured in separate experiments as described by Van Schaftingen et al. (1982).

Alkaline phosphatase activity was measured in 1 M-diethanolamine/HCl, pH 9.8, containing 0.5 mM-MgCl$_2$ and 10 mM-p-nitrophenyl phosphate as substrate (Hausamen et al., 1967).

Preparation of FBPase-2 and PFK-2 from yeast

Baker's yeast (300 g) from VEB Backhefe, Leipzig, G.D.R., was disrupted in a glass-bead homogenizer (VEB Maschinenwerk, Heidenau, G.D.R.) in 300 ml of 20 mM-Tris/HCl (pH 7.5)/30 mM-KCl/5 mM-mercaptoethanol (buffer A). After centrifugation (10000 g for 30 min), the extract was fractionated with poly(ethylene glycol) 6000. The precipitate between 5 and 11% poly(ethylene glycol) was dissolved in the original volume of buffer A and applied on a DEAE-Sephadex column (8 cm x 10 cm). Proteins were eluted with a linear gradient of KCl (0.03–0.50 m-KCl in 1000 ml). Only the

Abbreviations used: Fru-2,6-P$_2$, fructose 2,6-bisphosphate; Fru-6-P, fructose 6-phosphate; Glc-6-P, glucose 6-phosphate; FBPase-2, fructose 2,6-bisphosphatase (EC 3.1.3.46); PFK-2, 6-phosphofructo-2-kinase (EC 2.7.1.105).
Table 1. Purification of PFK-2 and FBPase-2 from yeast

For details, see the Experimental section. Alkaline phosphatase was measured with p-nitrophenyl phosphate as substrate. Abbreviation: n.d., not detectable.

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein (mg)</th>
<th>Total activity (munits)</th>
<th>Specific activity (munits/mg)</th>
<th>Yield (%)</th>
<th>Alkaline phosphatase (units)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PFK-2</td>
<td>FBPase-2</td>
<td>PFK-2</td>
<td>FBPase-2</td>
</tr>
<tr>
<td>Extract after gel filtration</td>
<td>20900</td>
<td>125</td>
<td>n.d.</td>
<td>0.006</td>
<td>n.d.</td>
</tr>
<tr>
<td>Poly(ethylene glycol) 6000 precipitate</td>
<td>4300</td>
<td>102</td>
<td>108</td>
<td>0.024</td>
<td>0.025</td>
</tr>
<tr>
<td>DEAE-Sephacel chromatography</td>
<td>700</td>
<td>52</td>
<td>56</td>
<td>0.074</td>
<td>0.080</td>
</tr>
<tr>
<td>Sephacryl Blue chromatography</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PFK-2 pool</td>
<td>6.0</td>
<td>20</td>
<td>5</td>
<td>3.3</td>
<td>0.8</td>
</tr>
<tr>
<td>FBPase-2 pool</td>
<td>9.6</td>
<td>3</td>
<td>22</td>
<td>0.3</td>
<td>2.3</td>
</tr>
<tr>
<td>CM-Sephadex chromatography</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PFK-2 pool</td>
<td>1.5</td>
<td>12</td>
<td>2</td>
<td>8.0</td>
<td>1.3</td>
</tr>
<tr>
<td>FBPase-2 pool</td>
<td>2.6</td>
<td>1.5</td>
<td>13</td>
<td>0.6</td>
<td>5.0</td>
</tr>
</tbody>
</table>

fractions containing activities of PFK-2 higher than 0.3 munits/ml were pooled and further chromatographed on a Sephacryl S-300 Blue column (1 cm x 8 cm) equilibrated with buffer A containing 0.3 M-KCl. The column was extensively washed with the same buffer and developed with a linear gradient of KCl (0.3-2.0 M-KCl in 30 ml). FBPase-2 activity was eluted at 0.6 M-KCl and PFK-2 activity at 1.0 M-KCl. The FBPase-2 and PFK-2 fractions were individually collected and extensively dialysed against 50 mM-potassium phosphate (pH 6.8)/5 mM-MgCl₂/5 mM-mercaptoethanol (buffer B). The fractions containing the FBPase-2 and the PFK-2 were applied on separate CM-Sephadex C-50 columns (1 cm x 4 cm), previously equilibrated with buffer B. Both columns were washed with the same buffer, and each of the two enzymes was eluted by a linear gradient of KCl (0-0.6 M-KCl) in 10 ml). The active fractions were pooled and stored at -20°C in the presence of 20% (v/v) glycerol.

Inactivation and re-activation of FBPase-2

Purified FBPase-2 (30 μg) was incubated at 30°C (final volume 0.1 ml) in 10 mM-potassium phosphate (pH 7.5), containing 3 mM-ATP, 10 mM-MgCl₂ and 3 μg of catalytic subunit of cyclic-AMP-dependent protein kinase. After 30 min, samples of this mixture were used for the FBPase-2 assay. Re-activation was performed by addition of an excess (9 μg) of the catalytic subunit of protein phosphatase 2A. Then, after further incubation for 30 min at 30°C FBPase-2 was measured again.

RESULTS AND DISCUSSION

In accordance with the findings of François et al. (1984, 1986), neither in cell-free yeast extracts nor in gel filtrates of the yeast extracts could appreciable amounts of FBPase-2 be detected. However, after poly(ethylene glycol) fractionation of the yeast extract, FBPase-2 activity became evident (Table 1). PFK-2 and FBPase-2 were co-purified in the first two steps of the purification procedure, i.e. by poly(ethylene glycol) precipitation and DEAE-Sephacel chromatography. The two enzyme activities could be separated by Sephacryl Blue chromatography (Fig. 1). These activities are therefore different from the bifunctional PFK-2/FPase-2 of Mammalian tissues (Van Schaftingen et al., 1982; Kountz et al., 1985). When, after separation of the two activities on the Sephacryl Blue column, PFK-2 and FBPase-2 were chromatographed separately on CM-Sephadex, their elution patterns were the same, both being eluted at 0.18 M-KCl.

The yeast extract as well as the poly(ethylene glycol) 6000 precipitate contain considerable amounts of alkaline phosphatase. Less than 50% of the alkaline phosphatase activity of the poly(ethylene glycol) 6000 precipitate binds to the DEAE-Sephacel column. From this column alkaline phosphatase is eluted at 0.075 M-KCl, whereas

![Fig. 1. Elution of FBPase-2 (O) and PFK-2 (●) from Sephacryl Blue S-300](image-url)

Procedure is described in the Experimental section. ----, A₄₅₀; ———, KCl gradient.
Fig. 2. Release of \(^{32}\)P\(_2\)P\(_1\) from Fru-2,6-[2-\(^{32}\)P]P\(_2\) by FBPase-2 from yeast

Purified FBPase-2 (0.5 munits/ml) was incubated at 30 °C in the Tris/acetate medium as described in the Experimental section, containing 5 \(\mu\)M-Fru-2,6-\(P_2\) (3000 c.p.m. of Fru-2,6-[2-\(^{32}\)P]P\(_2\)). \[^{32}\)P\(_2\)P\(_1\) radioactivity (■) was counted in alkali-treated samples after separation from Fru-2,6-[2-\(^{32}\)P]P\(_2\) on Dowex AG1 \(\times\) 8 (Van Schaftingen et al., 1982). Disappearance of labelled Fru-2,6-[2-\(^{32}\)P]P\(_2\) (radioactivity counted after elution with 2 M-HCl from Dowex AG1 \(\times\) 8; ○) parallels the disappearance of Fru-2,6-\(P_2\) (○) as measured by the activation of pyrophosphate:Fru-6-P phosphotransferase (Van Schaftingen & Hers, 1983).

Fig. 3. Specificity of FBPase-2 from yeast: no release of phosphate from the 6-position of Fru-2,6-\(P_2\)

Purified FBPase-2 (0.2 munit/ml) was incubated with 5 \(\mu\)M-Fru-2,6-\(P_2\) (2250 c.p.m. of Fru-2,6-[6-\(^{32}\)P]P\(_2\)) under the same conditions as in Fig. 2, but mercaptoethanol was omitted because it interferes with the phosphate extraction. \[^{32}\)P\(_2\)P\(_1\) (■) was extracted as the phosphomolybdate complex (Pilkis et al., 1981). Radioactive phosphate remaining in the 6-position (○) was counted in the aqueous phase after extraction of \(P_1\), Fru-2,6-\(P_2\) (○) was assayed enzymatically in alkali-treated samples as described by Van Schaftingen & Hers (1983).

80% of the total FBPase-2, the alkaline phosphatase activity is less than 20% that of FBPase-2, whereas in the poly(ethylene glycol) 6000 precipitate the alkaline phosphatase has an approx. 1000-fold higher activity than FBPase-2.

Fru-2,6-\(P_2\) in micromolar concentrations is practically not hydrolysed by the alkaline phosphatase as measured in the peak fractions of this enzyme after DEAE-Sepharose chromatography.

The specificity of FBPase-2 was studied as follows. Incubation of Fru-2,6-[2-\(^{32}\)P]P\(_2\) with the purified enzyme results in a release of \[^{32}\)P\(_2\)P\(_1\) which is stoichiometric with the disappearance of Fru-2,6-\(P_2\) (Fig. 2). No \[^{32}\)P\(_2\)P\(_1\) is liberated from Fru-2,6-[6-\(^{32}\)P]P\(_2\) (Fig. 3). Furthermore, the enzyme catalyses the formation of Fru-6-P from Fru-2,6-\(P_2\), but not from Fru-1,6-\(P_2\) (Fig. 4).
Fig. 5. Influence of the concentration of Fru-2,6-P₂ on the activity of FBPase-2 in the absence (○) and the presence (●) of 50 μM-Fru-6-P

The insert gives the corresponding Lineweaver–Burk plot. Calculated constants (absolute error minimum): ○, V = 1.67 ± 0.06 (munits/ml), Kₘ = 0.32 ± 0.05 (μM); ●, V = 0.49 ± 0.03 (munits/ml), Kₘ = 0.17 ± 0.05 (μM).

Fig. 6. (a) Inhibition of FBPase-2 by Fru-6-P at (●) 3 μM- and (○) 0.3 μM- Fru-2,6-P₂, and (b) release of the Fru-6-P inhibition by L-glycerol 3-phosphate in the presence of 50 μM-Fru-6-P

In (a) the broken lines indicate the activity in the absence of Fru-6-P. In (b), the broken lines indicate the enzyme activity in the absence of L-glycerol 3-phosphate (lower line) and of L-glycerol 3-phosphate and Fru-6-P (upper lines). Symbols: ○, 0.4 μM-Fru-2,6-P₂; ●, 1.0 μM-Fru-2,6-P₂.

1984b). Hence, in yeast and liver the two activities are reciprocally affected by phosphorylation. However, in liver phosphorylation decreases formation of Fru-2,6-P₂, whereas in yeast the opposite is true.

It should be pointed out that phosphorylation of yeast PFK-2 (François et al., 1984; Yamashoji & Hess, 1984b) and FBPase-2 was obtained with a mammalian protein kinase, and might therefore not be relevant in yeast. Its significance in vivo remains to be established.

The FBPase-2 from yeast as described in the present paper differs from the enzyme characterized by Purwin et al. (1987) in the following properties: (i) it is separable from the bulk of alkaline phosphatases; (ii) it forms Fru-6-P and therefore constitutes, together with PFK-2,
Table 2. Inactivation of FBPase-2 by cyclic-AMP-dependent protein kinase and reactivation by protein phosphatase 2A

The conditions are described in the Experimental section. Values represent means ± S.D. for four experiments.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Degradation of Fru-2,6-P₂ (nmol/min per mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>4.9 ± 0.6</td>
</tr>
<tr>
<td>ATP (3 mM)</td>
<td>5.2 ± 0.5</td>
</tr>
<tr>
<td>Catalytic subunit of cyclic-AMP-dependent protein kinase (30 µg/ml)</td>
<td>4.7 ± 0.4</td>
</tr>
<tr>
<td>Catalytic subunit of protein phosphatase 2A (90 µg/ml)</td>
<td>4.3 ± 0.4</td>
</tr>
<tr>
<td>ATP (3 mM) + catalytic subunit of cyclic-AMP-dependent protein kinase (30 µg/ml)</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>ATP (3 mM) + catalytic subunit of cyclic-AMP-dependent protein kinase (30 µg/ml) + catalytic subunit of protein phosphatase 2A (90 µg/ml)</td>
<td>4.4 ± 0.5</td>
</tr>
</tbody>
</table>

a substrate cycle; (iii) it has a considerably higher affinity for its substrate, Fru-2,6-P₂ (Kₘ = 0.3 µM) than the phosphatase described by Purwin et al. (1987) (Kₘ = 6 µM).

REFERENCES


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