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Properties of Pyrophosphate:Fructose-6-Phosphate Phosphotransferase from Endosperm of Developing Wheat (*Triticum aestivum* L.) Grains [1]

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ABSTRACT

Pyrophosphate:fructose-6-phosphate phosphotransferase (PFP, EC 2.7.1.90) from endosperm of developing wheat (*Triticum aestivum* L.) grains was purified to apparent homogeneity with about 52% recovery using ammonium sulfate fractionation, ion exchange chromatography on DEAE-cellulose and gel filtration through Sepharose-CL-6B. The purified enzyme, having a molecular weight of about 170,000, was a dimer with subunit molecular weights of 90,000 and 80,000, respectively. The enzyme exhibited maximum activity at pH 7.5 and was highly specific for pyrophosphate (PPi). None of the nucleoside mono-, di- or triphosphate could replace PPi as a source of energy and inorganic phosphate (Pi). Similarly, the enzyme was highly specific for fructose-6-phosphate. It had a requirement for Mg^{2+} and exhibited hyperbolic kinetics with all substrates including Mg^{2+}. K_m values as determined by Lineweaver-Burk plots were 322, 31, 139, and 129 micromolar, respectively, for fructose-6-phosphate, PPi, fructose-1,6-bisphosphate and Pi. Kinetic constants were determined in the presence of fructose-2,6-bisphosphate, which stimulated activity about 20-fold and increased the affinity of the enzyme for its substrates. Initial velocity studies indicated kinetic mechanism to be sequential. At saturating concentrations of fructose-2,6-bisphosphate (1 micromolar), Pi strongly inhibited PFP; the inhibition being mixed with respect to both fructose-6-phosphate and PPi, with K_i values of 0.78 and 1.2 millimolar, respectively. The inhibition pattern further confirmed the mechanism to be sequential with random binding of the substrates. Probable role of PFP in endosperm of developing wheat grains (sink tissues) is discussed.

Pyrophosphate:fructose-6-phosphate 1-phosphotransferase (PPi-PFP,[2] EC 2.7.1.90), first discovered in *Entamoeba histolytica* (18), is now known to occur in a wide range of photosynthetic and nonphotosynthetic tissues of higher plants (7, 9, 22, 26). The enzyme located in cytoplasm (5, 8, 14, 16) catalyses reversibly the interconversions of Fru-6-P/Fru-1,6-P_2 and PPi/Pi (Fru-6-P + PPi⇌Fru-1, 6-P_2 + Pi) and has been partially purified and characterized from a number of plant sources including mung bean (1), potato tubers (12, 13, 27), wheat seedlings (30), spinach leaves (2, 8), castor bean endosperm (11), cucumber (6), *Phaseolus* (5) and pea (28) seeds, pineapple leaves (3), and soybean suspension culture cells (16). The kinetic properties of PFP studied from these sources have suggested that the enzyme is activated by low concentrations of Fru-2,6-P_2 and changing levels of this metabolite strongly regulate glycolytic and gluconeogenic carbon flow in the cytoplasm of plant cells (4, 25, 26). Accordingly, plants can direct the flow of carbon upon being fed sucrose in specific tissues and cells through several alternative enzymes and set of reactions as suggested by Sung *et al.* (26).

In sink tissues such as developing grains, translocated sucrose is ultimately utilized for the biosynthesis of starch in amyloplasts (23). How the regulation of carbon partitioning is achieved in these tissues is still under debate. However, evidence is available that even in sink tissues, Fru-2,6-P_2 is present (24, 29) and activates the enzyme PFP present in the cytosol and strongly inhibits Fru-1,6-bisphosphatase. However, no explanation is yet available to account for the ability of different sink tissues to metabolize incoming sucrose in different ways. Moreover, the properties of the enzyme PFP from sink tissues, particularly developing grains, have not yet been reported (15). Here we report the kinetic properties of PFP purified from endosperm of developing wheat grains.

MATERIALS AND METHODS

Plant Material

Immature wheat (*Triticum aestivum* L.) grains were harvested from the field grown wheat crop (cv WH-157) at d 25 after anthesis, endosperms removed and stored in liquid nitrogen for further use. To know the age of the grains, samples were taken from ears which were tagged at the day of anthesis.

Chemicals

All biochemicals used were purchased from Sigma Chemical Co., St. Louis, MO. All other chemicals were of analytical grade (B.D.H., India).

Enzyme Purification

Unless otherwise stated, all steps of enzyme purification were carried out at 0 to 4°C.

Immature wheat endosperms (50 g) were hand homoge-

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[2] Abbreviations: PFP, pyrophosphate:fructose-6-phosphate phosphotransferase (EC 2.7.1.90); Fru-6-P, fructose 6-phosphate; Fru-1,6-P_2, fructose 1,6-bisphosphate; Fru-2, 6-P_2, fructose 2,6-bisphosphate.

nized in a prechilled mortar and pestle in 200 mL of 50 mM Hepes buffer (pH 7.5) containing 2 mM EDTA, 1 mM $MgCl_2$, 10 mM KCl, 10 mM 2-mercaptoethanol, 1% PVP, and 30% ethylene glycol. The homogenate was squeezed through four layers of cheese cloth and the filtrate centrifuged at 15,000*g* for 30 min. The supernatant so obtained was referred to as crude extract. PFP in the crude extract was precipitated between 30 and 60% saturation of $(NH_4)_2SO_4$. The precipitates obtained after centrifuging at 15,000*g* for 30 min, were dissolved in 50 mM Hepes buffer (pH 7.5) containing 2 mM EDTA, 1 mM $MgCl_2$, 10 mM KCl, 10 mM 2-mercaptoethanol, and 30% ethylene glycol and dialyzed against the same buffer for 24 h with at least three changes of buffer.

DEAE-Cellulose Chromatography

An aliquot of the dialyzed 30 to 60% $(NH_4)_2SO_4$ fraction was layered onto a DEAE-cellulose column (38 × 2.2 cm) previously equilibrated with 10 mM Hepes (pH 7.5) containing 0.5 mM EDTA, 1 mM $MgCl_2$, 5 mM KCl, 10 mM 2-mercaptoethanol, and 30% ethylene glycol. The column was washed with three bed volumes of the above medium. The enzyme was eluted with a linear gradient of NaCl (0–0.4 M) in the above medium at a flow rate of 30 mL h^{-1} and fractions of 5 mL each were collected. The active factions eluted as a single peak were pooled and concentrated to about 4 mL by osmosis against solid sucrose.

Sepharose CL-6B Chromatography

The concentrated enzyme obtained from the above step was applied onto a Sepharose CL-6B column (60 × 2 cm) previously equilibrated with 50 mM Hepes buffer (pH 7.5). The column was eluted with this buffer at a flow rate of 25 mL h^{-1} and fractions of 3 mL each were collected. The above fractions were pooled and stored at 4°C for further use.

Determination of Purity, Mol Wt, and Mol Wt of Subunits of Enzyme

The purity of the enzyme preparation obtained from gel filtration through Sepharose CL-6B was judged by PAGE at 4°C in 7.5% gel, using Tris-glycine buffer (pH 8.3) as described earlier (21). Fifty μg enzyme protein were loaded onto the top of the gel and a current of 3 mA per gel tube was maintained throughout the process. The mol wt of the purified enzyme was estimated by passing it through a column of Sepharose-CL-6B which had previously been calibrated with β-amylase (M_r 200,000), alcohol dehydrogenase (M_r 150,000), BSA (M_r 66,000), and carbonic anhydrase (M_r 29,000). The subunit mol wt was determined by SDS-PAGE carried out in 10% gel at 4°C according to the method described previously (21). β-Galactosidase (M_r 116,000), phosphorylase *b* (M_r 97,400), BSA (M_r 66,000), egg albumin (M_r 45,000), and carbonic anhydrase (M_r 29,000) were used as reference proteins. The protein bands were detected by staining with Coomassie brilliant blue.

Enzyme Assays

All assays were carried out in a total volume of 1.5 ml according to the method of Kombrink *et al.* (11). Auxiliary enzymes were dialyzed against 10 mM Hepes-NaOH (pH 7.5) before use. NADH oxidation and $NADP^+$ reduction was recorded at 340 nm with an Enzymeter (Calbiochem, San Diego, CA). The enzyme activity in the forward direction was measured by the production of Fru-1,6-P_2. The standard assay mixture contained 50 mM NADH, 1 mM PPi, 1 μM Fru-2,6-P_2, 1 IU aldolase, 1 IU glycerol-3-phosphate dehydrogenase, 10 IU triosephosphate isomerase, and the enzyme preparation. The reaction was started with PPi. The activity in the reverse direction was measured by the production of Fru-6-P in a mixture that contained 50 mM Hepes (pH 7.5), 5 mM $MgCl_2$, 0.5 mM Fru-1,6-P_2, 1 mM $NADP^+$, 5 mM NaH_2PO_4, 2 IU hexosephosphate isomerase, and 1 IU glucose-6-phosphate dehydrogenase . The assay was started by the addition of Pi. For kinetic studies, the concentrations of substrates and effectors were varied as indicated in "Results."

Protein Estimation

Protein in various fractions from DEAE-cellulose and Sepharose-CL-6B column was monitored by measuring the extinction at 280 nm. Quantitative estimation of protein at each step of purification, however, was done as described previously (21).

RESULTS

Enzyme Purification

The results of the enzyme purification are given in Table I. The enzyme was purified to apparent homogeneity as judged by PAGE with about 52% recovery using $(NH_4)_2SO_4$ fractionation, ion exchange chromatography on DEAE-cellulose and gel filtration through Sepharose-CL-6B. It was essential to include ethylene glycol during the purification. Attempts to purify PFP in absence of ethylene glycol resulted in almost complete loss of activity before the final step. During all the column chromatography steps, PFP activity eluted as a single

Table I. *Purification of PFP from Endosperm of Developing Wheat Grains*

Fraction	Total Activity	Protein	Specific Activity	Recovery	Overall Purification
	nmol/min	*mg*	*nmol/min/mg protein*	%	*-fold*
Crude extract	28380	2416.66	11.74	100	
$(NH_4)_2SO_4$ fraction	29390	1088.92	26.98	104	2.3
DEAE-cellulose	18450	53.26	346.41	65	29.5
Sepharose-CL-6B	14670	9.81	1495.41	52	127.4

peak. The purified enzyme could be kept at −20°C in 50 mM Hepes buffer (pH 7.5), 10 mM 2-mercaptoethanol, 0.5 mM EDTA, 1 mM $MgCl_2$, 5 mM KCl, and 30% ethylene glycol for more than 1 month without any loss in activity. The final enzyme preparation was essentially free of the activities of aldolase, fru-1,6-bisphosphatase, phosphofructokinase, hexosephosphate isomerase, phosphoglucomutase, and inorganic pyrophosphatase. However, the specific activity (1.5 μmol min^{-1} mg^{-1} protein) of the final preparation was much less than that reported for PFP purified from potato tubers (12, 27), castor bean endosperm (11), cucumber seeds (6) and suspension-cultured cells of soybean (16).

Mol Wt and Subunit Mol Wt of Enzyme

The mol wt of purified PFP, as determined from gel filtration through Sepharose-CL-6B was found to be about 170,000 (Fig. 1). On SDS-PAGE, the enzyme preparation yielded two protein bands with R_f (mobility relative to the tracking dye) values of 0.72 and 0.76, corresponding to the mol wt of 90,000 and 80,000, respectively. This indicated that PFP from developing endosperms of wheat grains is a dimer composed of two subunits with mol wt of 90,000 and 80,000. The observed value for the apparent mol wt is similar to that of the enzyme from suspension cultured cells of soybean (16), where a value of 183,000 was obtained by gel filtration chromatography.

Enzyme Stability and pH Optima

No loss of enzyme activity occurred upon incubation for 15 min at 30°C. However, at 50°C, the enzyme lost 50% of its activity after incubation for 15 min. The enzyme was completely inactivated at temperatures above 60°C. pH had a great influence on the enzyme activity in the forward direction, with maximum being at pH 7.5. On both sides of pH optimum, the activity was substantially lower. The activity

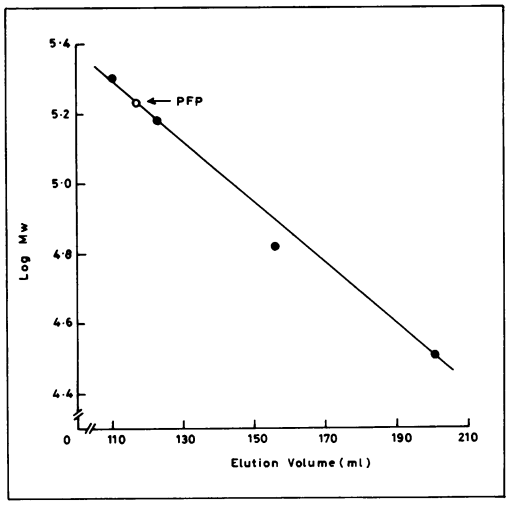

Figure 1. Estimation of the mol wt of native PFP. A Sepharose-CL-6B for gel filtration of the native protein was calibrated with β-amylase, alcohol dehydrogenase, BSA, and carbonic anhydrase (in descending mol wt).

of PFP from suspension cultured cells of soybean was also markedly dependent on pH and was optimal at pH 7.5 (16), whereas, the enzyme from cucumber seeds (6), and castor bean endosperm (11) had a broad pH optimum with maximum activity at pH 7.3 to 7.7. Stimulation by Fru-2,6-P_2 had no effect on the pH optimum in the forward direction.

Effect of Metal Ions

The enzyme had a requirement for Mg^{2+} with maximum activity obtained at concentrations greater than 3 mM. It displayed hyperbolic kinetics in response to increasing Mg^{2+} with a K_m of 181 μM in the forward direction. Other divalent and monovalent cations such as Co^{2+}, Mn^{2+}, Cu^{2+}, Ca^{2+}, Ba^{2+}, Sn^{2+}, Sr^{2+}, NH_4^+, Na^+, and K^+ were ineffective as activators. Such a hyperbolic response with increasing Mg^{2+} has also been reported for the enzyme from cucumber seeds (6), castor bean endosperm (11), and suspension cultured cells of soybean (16).

Effect of Anions

Since PFP from mungbean (1) and potato tubers (27) is known to be affected by several anions, we studied the effect of various anions on PFP in presence of Fru-2,6-P_2. Phosphate caused substantial inhibition and was investigated separately in detail. Sulfate, carbonate, acetate, and nitrate had no inhibition at 5 mM concentration, whereas, bicarbonate, tungstate, sulfite, thiosulfate, and chloride inhibited the enzyme to the extent of about 20 to 35%. These results could not be compared with those of earlier workers (11, 27), as they had used very high concentrations of anions (50–100 mM). Phosphate inhibition at higher concentration is common to PFP from all plant sources (22).

Effect of Substrates

In the forward direction, the enzyme activity was specifically dependent on Fru-6-P and PPi. None of the nucleoside mono-, di-, or triphosphates such as AMP, GMP, CMP, UMP, IMP, ADP, GDP, CDP, UDP, ATP, GTP, CTP, and UTP could replace PPi in the reaction. Similarly, the enzyme did not show any activity when Fru-6-P was replaced by any other phosphate ester. Moreover, the activity in the forward direction was strongly dependent on the presence of the activator Fru-2,6-P_2; the activation being around 20-fold at saturating concentrations of the substrates. This is true for PFP from all other plant sources as well (9, 22). The activation was again caused by increasing V_{max} for the reaction and apparent affinity of the enzyme for the two substrates. This now appears to be a general effect of Fru-2,6-P_2 in plant carbohydrate metabolism (4, 26).

As is true for PFP from castor bean endosperm (11), suspension cultured cells of soybean (16), cucumber seeds (6) and germinating *Phaseolus* seeds (5), the enzyme from wheat endosperm also exhibited hyperbolic kinetics with Fru-6-P, PPi, Fru-1,6-P_2, and Pi both in presence and absence of Fru-2,6-P_2. There was no evidence of sigmoid kinetics in absence of Fru-2,6-P_2 as reported earlier by Cseke *et al.* (8) and Van Schaftingen *et al.* (27). K_m values for Fru-6-P, PPi, Fru-1,6-

P_2, and Pi as determined by Lineweaver-Burk plots were found to be 322, 31, 139, and 129 μM, respectively.

Initial Velocity Studies

The enzyme activity was determined at four fixed concentrations of one of the substrates, while the concentration of the other substrate was varied. Double reciprocal plots of varying concentrations of PPi *versus* velocity at four fixed concentrations of Fru-6-P gave a set of lines which intersected to the left of the ordinate (Fig. 2). A qualitatively similar Lineweaver-Burk plot, with intersection of curves in between the two axes, was obtained when the enzyme activity was determined at four fixed concentrations of PPi with Fru-6-P as the varied substrate (Fig. 3). This indicates the kinetic mechanism to be sequential, which is in agreement with that of the enzyme from castor bean endosperm (11) and from cucumber seeds (6). However, the enzyme from pineapple leaves has been shown to catalyze the reaction by a Ping-Pong mechanism (7).

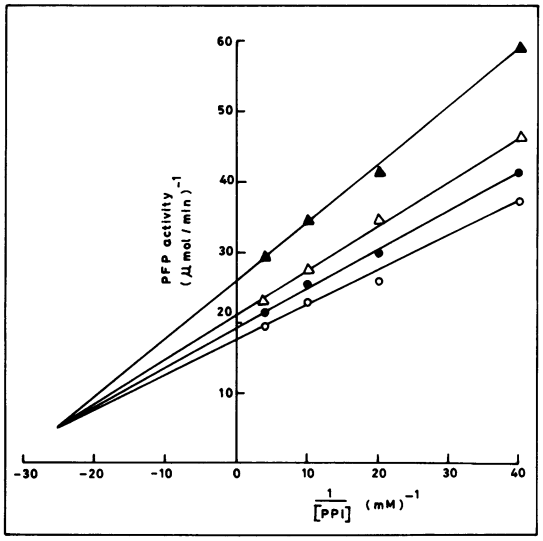

Figure 2. Substrate interaction kinetics of PFP with respect to PPi in the presence of 0.5 (▲), 1.0 (△), 2.5 (●), and 5.0 (○) mM Fru-6-P.

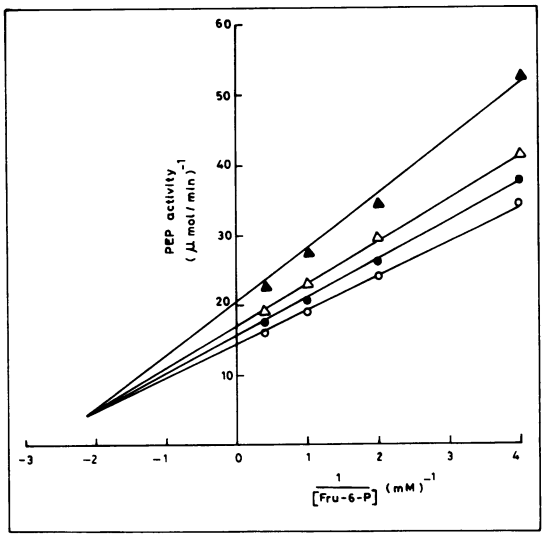

Figure 3. Substrate interaction kinetics of PFP with respect to Fru-6-P in the presence of 0.1 (▲), 0.25 (△), 0.5 (●), and 1.0 (○) mM PPi.

Inhibition by Pi

At saturating concentrations of Fru-2,6-P_2 (1 μM), Pi strongly inhibited PFP; the inhibition being mixed with respect to both Fru-6-P (Fig. 4) and PPi (Fig. 5) with K_i values of 0.78 and 1.2 mM, respectively. Similar pattern of inhibition with almost identical K_i values was obtained for the enzyme from castor bean endosperm (11). However, the enzyme from cucumber seeds (6) was inhibited noncompetitively by Pi with respect to both Fru-6-P and PPi with much higher K_i values. The inhibition pattern is again consistent with the sequential mechanism with random binding of the substrates to the enzyme (20). These results further suggest that PFP *in vivo* could be modulated by changes in the concentrations of Fru-6-P, Pi, and Fru-2,6-P_2. An increase in the ratio of Fru-6-P/Pi would increase the activation of PFP at a fixed, limiting concentration of Fru-2,6-P_2. Pi could also function by directly inhibiting the enzyme. At present, there are insufficient data on the levels of these metabolites to assess their importance in the regulation of PFP in developing endosperm of wheat grain.

DISCUSSION

The purification procedure described here yielded an enzyme preparation that was essentially free from other enzymes capable of metabolizing either the substrates or products of PFP. Furthermore, the enzyme from developing wheat grain endosperm, in general, resembled the enzyme from other

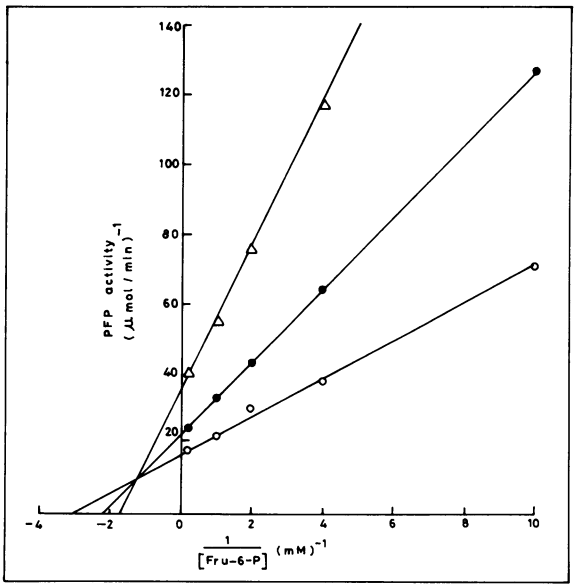

Figure 4. Pi inhibition of PFP at saturating concentration of PPi and varying concentrations of Fru-6-P. The standard assay mixture in addition contained 0 (○), 1.0 (●), and 2.0 (△) mM Pi. PPi concentration was 1.0 mM.

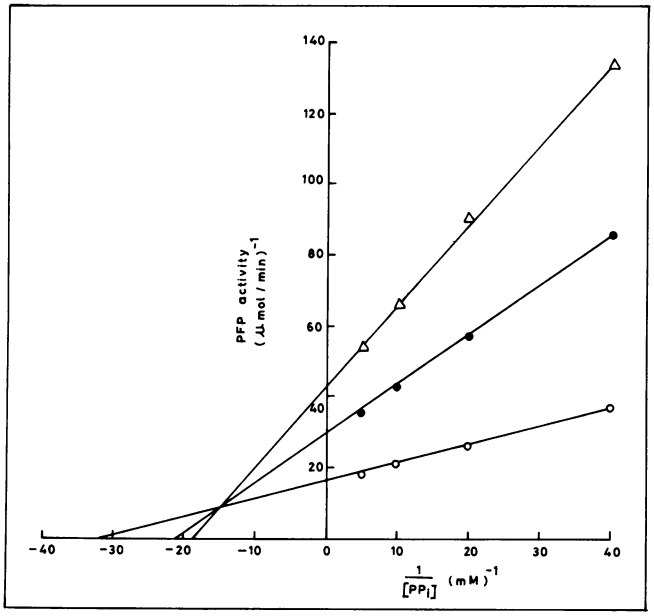

Figure 5. Pi inhibition of PFP at saturating concentration of Fru-6-P and varying concentrations of PPi. The standard assay mixture in addition contained 0 (○), 1.0 (●), and 2.0 (△) mM Pi. Fru-6-P concentration was 5.0 mM.

plant sources in its metal specificity, pH optimum, substrate kinetics, and inhibition by Pi (1, 6, 8, 11, 26, 27). The most striking feature again was the marked stimulation of the enzyme by micromolar concentrations of Fru-2,6-P_2. Contrary to the enzyme from spinach leaf (8) and potato tubers (27), the enzyme from wheat endosperm exhibited hyperbolic kinetics with all the four substrates of forward and reverse reactions.

The kinetic properties of PFP reported here do not suggest an obvious physiological role for the enzyme in wheat endosperm. Macdonald and Preiss (16) suggested that in tissues engaged in conversion of sucrose into starch, PFP might be more active in the direction of Fru-6-P and PPi synthesis. This activity could provide a source of PPi that would enable the further metabolism by UDP-glucose pyrophosphorylase of UDP-glucose produced by sucrose synthase during the utilization of incoming sucrose in the endosperm (23). Both sucrose synthase and UDP-glucose pyrophosphorylase are known to be localized in the cytoplasm (17). PFP again being in cytosol (16) could interact with these two enzymes to ensure the quick metabolism of sucrose. Higher affinity of PFP for Fru-1,6-P_2 than that for Fru-6-P, as observed in the present case, further indicates that the enzyme in sink tissues might be operative in gluconeogenic direction utilizing the reverse reaction for the formation of PPi, which could ensure continuous utilization of sucrose through the action of sucrose synthase. These views are further supported by the fact that the reactions catalyzed by sucrose synthase, UDP-glucose pyrophosphorylase, and PFP are close enough to equilibrium *in vivo* for the above scheme of sucrose breakdown to operate. However, in a recent report, Doehlert *et al.* (10) have indicated that in developing maize endosperm, PFP is present in activities much higher than the rate of starch accumulation, indicating that the enzyme might also function in the glycolytic direction in developing maize endosperm. A final decision on the metabolic role of PFP in these tissues will have to await further definitive evidence.

The proposal for the role of PFP in PPi synthesis as suggested above, requires that ATP-phosphofructokinase generates enough Fru-1,6-P_2 for PPi production, as well as for the formation of triosephosphates. However, ATP-phosphofructokinase activity as measured in developing endosperm tissues is not enough to allow it to function in this way (10). Thus, it is unlikely that PFP could function to produce PPi for UDP-glucose pyrophosphorylase in this tissue. Another possible source of PPi in endosperm tissue could be from the reaction of ADP-glucose pyrophosphorylase (UTP + Glu-1-P→UDP-glucose + PPi). However, this reaction is known to occur in the amyloplast (17, 23). Based on the analogy of similarity between chloroplasts and amyloplasts, it could be assumed that PPi produced in the amyloplast may be exchanged with the cytosol via an adenylate/PPi exchanger that is known to exist in chloroplasts (19). However, it seems unlikely in view of the presence of alkaline pyrophosphatase in the plastids, which does not allow PPi to accumulate in sufficient quantities in these organelles. Pyrophosphate might also be supplied by a reversible PPi-dependent tonoplast proton pump. Pyrophosphate might also be produced by an ATP pyrophosphate hydrolase that would make PPi and AMP from ATP, but the existence of this enzyme has not been confirmed. Though the description given here is speculative, yet it is interesting and needs further investigations to confirm or reject the above viewpoints. Some of the possibilities described here are currently under investigation in our laboratory.

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