Note

Inhibition of *Euglena gracilis* Fructose-1,6-bisphosphatase by Fructose-2,6-bisphosphate

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Work in several laboratories during the past few years has demonstrated that fructose-2,6-bisphosphate (Fru-2,6-P₂) is essential in the regulation of carbohydrate metabolism in rat liver, yeast, and some plants.¹⁾ There is agreement on the allosteric nature of the activation of phosphofructokinase (EC 2.7.1.11) by Fru-2,6-P₂^{2~6)} but the inhibition mechanism of fructose-1,6-bisphosphatase (EC 3.1.3.11) (FBPase) by Fru-2,6-P₂ is controversial.^{1,7~14)}

Euglena gracilis, a protozoan, accumulates paramylon, a β -1,3-glucan, and trehalose as energy reserves, but information on participation of Fru-2,6-P₂ in the regulative mechanism of carbohydrate metabolism is not available. We have studied the effects of Fru-2,6-P₂ in this organism, especially in more detail on FBPase. This paper reports that, like FBPase in other organisms, the *E. gracilis* enzyme is inhibited by Fru-2,6-P₂ but not AMP.

The following materials were purchased from the sources indicated: NADP⁺, Fru-1,6-P₂, AMP, dithiothreitol, Fru-2,6-P₂ and Tris (Tris(hydroxymethyl)aminomethane) from Sigma; glucose-6-P dehydrogenase and glucose-6-P isomerase from Boehringer; phosphocellulose (P-11) from Whatman; protein assay Coomassie reagent from Bio-Rad. Other reagents were also purchased and were of analytical grade.

E. gracilis FBPase was partially purified with phosphocellulose as described by Black *et al.*¹⁵⁾ *E. gracilis* SM-ZK, a bleached mutant¹⁶⁾ was used throughout the experiments. *E. gracilis* SM-ZK was cultured in Koren-Hutner medium¹⁷⁾ under illumination (2000 lux) with shaking for 5 days at 27°C and harvested by centrifugation for 5 min at 3,000 $\times g$. One gram (wet weight) of cells was suspended in 2 ml of a buffer, pH 7.4, containing 20 mM Tris-acetate, 0.1 mM EDTA, and 1 mM dithiothreitol, and disrupted by sonication (10 kHz) for 1 min. Cell debris was removed by centrifugation, and the supernatant was dialyzed against 250 ml of the same buffer for 3 hr. After dialysis, the solution was mixed with phosphocellulose equilibrated with the same buffer.

The amount of ion exchanger used was that required to make a 2-ml column. The phosphocellulose suspension was poured into a 0.6-cm diameter column and washed with the same buffer until the absorbance at 280 nm of the eluate was 0.01. FBPase was then eluted with the same buffer containing 1 mM Fru-1,6-P₂. Enzyme activity appeared with the eluent and the first two tubes (4 ml in sum) contained FBPase of a specific activity of about 3 units/mg. The overall purification of FBPase was about 70-fold with 12% yield.

The FBPase activity was measured spectrophotometrically at 30°C by following the rate of formation of NADPH at 340 nm in the presence of excess glucose-6-P

FIG. 1. Double Reciprocal Plots of Fru-1,6-P₂ for FBPase in the Presence of Fru-2,6-P₂.

FBPase activity was measured with different concentrations of Fru-1,6-P₂ in the absence (●) and presence of 1 μ M (▲) Fru-2,6-P₂. Assay conditions were described in text and protein concentration was 6 μ g.

FIG. 2. Effects of Fru-2,6-P₂ and AMP on FBPase.

Enzyme activity was measured at 1 mM Fru-1,6-P₂ plus the indicated concentrations of Fru-2,6-P₂ and AMP. A value of 100% was given the specific activity (3 units) measured in the absence of Fru-2,6-P₂. ●, AMP (0~1 mM); ▲, Fru-2,6-P₂ (0~5 μ M); ○, AMP (fixed 1 mM) plus Fru-2,6-P₂ (0~5 μ M).

isomerase and glucose-6-P dehydrogenase. Unless otherwise stated, the reaction mixture (1 ml) contained 50 mM Tris-acetate (pH 7.2), 0.2 mM Fru-1,6-P₂, 10 mM MgSO₄, 1 mM dithiothreitol, 0.1 mM EDTA, 0.2 mM NADP⁺, and 1.5 unit each of glucose-6-P isomerase and glucose-6-P dehydrogenase. The reaction was started by the addition of Fru-1,6-P₂. A unit of FBPase activity is defined as the amount of enzyme which catalyzes the formation of 1 μ mol of Fru-6-P/min. Protein was measured by the Coomassie dye-binding assay.¹⁸⁾

The optimum pH of the partially purified FBPase was 7.2 under these assay conditions and this result suggests that this FBPase is like the neutral FBPases in many organisms.¹⁹⁾ The substrate saturation curve was hyperbolic either in the absence or presence of Fru-2,6-P₂ and a low concentration of Fru-2,6-P₂ inhibited FBPase as shown in Fig. 1. The inhibition by Fru-2,6-P₂ was competitive, as reported for many organisms.^{7~10,12,20~22)} The *K_i* value for Fru-2,6-P₂ was 0.8 μ M, considerably smaller than the *K_m* value for Fru-1,6-P₂ (30 μ M).

In contrast to the enzymes of other organisms, *E. gracilis* FBPase was not inhibited by AMP (●) and was not enhanced by AMP in the presence of inhibitory concentrations of Fru-2,6-P₂ (○) as shown in Fig. 2. As reported by Marcus *et al.*,²⁰⁾ the AMP inhibition of *E. coli* FBPase is not increased by the presence of an inhibitory concentration of Fru-2,6-P₂ and our results confirm their observation. Although Babul and Guixé²³⁾ reported that phosphoenolpyruvate released the AMP inhibition of *E. coli* FBPase, it did not affect the *Euglena* FBPase either in the absence or presence of Fru-2,6-P₂ (data not shown).

The sigmoid shape of the substrate saturation curves in the presence of Fru-2,6-P₂ has been used as an argument for an allosteric type of Fru-2,6-P₂-FBPase interaction.¹²⁾ However, competitive inhibition in a multisite system may explain the data as well without any need to assume a separate allosteric site.²⁴⁾ From our results, there is no need to postulate an allosteric site for Fru-2,6-P₂ in *Euglena* FBPase since the inhibition of FBPase by Fru-2,6-P₂ appears to be competitive. Another characteristic of Fru-2,6-P₂ interaction with most FBPase^{7,8,10,12,22)} is that the Fru-2,6-P₂ inhibition is synergistic with that of AMP, and this has also been used to emphasize that Fru-2,6-P₂ is an allosteric competitive inhibitor of FBPase.^{12,13)} However, this is not valid with the *E. gracilis* FBPase since in this enzyme, AMP does not affect the Fru-2,6-P₂ inhibition and there is no need to postulate an allosteric site for Fru-2,6-P₂ or AMP. In conclusion, our results show that Fru-2,6-P₂ inhibits *Euglena* FBPase, most likely by interaction at the active site of the enzyme, like other substrate analogs of FBPase such as methyl-D-fructofuranoside-1,6-P₂.¹¹⁾

We have reported that Fru-2,6-P₂ inhibits trehalose phosphorylase and proposed that Fru-2,6-P₂ plays a key effector in carbohydrate metabolism in *Euglena*.²⁵⁾ The inhibition of the FBPase from *Euglena* suggests that gluconeogenesis in this protozoon, as well as glycolysis,

may be regulated by Fru-2,6-P₂ as in the liver.¹⁾

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