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PYROPHOSPHATE-DEPENDENT 6-PHOSPHOFRUCTOKINASE, A NEW GLYCOLYTIC ENZYME IN PINEAPPLE LEAVES

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SUMMARY. Pineapple leaves contain a pyrophosphate-dependent 6-phosphofructokinase which has been partially purified and characterized. In crude extracts the pyrophosphate-dependent activity is 10 to 20-fold higher than the ATP-dependent activity. The partially purified activity is near 2.5 μmol Fru-1,6-P_2 formed/min/mg protein. In the reaction 1 Fru-1,6-P_2 is formed per 1 pyrophosphate consumed. The enzyme exhibits a pH optimum of 8.0 and the activity is stimulated by Mg^{++}. The discovery of a pyrophosphate-dependent 6-phosphofructokinase in pineapple leaves indicates pyrophosphate can serve as an energy source for synthetic reactions in pineapple and perhaps in other plants as well.

Traditionally pyrophosphate is viewed as a reaction product with the hydrolysis of PPi† furnishing a favorable thermodynamic environment for a variety of synthetic processes. Recently, however, the direct use of PPi as an energy source in metabolic reactions has been demonstrated in the eukaryote *Entamoeba histolytica* and in several prokaryotes (1). *Entamoeba* contains four enzymes which utilize PPi as the phosphate donor instead of a nucleoside triphosphate (1-3). Likewise, PPi serves as an energy source in glucose fermentation by *Propionibacterium shermanii* (4,5). In the photosynthetic bacterium *Rhodospirillum rubrum* the synthesis of PPi is coupled to light-driven electron transport (6) and PPi is utilized as an

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†Abbreviations used are: PPi, inorganic pyrophosphate; Fru-1,6-P_2 (FDP), fructose-1,6-bisphosphate; Pi, inorganic phosphate; CAM, Crassulacean acid metabolism; PVP, polyvinyl-pyrrolidone; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; Fru-6-*P*, fructose-6-phosphate; PFK, phosphofructokinase; PEP, phosphoenolpyruvate.

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energy source for an energy-linked transhydrogenase, succinate-linked NAD^+ reduction, cytochrome reduction, proton uptake, and even to drive ATP synthesis (7).

Reeves and coworkers discovered the use of PPi as a phosphate donor to Fru-1,6-P_2 via the key glycolytic enzyme 6-phosphofructokinase in *Entamoeba histolytica* (2). The latter activity now has been found in four bacterial species i.e. *Propionibacterium shermanii* (4) a marine *Alcaligenes* species (8), *Pseudomonas marina* (8), and *Bacteroides fragilis* (9). There are no reports on the use of PPi as an energy source for plant enzymes. We have utilized the work of Reeves, Wood, and coworkers to provide the framework for the present report of a PPi-dependent 6-phosphofructokinase in pineapple leaves. The enzyme catalyzes the following reaction: Fructose 6-*P* + PPi $\rightleftarrows$ Fructose 1,6-bisphosphate + Pi. We will present some identifying properties of the enzyme and consider the implications of PPi as a phosphate donor in plants.

MATERIALS AND METHODS

Pineapple, *Ananas comosus* (L.) Merr., plants were propogated as previously described (10). The leaves exhibited a diurnal fluctation of malic acid indicative of CAM (10). Leaf samples were harvested about 4 p.m. when the acid content was lowest. Crude leaf protein extracts were prepared from 60 g of expanded or nearly fully expanded leaves. The leaves were washed in distilled H_2O, dried, sliced into pieces $\sim$ 1 X .2 cm, frozen in liquid N_2, and ground to a powder in a mortar and pestle. 1% wt/wt PVP-40 was ground into the resulting tissue powder. Three volumes of extraction buffer (100 mM HEPES, pH 8.0, 30 mM mercaptoethanol, 5 mM $MgCl_2$, 1 mM EGTA, 150 mM potassium acetate) were added to the tissue powder and grinding was continued. The homogenate was filtered through 8 layers of cheesecloth and centrifuged at 22500 x g for 20 min. A 25-45% $(NH_4)_2SO_4$ precipitate of the supernatant was prepared. The protein was resuspended in 5 ml of extraction buffer and desalted on a Sephadex G-25 column.

Fru-1,6-P_2 formation was assayed by following the oxidation of NADH at 340 nm in a 1 ml reaction mixture containing 100 mM HEPES buffer pH 8.0, 30 mM mercaptoethanol, 0.08 mM NADH, 6 mM $MgCl_2$, 91.7 µg aldolase, 1.0 µg triose isomerase, 6.7 µg α-glycerol-P dehydrogenase, 10 mM Fru-6-*P*, 1 mM PPi or 1 mM ATP, and leaf protein extract. The reaction was initiated with PPi or ATP. Assays were conducted at 30°C.

[^{32}P]PPi consumption was assayed as described by Chartier and Thuillier (11). The reaction mixture contained 25 µmoles Tris-HCl pH 8.0, 1 µmole $MgCl_2$, 5 µmoles Fru-6-*P*, 2 µmoles [^{32}P]PPi ($\sim$ 4.5 x 10^5 cpm/µmole) in 0.5 ml. The reactions were run at 30°C, initiated by addition of the

enzyme, and terminated at 5 min intervals over a period of 30 min by the addition of 0.2 ml of 40 mM ammonium molybdate in 2.5 N H_2SO_4 plus 0.1 ml of unlabeled 100 mM K_2HPO_4/KH_2PO_4, pH 8.0. Following vigorous mixing of the preparation, 0.2 ml of 100 mM triethylamine was added and mixed; the solution was centrifuged for 5 min to precipitate the Pi-complex, and 0.1 ml aliquots of the supernatant were counted. The amount of Fru-1,6-*P*$_2$ formed was independently determined in reactions run with unlabeled PPi using the α-glycerol-P dehydrogenase assay given above.

[^{32}P]PPi was obtained from New England Nuclear. All coupling enzymes, PVP, and HEPES were purchased from Sigma. EGTA was purchased from J. T. Baker Co. Protein was determined by the method of Bradford (12) and chlorophyll was determined as described by Arnon (13).

RESULTS

Enzyme detection and partial purification - Leaves characteristically contain an ATP-dependent 6-phosphofructokinase. Substitution of 1 mM PPi for ATP in the standard phosphofructokinase assay revealed the presence of a PPi-dependent activity which was 10 to 20-fold higher than the ATP-dependent activity in crude pineapple leaf extracts. The specific activity of the PPi-dependent activity in crude extracts ranged between 0.2 and 0.6 μmol/min/mg protein and on a chlorophyll basis between 6 and 12 μmol/min/mg chlorophyll. The PPi-dependent activity was purified approximately ten-fold and separated from the ATP dependent activity by an initial centrifugation at 22,500 x g for 20 min followed by $(NH_4)_2SO_4$ fractionation. About 95% of the PPi-PFK activity was localized in the 30-40% fraction whereas the ATP activity was found primarily in the 60-70% fraction. Routinely a 25-45% fraction was prepared, desalted on a Sephadex G-25 column, and used to characterize properties of the enzyme.

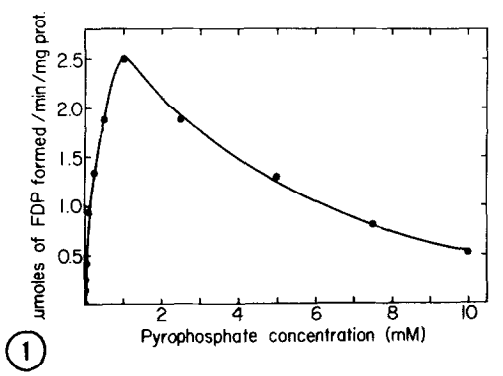
Properties of the enzyme - In the direction of Fru-1,6-*P*$_2$ formation the PPi-dependent PFK has an absolute requirement for PPi. Neither ATP, ITP, GTP, UTP, ADP, AMP, nor Pi at 1 mM will substitute for PPi as the phosphate donor (Table 1). The optimal PPi concentration at either 2 mM Fru-6-*P* or 10 mM Fru-6-*P* is 1 mM PPi. Figure 1 shows the influence of PPi upon PFK activity at 10 mM Fru-6-*P* and 6 mM $MgCl_2$. We have not studied the reasons for the decline in enzymatic activity at PPi concentrations above 1 mM PPi (Figure 1). The indicated apparent Km(PPi) and V_{max} at saturating

TABLE 1

IDENTIFYING PROPERTIES OF THE PPi-DEPENDENT 6-PHOSPHOFRUCTOKINASE FROM PINEAPPLE LEAVES DURING THE SYNTHESIS OF FRUCTOSE 1,6-BISPHOSPHATE

Reaction Mixture Component Varied	μmoles of Fru-1,6-P_2 formed/min/mg prot
Complete	2.32
-PPi	0.00
-PPi, +ATP (1mM)	0.01
+PPi (1mM), +ATP (1mM)	2.10
-Mg^{++}	0.85
-PPi, +Pi (1mM)	0.00
-Fru-6-*P*	0.00
-Enzyme*	0.00

*This enzyme preparation had been subjected to $(NH_4)_2SO_4$ fractionation to remove the ATP dependent activity.



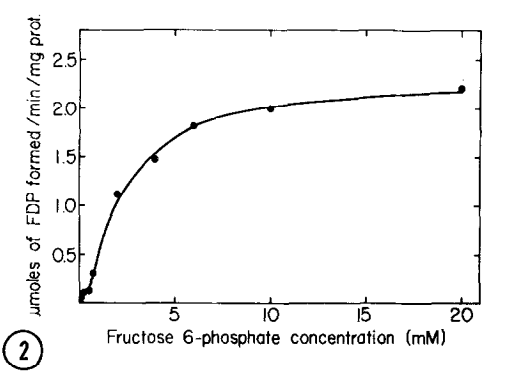


Figure 1. The responses of pineapple leaf phosphofructokinase to pyrophosphate concentration.

Figure 2. The responses of pineapple leaf phosphofructokinase to fructose 6-phosphate concentration.

Fru-6-*P* and Mg^{++} concentrations for this enzyme are near 0.1 mM PPi and 2.9 μmoles Fru-1,6-P_2 formed/min/mg protein respectively, however, a detailed kinetic analysis requires further enzyme purification. Figure 2 illustrates the dependence of PPi-PFK upon Fru-6-*P* concentration. The

enzyme approaches saturation at 10 mM Fru-6-*P* (Fig. 2). The apparent Km(Fru-6-*P*) is 2.6 mM. Although Mg^{++} is not absolutely required, the presence of 1 mM Mg^{++} (or higher) stimulates the PPi-PFK activity approximately three-fold (Table 1). Half-maximal stimulation is achieved near 0.2 mM Mg^{++}. The pH optimum for the enzyme is pH 8.0. Additional features of the PPi-dependent PFK are given in Table 1. No reaction is observed when Fru-6-*P*, PPi, or the enzyme is omitted from the reaction mix. Activity with ATP is less than 1% of the activity with PPi.

Stoichiometry of the PPi-PFK reaction - To demonstrate the quantitative conversion of Fru-6-*P* plus PPi to Fru-1,6-P_2 plus Pi we utilized the radiochemical assay of Chartier and Thuiller (11) with the modifications described in Materials and Methods. This enzyme preparation was desalted in 100 mM Tris·HCl pH 8.0 containing 5 mM $MgCl_2$ since we found that HEPES buffer and PPi precipitated with the reagents used to terminate the reaction. Enzyme activity was not affected by the buffer change. We calculated from the rate determined spectrophotometrically that 1.2 µmol of PPi would be consumed in a 30 min incubation period. We determined that 1.4 µmol of PPi were consumed and 1.3 µmol of Fru-1,6-P_2 were produced. This nearly stoichiometric consumption of PPi and synthesis of Fru-1,6-P_2 confirms the predicted substrate and product relationship for a PPi-dependent PFK.

DISCUSSION

Pyrophosphate is produced in a variety of processes in plants including the synthesis of proteins, nucleic acids, starch, and sucrose. It is generally felt that PPi is hydrolyzed by pyrophosphatases to poise these processes in the synthetic direction. Data are not available on PPi production or levels in plant tissues or cells although large variability in pyrophosphatase activity has been reported in various plants (14). Therefore, it seems reasonable to propose that PPi levels could be sufficient for PPi

to serve as an energy source for the phosphorylation of Fru-6-*P* via the PPi-dependent PFK in pineapple leaf extracts. In *Entamoeba* and bacterial species, where a similar enzyme has been detected, PPi does provide the energy for this phosphorylation (1,5).

6-Phosphofructokinase is a key enzyme in the regulation of glycolytic carbon flow (1,5). CAM plants such as pineapple are characterized by a massive reciprocal daily turnover of starch and organic acids amounting to as much as 20% of the leaf dry weight (15). At night starch is degraded via glycolysis to PEP (10). PEP subsequently is carboxylated to form oxalacetate and converted to malic acid which accumulates in vacuoles at night (16). During the day malic acid is decarboxylated to provide CO_2 for photosynthesis; the 3-carbon product of decarboxylation ultimately returns to the starch pool via gluconeogenic reactions (10,15-17). The latter cycle has been verified recently by noting that the $\delta^{13}C$ values of starch and organic acids over twenty-four hours are identical but different from $\delta^{13}C$ values of other organic substituents (17). The presence of a PPi utilizing phosphofructokinase in pineapple leaves with its potential role in daily carbohydrate pool turnover will necessitate a revision of our concepts about the energetics and regulation of glycolysis in plants.

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