Volume 226, number 1, 121-124 FEB 05426 December 1987

Identification of two forms of 6-phosphofructo-2-kinase in yeast

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Received 2 November 1987

Two forms of 6-phosphofructo-2-kinase have been identified in *Saccharomyces cerevisiae* by their different chromatographic behaviour on CM-Sephadex C-50. One of them was not adsorbed and represented ~30% of the eluted activity. The other one emerged at about 120 mM KCl. A molecular mass of 120 kDa was found for both of them. No differences in kinetic behaviour in susceptibility to activation by cAMP-dependent protein kinase were found between the two forms.

6-Phosphofructo-2-kinase; Fructose 2,6-bisphosphate; (Yeast)

1. INTRODUCTION

Fructose 2,6-bisphosphate, a metabolite formed from fructose 6-P by a specific 6-phosphofructo-2-kinase (PF2K), appears to play an important role in the regulation of glycolysis and gluconeogenesis in different organisms (review [1,2]). Liver and heart exhibit different isozymes of PF2K [3,4]. In yeast the enzyme is activated by phosphorylation after addition of glucose to the culture [5]. While studying the role of fructose 2,6-bisphosphate in yeast we observed that there are two different forms of PF2K in *Saccharomyces cerevisiae*. We present in this article biochemical evidence in support of this finding.

2. MATERIALS AND METHODS

2.1. *Strains and chemicals*

Commercial baker's yeast (Hércules, Madrid) was used in most of the work. One isolate from it (HER-1) was used to grow the yeast in different culture conditions. A mutant with disruptions in each of the two genes for phosphofructokinase (H48-7D) was used in some experiments (courtesy of Dr J. Heinisch, Darmstadt, FRG). One strain lacking the main proteases B, Y and S was kindly provided by Professor D.H. Wolf (Freiburg i.B., FRG). Commercial yeast was washed with water and stored frozen at −70°C. Strain H48-7D was grown with 3% glycerol and 3% ethanol, harvested by centrifugation and stored frozen at −70°C. The proteinase-less strain was grown on rich medium-glucose and treated as H48-7D. Biochemicals were from Sigma and chromatographic reagents from Pharmacia.

2.2. *Preparation and assay of PF2K*

30 g of yeast were resuspended in 60 ml of buffer A (50 mM potassium phosphate, 0.5 M KCl, 2 mM EDTA, 1 mM dithiothreitol, 20 mM NaF, 1 mM phenylmethylsulfonyl fluoride, pH 7.5), mixed with 170 ml glass beads and shaken in a refrigerated mill for 5 min. After filtration the beads were washed with 30 ml of the same buffer. The filtrate was centrifuged at 27000 × *g* for 30 min. Protamine sulphate dissolved in buffer A was added to the supernatant to a final concentra-

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

tion of 0.2%. After 30 min stirring the mixture was centrifuged as before. Solid polyethylene glycol (M_r = 8000) was added to the supernatant to a final concentration of 18% and after 30 min stirring the mixture was centrifuged at 27000 × g for 30 min. The precipitate was dissolved in 22 ml of buffer B (50 mM sodium phosphate, 5 mM $MgCl_2$, 1 mM EDTA, 5 mM 2-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride, 20% glycerol, pH 6.8). The solution was chromatographed on a CM-Sephadex C-50 column (2.3 × 14 cm) equilibrated with buffer B. The column was washed with buffer B and then eluted with 280 ml of a linear gradient of KCl (0–500 mM in buffer B). Fractions were collected from the beginning of the wash. Peaks with PF2K activity were separately pooled and each one applied to a Blue Sepharose column (1.3 × 3.8 cm). The column was washed with 50 ml buffer B, eluted with 50 ml of a linear KCl gradient (0–200 mM in buffer B) and then with another one from 200 mM to 2 M. The activity eluted at about 1 M KCl. The active pools were dialyzed for several hours against 10 mM Hepes, 20% glycerol, pH 7. The specific activities of these preparations were about 0.2 and 1.3 mU/mg protein for peak I and peak II, respectively.

PF2K was assayed essentially as described by François et al. [5]. Protein was measured by the method of Lowry et al. [6].

3. RESULTS AND DISCUSSION

The elution profile of PF2K from the CM-Sephadex column is shown in fig.1. Two peaks with PF2K activity came out from the column. The first one, peak I, represented about 30% of the total eluted activity and emerged with the unadsorbed protein while the second one, peak II, emerged at about 120 mM KCl. The total activity (sum of both peaks) recovered after this step was 80% of the activity measured in the crude extract.

Phosphofructokinase was not adsorbed to the column in accordance with the results of Yamashoji and Hess [7]. Several trivial explanations may be offered for the appearance of the two peaks of PF2K activity. We considered the following ones: (i) the column is overloaded; (ii) peak I is artefactual and connected with some activity of phosphofructokinase; (iii) the two peaks are produced by proteolysis of one protein; (iv) there is an equilibrium between different aggregation states of the enzyme; (v) the peaks correspond to the non-phosphorylated and the phosphorylated states of the protein [5]. We performed several experiments to decide which, if any, of these possibilities could account for the observed result.

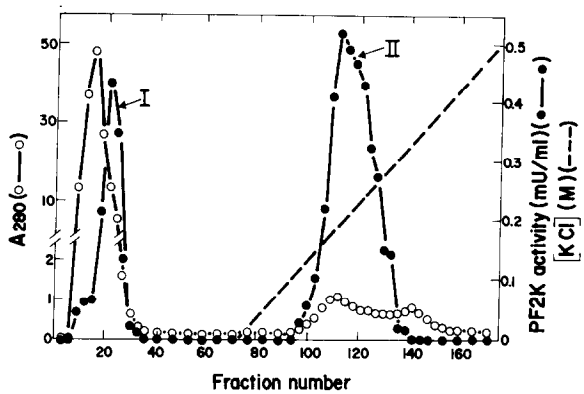

Fig.1. Chromatography of yeast PF2K on CM-Sephadex C-50. Chromatography was as described in section 2. Fractions of 3 ml were collected.

Chromatography was carried out with samples containing 5- and 10-times less protein concentration as well as with samples that were not treated with polyethylene glycol. The same behaviour was observed in all cases. This result discards in our opinion explanation (i).

To discard possibility (ii) we used mutants with non-functional phosphofructokinase due to disruption of genes pfk1 and pfk2 [8]. No change in the chromatographic behaviour was observed.

The idea of proteolysis seems unlikely due to the following results. The same elution profile and the same ratio of activities were observed in different batches. No changes were found during harvesting of the yeast in the logarithmic or stationary phases of growth. The use of a mutant lacking proteases B, Y and S did not change the chromatographic behaviour. In addition rechromatography of each peak after incubation for 2 h at 28°C with a small amount of freshly prepared extract did not change significantly the corresponding profile.

To test the hypothesis of an equilibrium between different aggregation states each peak was rechromatographed separately on CM-Sephadex. As can be seen in fig.2, each peak emerges at the same position as initially and no appearance of

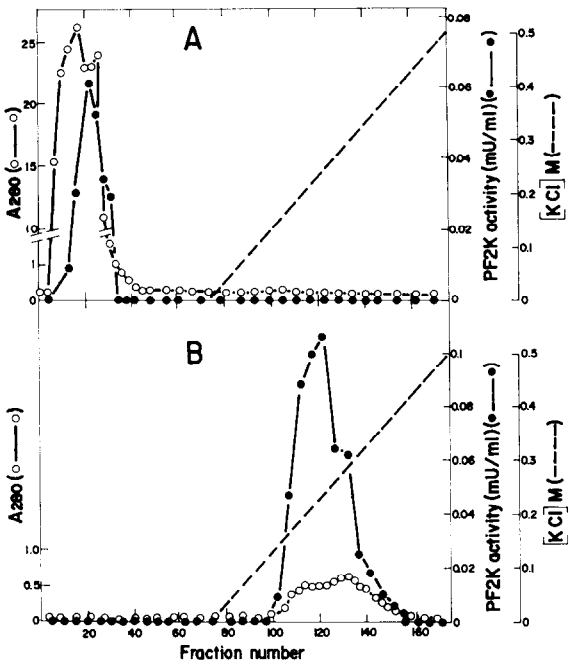

Fig.2. Rechromatography on CM-Sephadex C-50 of peak I and peak II of yeast PF2K. Active fractions containing peaks I and II of fig.1 were pooled separately, dialyzed for several hours against buffer B (see section 2) and chromatographed again in the same conditions as in fig.1. (A) Peak I, (B) peak II.

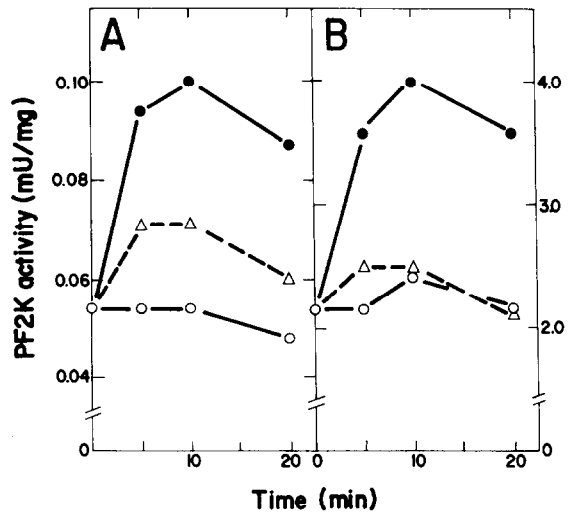

Fig.3. Activation of peak I and peak II of yeast PF2K by cAMP-dependent protein kinase. The reaction was carried out with peak I (A) and peak II (B) purified to the step of Blue Sepharose (see section 2). The reaction mixture contained in a final volume of 1 ml: 5 mM potassium phosphate, 10 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 1 mM ATP, and as indicated 2.5 μg of catalytic subunit of cAMP-dependent protein kinase and 420 μg of protein kinase inhibitor. PF2K I was at a concentration of 19 μU and PF2K II at 240 μU. The temperature was 30°C. (○---○) Control; (●---●) with catalytic subunit; (△---△) with catalytic subunit and protein kinase inhibitor.

another new peak could be observed. We believe therefore that the idea of an equilibrium between different aggregation states is not adequate. This experiment also argues strongly against the hypothesis of overloading of the column.

Yeast PF2K may exist in two forms with different activities as a consequence of the activity of a cAMP-dependent protein kinase [5]. It could be thought that the two peaks correspond to these two forms. To test this possibility both peaks were further purified by Blue Sepharose chromatography and subjected to treatment with commercial cAMP-dependent protein kinase. As shown in fig.3 both peaks were similarly stimulated by the treatment. In our opinion this result rules out the possibility considered.

Taken together these results are best interpreted as indicative of the existence in yeast of two forms of PF2K with different chromatographic behaviour.

The molecular mass of both peaks was determined separately after chromatography on Blue Sepharose with a Superose column and the adequate markers (range from chymotrypsinogen A, 25 kDa to thyroglobulin, 669 kDa). The dimensions of the column were 1.6 × 58 cm and the buffer was 50 mM potassium phosphate, 0.1 M KCl, 5 mM $MgCl_2$, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 20% glycerol, pH 6.8. Both forms showed a similar molecular mass of ~120 kDa. This molecular mass differs from that reported by Yamashoji and Hess [7] who observed that the elution from the CM-Sephadex column gave a major peak and a minor shoulder of PF2K activity and found a molecular mass of 250 kDa. The difference between their result and ours is not easy to explain. One possibility is that the composition of the buffer and the previous treatment influence the behaviour of the enzyme on the gel filtration column. Since the molecular mass found in this work is about one half of that reported by

Table 1

K_m values of partially purified yeast PF2K

Substrate	K_m (mM)	
	Peak I	Peak II
Fructose 6-P[a]	0.4	0.33
Mg ATP[b]	0.38	0.29

[a] In these experiments Mg ATP was kept at 5 mM
[b] In these assays fructose 6-P was 1 mM

Peaks I and II from CM-Sephadex chromatography were further purified by treatment with Blue Sepharose as described in section 2

Yamashoji and Hess [7] it could be thought that in their conditions the enzyme could dimerize.

As shown in table 1 the kinetic properties of PF2K from both peaks, studied after passage through Blue Sepharose, were not significantly different. No significant difference was also observed in their behaviour towards potential effectors when the enzyme was assayed at 5 mM ATP and 0.5 mM fructose 6-P. Under these conditions both forms were inhibited about 35% by 2 mM *sn*-glycerol 3-P and about 60% by 2 mM P-*enol*pyruvate. Other effectors tested (citrate, AMP, inorganic phosphate) were without significant effect.

Van Schaftingen and Hers [9] noted that in some preparations of rat liver two peaks of PF2K activity eluted from DEAE-cellulose, however, in some experiments one of the peaks was barely detectable. El-Mahgrabi et al. [10] also observed the two peaks and reported a similar molecular mass for them (90 kDa). Some differences in kinetic properties between the two peaks were observed by this last group [10].

Yeast is an organism that exhibits a high number of isozymes and repeated genes. In the glycolytic pathway there are three glucose phosphorylating activities, at least two phosphofructokinases, two enolases and tandemly repeated genes for glyceraldehyde-3-P dehydrogenase (review [11]). It is therefore not surprising that two isozymes of PF2K may exist. One could speculate that two genes exist or that a unique gene is transcribed from two different starting points as in the case of histidine-tRNA synthetase [12] or of invertase [13]. Genetic evidence is needed to clarify the significance and origin of the two peaks found in this work.

ACKNOWLEDGEMENTS

Financial help from the Comisión Asesora de Investigación Científica y Técnica and the Fondo de Investigaciones Sanitarias de la Seguridad Social is greatly acknowledged. We acknowledge the help of M.A. Blázquez, M.J. Vaquerizo and M.A. Chávez in some experiments.

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