

Yeast 6-phosphofructo-2-kinase

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Received 9 April 1984

Yeast 6-phosphofructo-2-kinase (PFK2) was purified about 4000-fold by CM-Sephadex and Blue Sepharose chromatographies. Yeast contained two kinds of PFK2, both with apparent M_r values of 250 000 as estimated by gel filtration. The apparent K_m values of the major components for fructose 6-phosphate (F6P) and ATP were estimated as 0.5 and 0.7 mM, respectively.

Yeast 6-Phosphofructo-2-kinase Fructose 2,6-bisphosphate

1. INTRODUCTION

Recently F26P was identified as the most powerful stimulator for PFK1 among the glycolytic metabolites [1–3]. Upon the addition of glucose to isolated hepatocytes [4–6] and yeast [7], the level of F26P rapidly increases enough to stimulate PFK1 and inhibit FBPase. In metabolism, F26P is synthesized from ATP and F6P [8] by the enzyme PFK2, which has been purified from rat liver. PFK2 is known to be inactivated by protein kinase and reactivated by alkaline phosphatase [9]. PFK2 from rat liver is stimulated by AMP and P_i [8], and inhibited by citrate and PEP [10]. Furthermore, PFK2 from rat liver is also found to express the activity of fructose-2,6-bisphosphatase in the same protein [11], which is regulated by cAMP-dependent phosphorylation [11]. PFK2 from yeast has not yet been isolated. In this paper, the isolation and the properties of this enzyme are described.

Abbreviations: F6P, fructose 6-phosphate; F26P, fructose 2,6-bisphosphate; PFK1, 6-phosphofructo-1-kinase; PFK2, 6-phosphofructo-2-kinase; FBPase, 1,6-bisphosphatase; PEP, phosphoenolpyruvate; PMSF, phenylmethanesulfonyl fluoride

2. MATERIALS AND METHODS

F6P and F26P were purchased from Sigma. All enzymes were products of Boehringer Mannheim. Other chemicals were reagent grade and obtained from commercial sources.

2.1. Assay for F26P

F26P was assayed by using rabbit muscle PFK1 as in [12].

2.2. Assay for PFK2

PFK2 was assayed in 200 mM Tris-HCl (pH 7.5), 5 mM ATP, 5 mM F6P, 7.5 mM $MgCl_2$, 25 mM phosphate, 0.5 mM EDTA, 2.5 mM 2-mercaptoethanol and 0.1 mM PMSF (final volume, 0.08 ml). The mixture was incubated at 30°C. The mixture was transferred to 0.12 ml of 0.25 M NaOH solution, and was boiled for 10 min. Aliquots of the alkali-treated reaction mixture were assayed for F26P. One unit of the activity is defined as the amount of enzyme that catalyses the formation of 1 μ mol F26P per min under these conditions.

2.3. Preparation of PFK2

Baker's yeast was broken by sonication and suspended in buffer A: 50 mM sodium phosphate (pH 6.8), 5 mM 2-mercaptoethanol, 1 mM EDTA, 5 mM $MgCl_2$ and 0.2 mM PMSF. After the

removal of nucleic acids from the above yeast extract with protamine [13], ammonium sulfate was added to the yeast extract to give 50% saturation, and proteins were collected by centrifugation. The precipitate was dialyzed against buffer A overnight. The protein solution was applied to a CM-Sephadex C-50 column equilibrated with buffer A. The column was washed with buffer A, and PFK2 was eluted by a gradient of KCl (0–500 mM) in the same buffer. After concentrating the PFK2 fractions, the enzyme solution was applied to a Blue Sepharose column equilibrated with buffer A. After washing with buffer A containing 400 mM KCl, PFK2 was eluted by buffer A containing 800 mM KCl or 2 mM ATP. Protein concentrations were determined by the Coomassie brilliant blue G-250 dye-binding method using Bio-Rad dye reagent [14].

3. RESULTS

When F26P is synthesized in the presence of 5 mM ATP, 5 mM $MgCl_2$, 5 mM F6P and protein prepared by 80% $(NH_4)_2SO_4$ fractionation of yeast extract at pH 7.4, the production of F26P reaches a maximum in 20 min. The maximum concentration of F26P was estimated as 6 nmol per g protein of the intact cells, and was enough to activate PFK1 and to inhibit FBPase. These phenomena were observed in the intact cells after the addition of glucose to the culture of yeast [7]. Highest activities of PFK2, as well as PFK1 [13], are observed in the proteins prepared by 50% $(NH_4)_2SO_4$ fractionation.

When these protein fractions are applied to a CM-Sephadex C-50 column, PFK2 is adsorbed to the column, but PFK1 is not adsorbed. The elution of PFK2 from the column yields two peaks of the enzyme activity as shown in fig.1. PFK2 in the main peak and PFK2 in the small peak are eluted by buffer A containing 150 and 275 mM KCl, respectively. This observation shows that yeast contains two kinds of PFK2. PFK2 in the main peak and PFK2 in the small peak are designated as PFK2 (I) and (II), respectively.

When PFK2 (I) and (II) are applied to a gel filtration column, they are eluted as a single symmetrical peak on the column as shown in fig.2. PFK2 (I) and (II) seem to have similar M_r values. Their apparent M_r values are estimated approx-

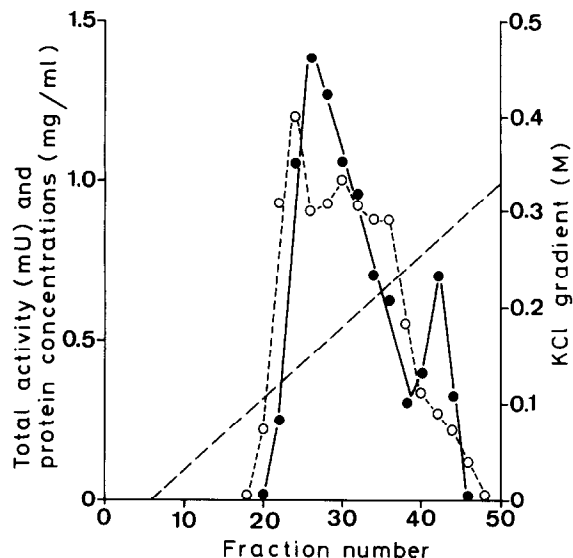


Fig.1. CM-Sephadex C-50 chromatography of yeast PFK2. PFK2 (24 munits) from the 50% $(NH_4)_2SO_4$ fractionation step was applied to a CM-Sephadex C-50 column (2.6×6 cm). Each fraction volume was 5 ml and the enzyme was eluted with a linear KCl gradient (---). (●) PFK2 activity, (○) protein concentration.

imately as 250000 (see fig.2), similar to catalase.

After fractionation of PFK2 with a CM-Sephadex C-50 column, PFK2 (I) is applied to a

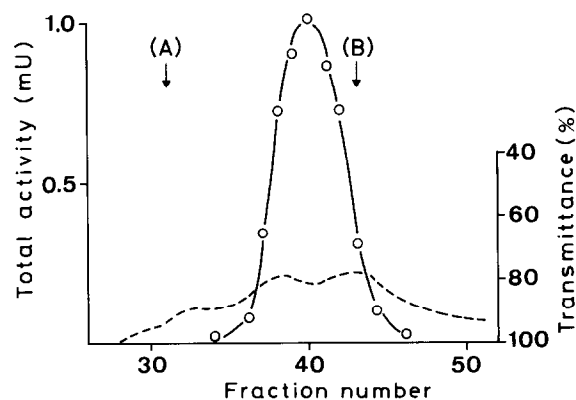


Fig.2. Gel filtration of PFK2 (I) and (II). The enzyme fractions [6 munits PFK2 (I) and 1 munit PFK2 (II)] pooled from the CM-Sephadex C-50 chromatography step were applied to an Ultro Gel AcA 34 LKB column (1.6×82 cm). Each fraction volume was 2.3 ml. (A,B) Elution of ferritin ($M_r=440000$) and catalase ($M_r=240000$), respectively. (---) Transmittance in the ultraviolet region, (○) PFK2 activity.

Table 1
Purification of PFK2

Step	Protein (mg)	Total activity (mU)	Specific activity (μ U)	Purification	Yield (%)
Sonification (yeast extract)	8100	ND ^b	ND		
80% (NH ₄) ₂ SO ₄ fractionation	3000	3.6	1.2	1	100
50% (NH ₄) ₂ SO ₄ fractionation	600	3.0	5	4.2	83
CM-Sephadex chromatography	12	2.5	210	175	69
Blue Sepharose ^a chromatography of PFK2 (I)	0.15	0.75	5000	4167	21

^a PFK2 (I) was eluted by buffer A containing 800 mM KCl from Blue Sepharose column

^b ND, not detectable

Blue Sepharose column, and eluted by buffer A containing 800 mM KCl or 2 mM ATP. F6P has no effect on the elution of PFK2 (I) from Blue Sepharose. The result of the partial purification of yeast PFK2 is summarized in table 1, which shows that PFK2 (I) is purified about 4000-fold. The partially purified PFK2 (I) is stable in buffer A at -20°C for about 1 month.

The K_m values of PFK2 (I) for F6P and ATP were estimated as 0.5 and 0.7 mM, respectively, as shown in fig.3. These values are similar to the physiological concentrations in growing yeast

[15,16]. The optimum pH for PFK2 (I) is pH 8.4. The activity of PFK2 (I) at 5 mM phosphate and pH 8.4 is similar to that at 25 mM phosphate and pH 7.5 (assay system for PFK2), indicating that the activity of PFK2 (I) increases with increasing concentration of phosphate at neutral pH.

4. DISCUSSION

Yeast PFK1 is inhibited by ATP, and is almost not stimulated by F6P, F16P and AMP at the physiological concentrations of these glycolytic metabolites [17,18]. For example, 200 μ M AMP which is above the physiological concentration [16], is required to give half-maximum stimulation for PFK1 [19]. On the other hand, F26P diminishes the inhibition of PFK1 by ATP at these physiological concentrations [17], and stimulates PFK1 by increasing the affinity of PFK1 for F6P [19]. Thus, F26P is considered to be a powerful initiator for yeast glycolysis and glycolytic oscillation [20], because F26P is rapidly synthesized to act as the initiator after the addition of glucose to the culture of yeast [7].

In the protein fraction prepared by 50% (NH₄)₂SO₄ precipitation, the specific activity of PFK2 (5 μ units/mg) was much lower than that of PFK1 (4 units/mg), but was nevertheless enough to account for the formation of F26P to stimulate PFK1.

The occurrence of two PFK2 fractions in yeast agrees with the two fractions of PFK2 observed in rat liver [8,21]. The difference between the two PFK2 fractions from either source has not yet been

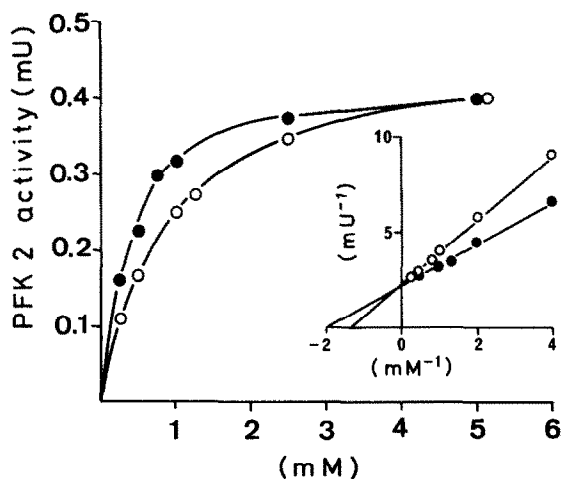


Fig.3. Effect of F6P and ATP concentration on PFK2 (I) eluted by buffer A containing 800 mM KCl from Blue Sepharose. The reaction mixtures contained 0.5 m units PFK2 (I), 5 mM phosphate, 5 mM MgCl₂, 500 mM Tris-HCl (pH 8.3) and 5 mM ATP (●) or 5 mM F6P (○). (●) F6P, (○) ATP.

investigated. The presence of two fractions suggests either two isomers of PFK2 or more probably the interconversion of PFK2 by protein kinase, which is under current investigation. Indeed, it has been described that PFK2 from rat liver is controlled by protein kinase in addition to other glycolytic metabolites such as citrate, AMP, phosphate and PEP [8,10].

Yeast PFK2, as well as PFK1 [15,22], is adsorbed on Blue Sepharose, and eluted by a buffer containing ATP at low concentration or KCl at high concentration. F6P has no effect on the elution of PFK2 and PFK1 [22]. Because Blue Sepharose is specific for the super-secondary structure called the dinucleotide fold [23–25], these facts suggest that yeast PFK1 and PFK2 have a similar dinucleotide fold. This fold forms NAD or ATP binding sites, and involves a β -sheet core composed of 5 or 6 parallel strands connected by α -helical loops located above and below the β -sheet. This suggests that yeast PFK1 and PFK2 may be derived from a common ancestor protein, because most glycolytic enzymes are known to have a dinucleotide fold [26,27].

The K_m value of PFK2 (I) for F6P was similar to that of PFK1 [15], but that of PFK2 (I) for ATP was higher than that of PFK1 [15]. As the K_m values of PFK2 (I) for F6P and ATP are similar to the physiological concentrations [15,16], the activity of PFK2 (I) seems to depend on the variation of the physiological concentrations of F6P and ATP. The regulatory mechanism of PFK1 in yeast is currently being analyzed especially under conditions of glycolytic oscillations.

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