Note

Fructose-2,6-bisphosphate Activates Pyrophosphate: D-Fructose 6-Phosphate 1-Phosphotransferase from *Euglena gracilis*

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Pyrophosphate: D-fructose 6-phosphate 1-phosphotransferase (PFP) (EC 2.7.1.90) occurs in several plant tissues,^{1~6)} *Entamoeba*⁷⁾ and some prokaryotes.⁷⁾ The enzyme in mung hypocotyl,³⁾ spinach leaf,⁴⁾ potato tuber⁵⁾ and castor bean⁶⁾ is strongly activated by fructose-2,6-bisphosphate (Fru-2,6-P₂), a potent activator of some mammalian phosphofructokinases (EC 2.7.1.11).⁸⁾ The observation made by Sabulase and Anderson³⁾ that the activity of the mung bean PFP is greatly increased by Fru-2,6-P₂ has opened a new field of investigation on the role of PFP in glycolysis and gluconeogenesis in plants or plantlike organisms. Greater parts of the biochemical studies previously reported in *Euglena* concern photosynthesis and related subjects. However, recent enzymological studies have found more and more animal-like features, or unique enzymatic characteristics different from bacterial, plant, and animal enzymes.

In a previous paper,⁹⁾ we reported the detection of PFP in *Euglena gracilis*, which can grow photoautotrophically like a plant as well as heterotrophically in the dark. No relationship between this enzyme and Fru-2,6-P₂ has been studied, and there is little evidence to suggest that PFP is physiologically significant in this interesting organism. This paper reports the PFP activation by Fru-2,6-P₂, and we propose that Fru-2,6-P₂-sensitive PFP has a possible key role in carbohydrate metabolism in *Euglena*.

The following materials were purchased from the sources indicated; NADH, Fru-6-P, Fru-2,6-P₂ and Tris (Tris(hydroxymethyl)aminomethane) from Sigma; Affi-Gel Blue, DEAE BioGel A, and protein assay Coomassie reagent from Bio-Rad; aldolase, triosephosphate isomerase, and glycerol-3-phosphate dehydrogenase were from Boehringer and were dialyzed before use; and phosphocellulose from Whatman. Other reagents were of analytical grade.

A streptomycin-bleached mutant of *E. gracilis*¹⁰⁾ was used throughout the experiments. Cells were cultured in

the Koren-Hutner medium¹¹⁾ under illumination (2000 lux) with shaking (90 strokes/min) at 27°C, harvested by centrifugation (1500 × g, 2 min) and washed with 50 mM Tris-acetate buffer, pH 6.5, containing 30% (v/v) propyleneglycol. Cells were disrupted by sonication (10 kHz) for 30 sec and the supernatant obtained by centrifugation of the sonicate at 10000 × g for 10 min was used as the crude enzyme. PFP was partially purified by chromatographies on DEAE BioGel, Affi-Gel blue, and phosphocellulose with different KCl concentrations. A crude enzyme was put on DEAE BioGel A equilibrated with Tris-acetate buffer, pH 6.5, containing 30% (v/v) propyleneglycol, and washed with the same buffer. The PFP activity was not retained by DEAE BioGel. Active fractions were pooled and further purified by Affi-Gel Blue and phosphocellulose chromatographies. The overall purification of PFP was 20-fold over cell homogenate with 17% yield and free from phosphofructokinase, pyrophosphatase, and fructose-1,6-bisphosphatase activities. Active fractions were mixed with equal volumes of cold propyleneglycol and kept at -20°C. Under these storage conditions, PFP lost less than 10% of its activity in one month.

All enzyme assays were done at 30°C in 1 ml total volume. NADH oxidation was recorded with a Hitachi 200 spectrophotometer. The reaction mixture for assaying the PFP activity contained 50 mM Tris-acetate buffer, pH 6.7, 5 mM MgCl₂, 10 mM Fru-6-P, 0.2 mM NADH, 1 mM pyrophosphate (PPi), 1 unit aldolase, 5 units triosephosphate isomerase, 1 unit glycerol-3-phosphate dehydrogenase, and an effector (Fru-2,6-P₂) at the concentration indicated. The reaction was started by the addition of PPi. One unit of the enzyme is the amount which catalyzes the formation of 1 μmol of Fru-1,6-P₂ per min under these conditions. Protein was measured by the Bradford method.¹²⁾

The effects of Fru-6-P concentration on the activity of partially purified PFP are shown in Fig. 1. *Euglena* PFP showed a strong requirement for Fru-2,6-P₂. In the presence of 1 μM Fru-2,6-P₂, PFP had a Michaelis type saturation curve for Fru-6-P. In the absence of Fru-2,6-P₂, the saturation curve of PFP with Fru-6-P was sigmoidal with a Hill coefficient of 1.7 and the half maximal velocity, S_{0.5} was 2.5 mM (calculated from these results). Fru-2,6-P₂ increased V_{max} of the PFP reaction 4-fold and decreased S_{0.5} for Fru-6-P 5-fold. The saturation curve of the enzyme with PPi was hyperbolic, K_m was 100 μM, and it was not affected by Fru-2,6-P₂ (Fig. 2). PPi was an inhibitor at concentrations as 5 mM in the absence of 1 μM of Fru-2,6-P₂, but no such inhibition was found in the presence of Fru-2,6-P₂ even at a concentration of 10 mM of PPi. The data indicate that Fru-2,6-P₂ promotes the activation of PFP through relieving substrate inhibition by PPi and increasing its affinity to Fru-6-P.

PFP catalyzes the reversible reaction between Fru-6-P and Fru-1,6-P₂ and may therefore play a dual role in glycolysis and gluconeogenesis. PFP activity has been found in many plants^{2~6,13,14)} and the enzymes from

FIG. 1. Effects of Fru-6-P Concentration on Activity of PFP from *E. gracilis*.

The activity was assayed in the presence (○) and absence (●) of $1 \mu\text{M}$ Fru-2,6- P_2 . Fru-6-P was varied against a constant concentration of pyrophosphate (1 mM). The PFP concentration was $5 \mu\text{g}$.

FIG. 2. Effects of PPi Concentration on the Activity of PFP from *E. gracilis*.

The activity was assayed in the presence (○) and absence (●) of $1 \mu\text{M}$ Fru-2,6- P_2 . Pyrophosphate was varied against a constant concentration of Fru-6-P (10 mM). The PFP concentration was $5 \mu\text{g}$.

plants are reported to be activated by Fru-2,6- P_2 like phosphofructokinase in rat liver.⁸⁾ We have reported that *Euglena* fructose-1,6-bisphosphatase and trehalose phosphorylase are greatly inhibited by Fru-2,6- P_2 and suggest that *Euglena* carbohydrate metabolism is regulated by Fru-2,6- P_2 similarly to the hepatic cells in rats.^{15,16)} Reeves has demonstrated that PFP is a key glycolytic

enzyme.⁷⁾ The kinetic properties of Fru-2,6- P_2 -sensitive PFP from potato tuber showed that it worked in the direction of glycolysis *in vitro*.⁵⁾ In our previous paper, we showed that PFP activity in the cytoplasmic fraction was 10~30 times greater than that of phosphofructokinase throughout the growth of *Euglena*.⁹⁾ The results presented here demonstrated for the first time that the PFP in *Euglena*, a photosynthetic protozoan, is sensitive to Fru-2,6- P_2 , and it is worth emphasizing that *Euglena* PFP shows significant activation by Fru-2,6- P_2 like animal phosphofructokinase and plant PFP. In conclusion, *Euglena* Fru-2,6- P_2 -sensitive PFP appears to play a key role in glycolysis in this organism.

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