

Characterization of yeast fructose-2,6-bisphosphate 6-phosphatase

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To obtain information on the biological significance of yeast fructose-2,6-bisphosphate 6-phosphatase, kinetic data of the purified enzyme [(1987) Eur. J. Biochem. 164, 27–30] have been measured. Maximal activity was found between pH 6 and 7, the apparent Michaelis constant with fructose 2,6-bisphosphate was 7.2 μ M at pH 6.0 and 79 μ M at pH 7.0. Concentrations required for 50% inhibition of the enzyme at pH 6.0 were 8 μ M Fru2P, 45 μ M Glc6P, 80 μ M Fru6P and 200 μ M inorganic phosphate. The known intracellular steady-state level of about 10 μ M fructose 2,6-bisphosphate in the presence of glucose is likely to be the result of a balance between the rapid synthesis of fructose 2,6-bisphosphate catalyzed by 6-phosphofructose 2-kinase and a fructose 2,6-bisphosphate degrading activity. The biological function of fructose-2,6-bisphosphate 6-phosphatase with an apparent Michaelis constant between 7 and 79 μ M fructose 2,6-bisphosphate at pH 6–7 is therefore suggested to participate in the maintenance of a steady-state level of fructose 2,6-bisphosphate in a concentration range that fits well with the Michaelis constant of the enzyme.

Yeast; Fructose-2,6-bisphosphate 6-phosphatase; Kinetic parameter; Inhibitor

1. INTRODUCTION

A purified phosphatase from *Saccharomyces cerevisiae* has been shown to dephosphorylate Fru(2,6)P₂ to fructofuranose 2-phosphate (Fru2P) [1,2]. This Fru(2,6)P₂ 6-Pase can be completely separated from the previously described Fru6P 2-kinase [3,4]. Therefore, in contrast to the situation in liver tissue, where synthesis of Fru(2,6)P₂ by Fru6P 2-kinase and degradation of Fru(2,6)P₂ by Fru(2,6)P₂ 2-Pase are catalyzed by one and the same bifunctional polypeptide [5], in yeast synthesis and degradation of Fru(2,6)P₂ are catalyzed by different proteins. Recently, an additional phosphatase from yeast has been described which dephosphorylates Fru(2,6)P₂ not to Fru2P, but to Fru6P [6,7]. This Fru(2,6)P₂ 2-Pase can, similar to

the 6-phosphatase, be completely separated from Fru6P 2-kinase [6,7]. To evaluate the biological significance of the Fru(2,6)P₂ degrading phosphatases, it is necessary to know the affinity for the substrate Fru(2,6)P₂ and other kinetic constants. In the present work using purified Fru(2,6)P₂ 6-Pase the following parameters were measured: the pH dependence of the catalytic activity, the apparent Michaelis constant with Fru(2,6)P₂ and the influence of physiologically relevant sugar phosphates on the activity of the enzyme. On the basis of these results, the biological function of Fru(2,6)P₂ 6-Pase is discussed.

2. MATERIALS AND METHODS

Pyrophosphate-dependent phosphofructokinase from potato tubers (EC 2.7.1.90) and Fru(2,6)P₂ were purchased from Sigma (Deisenhofen, FRG). All other enzymes were purchased from Boehringer Mannheim (FRG). Chemicals were from commercial sources at highest purity available.

For the assay of Fru(2,6)P₂ 6-Pase the rate of disappearance of Fru(2,6)P₂ was measured. The assay was performed as described by Purwin et al. [1] with the exception that the reac-

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Abbreviations: Fru(2,6)P₂, fructose 2,6-bisphosphate; Fru(2,6)P₂ 6-Pase, fructose-2,6-bisphosphate-6-phosphatase

tion was stopped by alkalinisation not at 95°C, but at room temperature. The remaining Fru(2,6)P₂ was determined as described by Van Schaftingen et al. [8]. Protein concentration was determined by the method of Bradford [9].

3. RESULTS

The dependence of Fru(2,6)P₂ 6-Pase activity on pH is shown in fig.1. When assayed at a substrate concentration of 40 μ M Fru(2,6)P₂ the enzyme exhibits maximal activity between pH 6.0 and 6.5. Dependence of the activity of Fru(2,6)P₂ 6-Pase on the concentration of Fru(2,6)P₂ is shown at pH 6.0 in fig.2 and at pH 7.0 in fig.3, respectively. Evaluation of the Lineweaver-Burk plots (see insets in figs 2 and 3) results in apparent Michaelis constants (substrate concentration necessary for half-maximal velocity) of 7.2 μ M Fru(2,6)P₂ at pH 6.0 and 79 μ M Fru(2,6)P₂ at pH 7.0. Maximal activities (at substrate saturation) were 0.86 μ mol \cdot min⁻¹ \cdot mg⁻¹ at pH 6.0, and 0.94 μ mol \cdot min⁻¹ \cdot mg⁻¹ at pH 7.0. At substrate saturation, the maximal activity is slightly higher at pH 7.0 as compared to pH 6.0. The pH dependence of activity with 40 μ M Fru(2,6)P₂ (i.e. substrate saturation at pH 6.0 and below saturation at pH 7.0) is shown in fig.1.

Inhibition of Fru(2,6)P₂ 6-Pase by the product

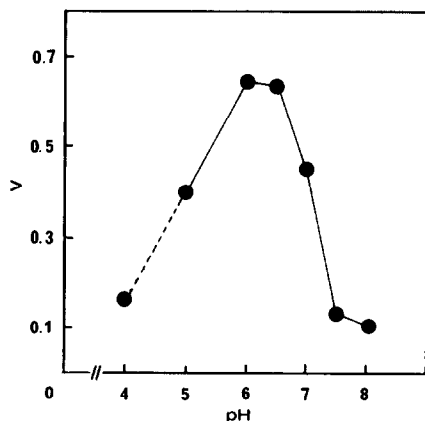


Fig.1. Effect of pH on the activity of Fru(2,6)P₂ 6-Pase. The activity was measured in test mixtures containing 40 μ M Fru(2,6)P₂, 5 mM MgCl₂ and 50 mM Na-acetate (pH 4–6) or 50 mM imidazole/HCl (pH 6–8), respectively, as described in section 2. At pH 6.0, activity in acetate buffer was the same as in imidazole buffer. Values for V are expressed in μ mol \cdot min⁻¹ \cdot mg⁻¹. The value at pH 4.0 is somewhat uncertain because of the relative instability of Fru(2,6)P₂ and of the enzyme at this pH value.

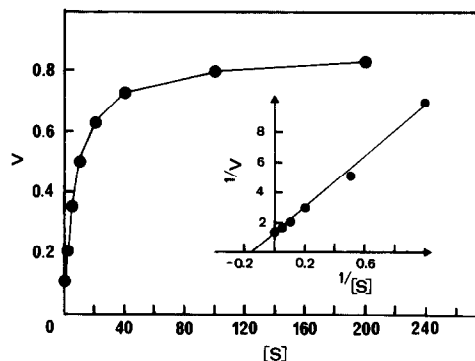


Fig.2. Determination of kinetic parameters of Fru(2,6)P₂ 6-Pase at pH 6.0. Fru(2,6)P₂ 6-Pase was assayed in imidazole/HCl buffer, pH 6.0, using increasing concentrations of Fru(2,6)P₂ as substrate. Assay conditions are described in section 2. The S and K_m values are expressed in μ M, V and V_{max} values in μ mol \cdot min⁻¹ \cdot mg⁻¹. $K_m = 7.2 \mu$ M Fru(2,6)P₂; $V_{max} = 0.86 \mu$ mol \cdot min⁻¹ \cdot mg⁻¹.

of the reaction, Fru2P, and by the related sugar phosphates Fru6P and Glc6P, as well as by inorganic phosphate is shown in fig.4. The apparent inhibitor constants K_i (concentration of inhibitor for half-maximal activity) are found to be 8 μ M for Fru2P, 45 μ M for Glc6P, 80 μ M for Fru6P and 200 μ M for inorganic phosphate (all measurements at pH 6.0). Fru, also found as a product of the

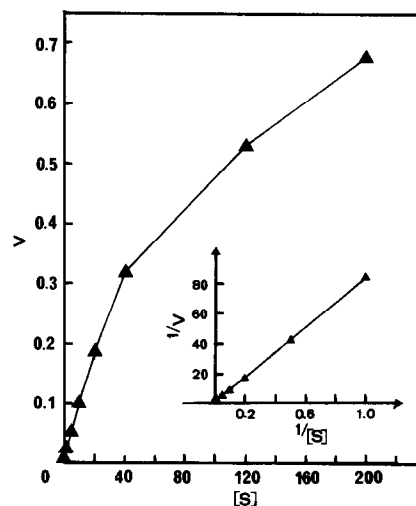


Fig.3. Determination of kinetic parameters of Fru(2,6)P₂ 6-Pase at pH 7.0. Enzyme activity was assayed in 50 mM imidazole/HCl, pH 7.0. Experimental details are described in the legend to fig.2 and in section 2. $K_m = 79 \mu$ M Fru(2,6)P₂; $V_{max} = 0.94 \mu$ mol \cdot min⁻¹ \cdot mg⁻¹.

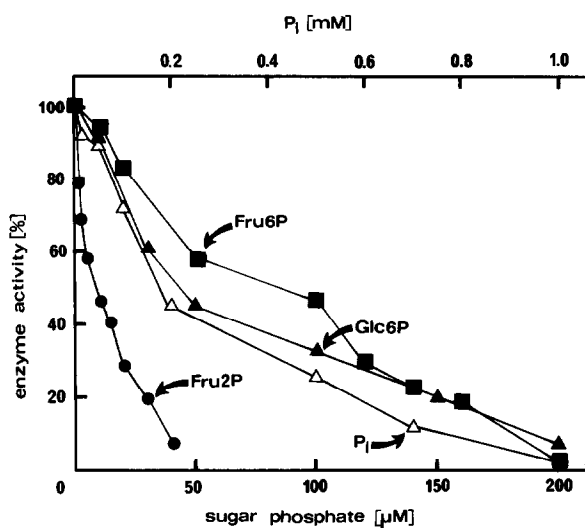


Fig.4. Effect of the reaction products Fru2P and P_i , and of the sugar phosphates Fru6P and Glc6P on the activity of Fru(2,6) P_2 6-Pase. Fru(2,6) P_2 6-Pase was assayed as described in section 2 with increasing concentrations of Fru2P, P_i , Fru6P and Glc6P. K_i values: Fru2P, 8 μ M; Glc6P, 45 μ M; Fru6P, 80 μ M; P_i , 200 μ M.

dephosphorylation of Fru(2,6) P_2 [1], does not inhibit at 10 mM concentration (not shown).

4. DISCUSSION

The time course of the disappearance of Fru(2,6) P_2 during the first 10 min of incubation with Fru(2,6) P_2 6-Pase was shown in a previous publication [1] to parallel almost exactly the appearance of fructose 2-phosphate. Starting the reaction with an initial concentration of 250 μ M Fru(2,6) P_2 a continuous decrease in the rate of disappearance of Fru(2,6) P_2 was observed [1]. This fact was discussed by Francois et al. [7] as indicating an apparent Michaelis constant of Fru(2,6) P_2 6-Pase with Fru(2,6) P_2 higher than 50 μ M which would be far beyond the steady-state level of about 10 μ M and therefore question the physiological significance of Fru(2,6) P_2 6-Pase. This conclusion cannot be drawn for the following reasons: (i) the rates to be used for calculation of the apparent Michaelis constant have to be by definition 'initial rates' and not the rates observed in an incubation mixture in which the substrate concentration continuously decreases; (ii) as

shown in fig.4, very low concentrations of the dephosphorylation product of Fru(2,6) P_2 , i.e. Fru2P, inhibit the reaction (K_i = 8 μ M at pH 6.0). The accumulating product has therefore a strong influence on the time course of the disappearance of Fru(2,6) P_2 ; (iii) assays of the initial rate of activity in samples taken from the incubation mixture described in [1] showed that the Fru(2,6) P_2 6-Pase loses a significant part of its activity during the incubation (not shown).

For the discussion of the biological function of Fru(2,6) P_2 6-Pase with respect to the very low concentrations of Fru(2,6) P_2 found in yeast cells [10–12], the apparent Michaelis constants calculated from measurements of the initial rates of activity at different concentrations of Fru(2,6) P_2 (cf. figs 2 and 3) have to be considered. In stationary phase cells, the concentration of Fru(2,6) P_2 is below 1 μ M [12]. After addition of glucose, Fru(2,6) P_2 increases rapidly to steady-state concentrations of 5 to 15 μ M. The reason for this increase of Fru(2,6) P_2 is the rapid formation of Fru6P via Glc6P from glucose and the subsequent phosphorylation of this sugar to Fru(2,6) P_2 by Fru6P 2-kinase [3,4], which in turn is activated by the addition of glucose via a transient increase in cAMP [13]. To yield the steady-state concentration of about 10 μ M Fru(2,6) P_2 , the synthesis of Fru(2,6) P_2 therefore must be counteracted by Fru(2,6) P_2 degradation. Because addition of glucose decreases the cytosolic pH from initially pH 7.0 to pH 6–6.5 [14], the Fru(2,6) P_2 6-Pase exhibiting an apparent K_m value of 7.2 μ M at pH 6.0 and of 79 μ M at pH 7.0 as described in this paper might well participate in balancing the increase of Fru(2,6) P_2 .

Inhibition of Fru(2,6) P_2 6-Pase by inorganic phosphate (K_i = 0.2 mM, see fig.4) would only play a role in stationary cells and not in glucose-metabolizing cells, where the concentration of 'free inorganic phosphate' is very probably much below 0.2 mM [15].

Glc6P is found in glucose-metabolizing yeast cells at a steady-state concentration of about 1 mM [13]. Because the apparent inhibitor constant has been determined to be K_i = 0.045 mM (see fig.4), the Fru(2,6) P_2 6-Pase should be inhibited almost completely in the presence of glucose. It might be that de-inhibiting factors or metabolites exist. A very similar problem has been observed with the

other Fru(2,6)P₂ degrading enzyme, Fru(2,6)P₂ 2-Pase. This enzyme is almost completely inhibited by physiological concentrations of Fru6P [6,7].

Another biological function of Fru(2,6)P₂ 6-Pase in addition to the control of the steady state concentration of Fru(2,6)P₂ might be given by regulatory functions of the reaction product Fru2P similar to the well known metabolic control effects of Fru(2,6)P₂ [16]. Search for regulatory functions of Fru2P is in progress in our laboratory.

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