Note

Detection and Subcellular Distribution of Pyrophosphate: D-Fructose 6-Phosphate Phosphotransferase (PFP) in *Euglena gracilis*

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Pyrophosphate: D-Fructose 6-phosphate 1-phosphotransferase (EC 2.7.1.90) (PFP) has been measured in several plant tissues,^{1~6} *Entamoeba*,⁷⁾ and some prokaryotes.⁷⁾ Information on PFP in *Euglena*, a protozoon, which can grow photoautotrophically like a plant as well as heterotrophically in the darkness has not been available. The present paper reports the occurrence and subcellular location of this enzyme in *E. gracilis*, and discusses its probable physiological functions.

E. gracilis Z and its streptomycin-bleached mutant SM-ZK⁸⁾ were used throughout our experiments. Cells were cultured in Koren-Hutner medium⁹⁾ under illumination (2,000 lux) with shaking at 27°C. The method of preparing vitamin B₁₂-limited cells was reported previously.¹⁰⁾ Cells were harvested by centrifugation (1,500 × *g*, 2 min), washed with 20 mM glycyl-glycine buffer (pH 7.4) containing 0.25 M sucrose, and disrupted by sonication (10 kc) for 30 sec. The supernatant obtained by centrifugation of the sonicate at 10,000 × *g* for 10 min was used as the crude enzyme. A cell homogenate was obtained by partial trypsin digestion of the pellicle followed by mild mechanical disruption and was fractionated by differential centrifugation by the method described in our previous paper.¹¹⁾

All enzyme assays were carried out at 30°C in 1 ml total volume unless specified otherwise. Coupling enzymes obtained from Boehringer or Sigma, were dialyzed before use. NADH oxidation was recorded at a Hitachi 200 spectrophotometer. The reaction mixture for PFP activity contained 50 mM Tris-acetate buffer, pH 6.5, 5 mM MgCl₂, 10 mM fructose 6-phosphate, 0.1 mM NADH, 1 mM PPi, 5 unit aldolase, 1 unit triosephosphate isomerase, and 1 unit glycerol-3-phosphate dehydrogenase. The reaction was started by the addition of PPi. Assay conditions for phosphofructokinase (EC 2.7.1.11) (PFK) were identical to those for PFP, except that 1 mM ATP replaced PPi at pH 8.0. Other marker enzymes were assayed as described previously.¹¹⁾ Protein was measured according to the method of Lowry *et al.*¹²⁾ with bovine serum albumin as a standard.

Table I shows the presence of PFP activity in crude *Euglena* extract. In the direction of fructose-1,6-diphosphate formation, the PFP showed an absolute requirement for PPi. Neither ATP, ITP, GTP, UTP, ADP, AMP, nor Pi at 1 mM substituted as the phosphate donor. No reaction was observed when fructose 6-phosphate, PPi, or crude enzyme was omitted from the reaction mixture; Mg⁺⁺ was required by the reaction as a divalent ion. The optimum pH of PFP was 6.5 while that of PFK was 8.0. The enzymes of plants,^{1~6)} bacteria⁷⁾ and *Entamoeba*⁷⁾ have optimum pHs between 7.3~8.0 and the optimum pH of *Euglena* PFP is slightly acidic. The specific activity of PFP in crude extracts ranged from 0.3~0.7 μmol/mg protein/min while PFK ranged from 0.02~0.03 μmol under their respective optimum conditions. In crude extracts of pineapple leaves,¹⁾ PFP activity was 10- to 20-fold higher than the PFK and the same relationships have been reported in many plants.^{2,3,5,6)} The saturation curve for PPi was hyperbolic and the *K_m* value for PFP was 250 μM while the curve for fructose 6-phosphate was sigmoidal and the *K_{0.5}* was 4.6 mM. The *K_m* values for PPi and fructose 6-phosphate of *Entamoeba* were 14 and 38 μM,⁷⁾ respectively and those for PPi and fructose 6-phosphate of bacterial PFP from *Propionibacterium shermanii*, were 7 and 100 μM,⁷⁾ respectively. The potato PFP showed a hyperbolic saturation curve for PPi and a sigmoidal one for fructose 6-phosphate and the *K_m* value for PPi and *K_{0.5}* for fructose 6-phosphate were 10 μM and 3 mM,⁵⁾ respectively. In the presence of fructose 2,6-bisphosphate, the affinity for fructose 6-phosphate of the potato PFP greatly increased and the kinetics became Michaelian⁵⁾ and the same effect of fructose 2,6-bisphosphate on plant

TABLE I. IDENTIFYING PROPERTIES OF THE PFP FROM *E. gracilis* DURING THE SYNTHESIS OF FRUCTOSE 1,6-DIPHOSPHATE

Extract prepared by sonicating a 5-day-old culture of *E. gracilis* was centrifuged at 10,000 × *g* for 10 min. Fru-6-P, fructose 6-phosphate.

Reaction mixture component	μmol of Fructose 1,6-diphosphate formed/min/mg protein
Complete (+PPi and Fru-6-P)	0.32
-PPi	0
-PPi + ATP (1 mM)	0.01
-PPi + GTP (1 mM)	0.01
-PPi + UTP (1 mM)	0.01
-PPi + ADP (1 mM)	0
-PPi + AMP (1 mM)	0
-PPi + Pi (1 mM)	0
-Mg ⁺⁺	0.02
-Fru-6-P	0
-Crude enzyme	0

TABLE II. SUBCELLULAR LOCALIZATION OF PFP IN *E. gracilis*, SM-ZK

Homogenate from a 5-day-old culture of *Euglena* cells by partial trypsin digestion method (see the text) was fractionated by centrifugation. Enzyme activities were measured in the unfractionated homogenate, mitochondria, microsomes, and supernatant. All values represent the average of two separate experiments.

Enzymes	Activity in unfractionated homogenate (nmol/min/mg protein)	Enzyme activity (% of activity in cell homogenate)		
		Mitochondria	Microsomes	Cytosol
Isocitrate dehydrogenase (NAD-dependent)	17.2	90.5	0.5	4.3
Succinic semialdehyde dehydrogenase	20.1	82.4	3.2	15.3
Glucose 6-phosphatase	0.2	6.7	75.4	11.3
Glutamate dehydrogenase (NADP-dependent)	45.1	0.1	1.8	95.3
Pyrophosphate: D-fructose 6-phosphate 1-phosphotransferase	302.6	0.5	0.3	98.6

PFP have been reported recently.²⁻⁶⁾ These results suggest that *Euglena* PFP is also activated by fructose 2,6-bisphosphate.

To determine the subcellular location of PFP in *Euglena* we measured the distribution of the activity in the pellets and supernatant after centrifugation of the cell homogenate (Table II). The $10,000 \times g$ pellet contained major portions of mitochondria as indicated by the distributions of isocitrate dehydrogenase and succinic semialdehyde dehydrogenase while the $100,000 \times g$ pellet contained the microsome fraction as indicated by the distribution of glucose 6-phosphatase. Contamination of the pellets by cytoplasm or whole cells was slight, as indicated by the distribution of glutamate dehydrogenase. The distribution of PFP was very similar to that of glutamate dehydrogenase showing that this enzyme also is located in the cytoplasm in *Euglena*. All PFP is apparently located in the cytoplasm, the site of glycolysis,¹³⁾ and *Euglena* PFP was not detected in the intact chloroplasts isolated by the controlled digestion technique.¹⁴⁾

Changes of the activities of PFP and PFK during cell growth of *E. gracilis* are presented in Fig. 1. The PFP activity was detected in all growth stages, and throughout the growth the PFP activity was 10~30 times greater than that of PFK.

PFP catalyzes an easily reversible reaction, and may therefore play a dual role in gluconeogenesis as well as in glycolysis.⁶⁾ The PFP reaction strongly depends on the concentration of PPi in the cell. Pyrophosphate is produced in a variety of processes in the cell in the synthesis of proteins, nucleic acids, and glycogen or starch. The PPi level is apparently sufficient to serve as an energy source for the phosphorylation of fructose 6-phosphate by the action of PFP in *Euglena*. Some investigators have reported that PFP is the key glycolytic enzyme and PPi provides energy for this phosphorylation in *Entamoeba*

FIG. 1. Enzyme Activities in *Euglena* during Culture.

Each value represents the mean value of measurements from two separately grown batches of *Euglena* cells.

(●), PFP activity; (○), PFK activity; (□), cell growth.

and some bacterial species.⁷⁾ Kinetic properties of PFP from potato tubers showed that it works in the direction of glycolysis *in vivo*.⁴⁾ PFK is a key enzyme in the regulation of glycolytic carbon flow¹⁵⁾ while its activity was 10~30 times smaller than that of PFP in *Euglena* (Fig. 1), throughout cell growth. The present result, together with some reports on PFP in other organisms,^{1~7)} may suggest that PFP plays an important role in the regulation of carbohydrate metabolism in *Euglena*.

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