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Purification and Structural and Kinetic Characterization of the Pyrophosphate:Fructose-6-Phosphate 1-Phosphotransferase from the Crassulacean Acid Metabolism Plant, Pineapple[1]

Karina E. J. Trípodi and Florencio E. Podestá*

Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario and Centro de Estudios Fotosintéticos y Bioquímicos (CONICET, Fundación M. Lillo), Suipacha 531, 2000 Rosario, Argentina

Pyrophosphate-dependent phosphofructokinase (PFP) was purified to electrophoretic homogeneity from illuminated pineapple (*Ananas comosus*) leaves. The purified enzyme consists of a single subunit of 61.5 kD that is immunologically related to the potato tuber PFP β subunit. The native form of PFP likely consists of a homodimer of 97.2 kD, as determined by gel filtration. PFP's glycolytic activity was strongly dependent on pH, displaying a maximum at pH 7.7 to 7.9. Gluconeogenic activity was relatively constant between pH 6.7 and 8.7. Activation by Fru-2,6-bisphosphate (Fru-2,6-P_2) was dependent on assay pH. In the glycolytic direction, it activated about 10-fold at pH 6.7, but only 2-fold at pH 7.7. The gluconeogenic reaction was only weakly affected by Fru-2,6-P_2. The true substrates for the PFP forward and reverse reactions were Fru-6-phosphate and Mg-pyrophosphate, and Fru-1,6-P_2, orthophosphate, and Mg^{2+}, respectively. The results suggest that pineapple PFP displays regulatory properties consistent with a pH-based regulation of its glycolytic activity, in which a decrease in cytosolic pH caused by nocturnal acidification during Crassulacean acid metabolism, which could curtail its activity, is compensated by a parallel increase in its sensitivity to Fru-2,6-P_2. It is also evident that the β subunit alone is sufficient to confer PFP with a high catalytic rate and the regulatory properties associated with activation by Fru-2,6-P_2.

The path of carbon in plants displaying CAM is characterized by a massive mobilization of reserve carbohydrates during the dark period to provide PEP for the primary carboxylation reaction, which eventually leads to the accumulation of malate in the vacuole (Ting, 1985). The source of the carbon skeleton of PEP may vary depending on the species; some CAM plants degrade primarily chloroplastic glucans or starch, whereas others make use of soluble carbohydrates stored in extrachloroplastic compartments (Ting, 1985; Carnal and Black, 1989; Christopher and Holtum, 1996). Obviously, there are marked differences in the enzyme complement involved in each pathway, and probably also in its regulation. In starch-degrading CAM plants, for instance, chloroplastic glycolysis produces 3-phosphoglycerate, which is later exported to the cytosol via the Pi-translocator, and then converted to PEP by enzymes of the glycolytic pathway (Ting, 1985). On the other hand, the degradation of soluble carbohydrates to PEP is an entirely cytosolic process that involves the complete set of cytosolic glycolytic enzymes (Carnal and Black, 1989). The regulation of this pathway upstream of PEP carboxylase in CAM plants has yet to be resolved.

The phosphorylation of Fru-6-P to Fru-1,6-P_2 is a major point of regulation for glycolysis in nonplant organisms, especially at the level of PFK (Fahrendorf et al., 1987; Stitt, 1990; Plaxton, 1996). Conversely, plants exert primary and secondary regulation of glycolysis at the level of PEP and Fru-6-P utilization, respectively (Plaxton, 1996). Additionally, in plants and some microorganisms, the conversion of Fru-6-P to Fru-1,6-P_2 is mediated by a second, PPi-dependent phosphofructokinase (pyrophosphate, Fru-6-P: 1-phosphotransferase, EC 2.7.1.90; PFP), a strictly cytosolic enzyme that catalyzes the following reaction:

$$\text{Fru-6-P} + \text{PPi} \overset{Mg^{2+}}{\rightleftharpoons} \text{Fru-1,6-P}_2 + \text{Pi}$$

Unlike plant PFK, PFP catalyzes a reversible reaction and is activated by Fru-2,6-P_2. Its role in plant carbohydrate metabolism is still uncertain, despite a plethora of studies attempting to elucidate its function. One common feature arising from these studies is that PFP appears to be an adaptative enzyme, the function of which depends on the metabolic needs of the parent tissue. In this way, PFP has been implicated in glycolysis (Hatzfeld et al., 1989; Stitt, 1990; Trevanion and Kruger, 1991; Theodorou and Plaxton, 1996), in gluconeogenesis (Botha and Botha, 1991; Hajirezai et al., 1994), in the interconversion of the pool of triosephosphates (Hatzfeld and Stitt, 1990), in supporting glycolytic flux under conditions of Pi deficiency (Duff et al., 1989; Theodorou and Plaxton, 1994).

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* Corresponding author; e-mail rncefobi@arcride.edu.ar; fax 54–41–370044.

Abbreviations: FBPase, Fru-1,6-bisphosphatase; FPLC, fast-protein liquid chromatography; Fru-6-P, Fru-6-phosphate; Fru-1,6-P_2, Fru-1,6-bisphosphate; Fru-2,6-P_2, Fru-2,6-bisphosphate; 2-ME, 2-mercaptoethanol; PFK, ATP-dependent phosphofructokinase; PFP, pyrophosphate-dependent phosphofructokinase; Q_{10}, ratio of rates temperatures differing by 10° C.

Most plant PFPs studied to date possess a two-subunit (α and β) type of structure, with heterotetrameric or heterooctameric arrangements (Kruger and Dennis, 1987; Botha and Botha, 1991; Moorhead and Plaxton, 1991; Nielsen, 1994; Podestá et al., 1994). The relative amount of the α and β subunits seems to play a role in the regulation of the holoenzyme's activity within the cell (Theodorou and Plaxton, 1994), and the relative concentration of the two subunit types depends on factors such as nutrient availability or developmental stage of the parent tissue (Podestá and Plaxton, 1994; Theodorou and Plaxton, 1994).

CAM plants such as pineapple (*Ananas comosus*) present a unique opportunity to study the role of PFP in more detail, because the leaf levels of this enzyme are more than 20-fold in excess over those of PFK (Carnal and Black, 1983). In fact, it was in this tissue that PFP was first described in plants (Carnal and Black, 1979), although scarce attention was devoted thereafter to the study of its kinetic, regulatory, and structural properties.

This paper presents a method for the rapid purification of pineapple leaf PFP to electrophoretic homogeneity using FPLC techniques, and reports on a number of kinetic and structural properties of the purified enzyme.

MATERIALS AND METHODS

Pineapple (*Ananas comosus* var. Havaiano) plants were purchased at a local market. Detached crowns were placed in pots with distilled water until rooting, and then transferred to pots with soil and watered with a 10% (v/v) dilution of Hoagland solution. Plants were grown in a greenhouse with an illumination regime of 16 h light/8 h dark. The temperature was 25 to 30°C during the day and 15°C during the night. These conditions were sufficient to promote titratable acidity fluctuations consistent with CAM metabolism.

Reagents

Fru-6-P, Fru-1,6-P_2, Fru-2,6-P_2, NADH, $NADP^+$, Q-Sepharose, Sephadex G-50, standard molecular weight markers for gel filtration, SDS-PAGE, and blue dextran were from Sigma. pI standards were purchased from Bio-Rad. Fru-6-P was acid-treated before use to remove traces of Fru-2,6-P_2. All other reagents were of analytical grade and were purchased from J.T. Baker. Solutions of reagents present in the assay media were brought to the corresponding pH.

Assay of Activity

PFP was assayed spectrophotometrically in the glycolytic direction in a medium containing 50 mM Tris-HCl, pH 7.7, 2 mM $MgCl_2$, 0.15 mM NADH, 1 mM PPi, 2 mM Fru-6-P, 5 units of triose-phosphate isomerase, 1 unit of aldolase, and 1 unit of glycerol phosphate dehydrogenase. Activity in the gluconeogenic direction was determined in a medium containing 50 mM Tris-HCl, pH 7.7, 2 mM $MgCl_2$, 0.2 mM $NADP^+$, 2 mM Fru-1,6-P_2, 1 mM Pi, 1 unit of phosphoglucoisomerase, and 1 unit of Glc-6-P dehydrogenase. The terms forward and reverse are used as synonyms for the glycolytic and gluconeogenic reactions catalyzed by PFP, respectively. Coupling enzymes were desalted before use according to the method of Penefsky (1977) in 2.5-mL columns equilibrated with 50 mM Hepes-NaOH, pH 7.0, plus 20% (v/v) glycerol. Activity was measured at 25°C. For the pH-dependence studies, 50 mM Bis-Tris propane was used instead of Tris-HCl. Reactions were started by the addition of PPi or Pi. One unit is defined as the amount of enzyme necessary to catalyze the consumption of 1 μmol Fru-6-P (or 2 μmol NADH) per minute in the glycolytic direction, or the use of 1 μmol Fru-1,6-P_2 (or 1 μmol NADPH produced) per minute in the gluconeogenic direction. Kinetic parameters, including activation kinetics, were determined using a nonlinear, least-squares-regression computer kinetics program kindly provided by Dr. Stephen Brooks (Brooks, 1992). The same program was used to analyze the data obtained by assaying PFP at different temperatures through the Arrhenius plot. The effect of temperature was also analyzed by obtaining Q_{10} values as described in Trevanion and Kruger (1991) according to the following equation:

$$Q_{10} = (V_2/V_1)^{10/(T_2 - T_1)}$$

where V_n is the enzyme activity at a temperature of T_n.

The nature of the true reactants for PFP's reverse reaction was evaluated by measuring gluconeogenic activity in the presence of different levels of Mg^{2+} and constant concentrations of the complexed or uncomplexed species of Fru-1,6-P_2 and Pi. The basis for the experiment has been described before (Podestá and Plaxton, 1992, and refs. therein). In summary, the reciprocal of the observed velocity v at a constant concentration of the substrates can be expressed as a polynomial equation in Mg^{2+}:

$$v^{-1} = \sum_x f_x(Mg^{2+})^i$$

where the power i affecting Mg^{2+} concentration is determined by the substrate form selected. This parameter assumes values of 0 or -1 (that is, linear graphs will be obtained) only when the true substrate combination is used. Dissociation constants were obtained from Bertagnolli and Cook (1984).

Buffers Used for the Purification of PFP

Buffer A: 100 mM Tris-HCl, pH 8.2, 20 mM sodium acetate, 20% (v/v) glycerol, 1 mM PMSF, 2 mM 2-ME, 1 mM EDTA, 2.5% (w/v) polyvinylpolypyrrolidone. Buffer B: 20 mM Tris-HCl, pH 7.5, 10% (v/v) glycerol, 2 mM 2-ME, 1 mM EDTA. Buffer C: 25 mM Pi, pH 7.0, 40% saturation $(NH_4)_2SO_4$, 2 mM 2-ME. Buffer D: 15 mM Pi, pH 7.0, 20% (v/v) glycerol, 2 mM 2-ME. Buffer E: 25 mM Tris-HCl, pH 7.0, 20% (v/v) glycerol, 2 mM 2-ME.

PFP Purification

For a typical purification, 70 g of fully developed pineapple leaves, harvested 7 h into the illumination period and with the first 2 cm from the tip and bottom removed, were used. Leaves were washed with distilled water, and finely chopped and ground in a blender at maximal speed with 2

volumes of buffer A. The homogenate was filtered through cheesecloth and centrifuged for 10 min at 10,000*g*. The pH of the supernatant fluid was adjusted to 7.2 with HCl after the addition of 0.989 g of Na_4PPi and 2 mL of 1 M $MgCl_2$ per liter of extract. The solution was brought to 35% saturation $(NH_4)_2SO_4$, stirred for 20 min, and centrifuged for 15 min at 12,000*g*. The supernatant fraction was made 60% saturated with $(NH_4)_2SO_4$, stirred for 20 min, and centrifuged for 15 min at 12,000*g*. All of the above procedures were carried out at 4°C. The pellet was resuspended in a minimal volume of buffer B and this solution was dialyzed overnight against 50 volumes of buffer B with one change of buffer. The dialysate was loaded at 0.5 mL min^{-1} onto a 1.5- × 7-cm Q-Sepharose column equilibrated in buffer B connected to an FPLC system (Pharmacia). After loading, the column was washed at 1 mL min^{-1} with buffer B until the A_{280} decreased below 0.2. Elution was performed by increasing the concentration of KCl in buffer B from 0 to 400 mM. PFP activity began to elute at 120 mM KCl and peaked at 220 mM. Active fractions were pooled, precipitated with 60% $(NH_4)_2SO_4$, and centrifuged for 15 min at 12,000*g*. The pellet was redissolved in 25 mM Pi, pH 7.0, plus 2 mM 2-ME, made 40% in $(NH_4)_2SO_4$, and centrifuged at 10,000*g* for 10 min. The clear supernatant fluid was loaded at 0.25 mL min^{-1} onto a prepacked phenyl Superose HR 5/5 column (Pharmacia). After loading, the column was washed with 5 mL of buffer C, and bound protein was eluted by increasing the percentage of buffer D by steps to 50 and 75% and a final wash with 100% buffer E that eluted bound PFP. The flow rate was decreased from 0.25 to 0.10 mL min^{-1} for the last step. Active fractions were collected and concentrated using Centricon PM30 tubes at 4°C and 3,000*g*. All chromatographic procedures were performed at 20°C. The final preparation was divided into aliquots and stored at −80°C until use. PFP was stable for at least 6 months when stored in this manner.

Electrophoretic Methods

SDS-PAGE was carried out according to the method of Laemmli (1970). Native PAGE was performed as described by Moorhead and Plaxton (1990). The final polyacrylamide concentration in the separating gels was 7% (w/v) for nondenaturing PAGE and 8% (w/v) for SDS-PAGE. The molecular mass of a given polypeptide was estimated by comparing its mobility to the following standards: myosin (205 kD), β-galactosidase (116 kD), phosphorylase *b* (97.4 kD), BSA (66 kD), ovalbumin (45 kD), and carbonic anhydrase (29 kD). Immunological detection by western blotting was done according to the method of Bollag and Edelstein (1991). Extracts obtained under denaturing conditions were processed as described by Plaxton (1989). Anti-potato tuber PFP antisera were the generous gift of Dr. W.C. Plaxton, Queen's University, Kingston, Ontario, Canada. Silver staining was done as described by Harlow and Lane (1988). Activity staining was performed as follows: after nondenaturing electrophoresis at 4°C, gels were soaked 10 min in a solution containing 200 mM Tris-HCl, pH 8.0, and 2 mM 2-ME, and then developed by adding 10 mL of the same solution plus 5 mM $MgCl_2$, 5 mM Fru-1,6-P_2, 2 mM Pi, 1 unit each of phosphoglucoisomerase and Glc-6-P dehydrogenase, 0.1 mg ml^{-1} of nitroblue tetrazolium, and 5 μg ml^{-1} of phenazine methosulfate. Development was complete after 30 min, and the reaction was stopped by thoroughly rinsing with cold distilled water. Denaturing IEF in slab gels was done in a Mini-Protean II cell according to the protocol described by Bollag and Edelstein (1991), using pI standards provided by Bio-Rad. Native IEF was carried out in horizontal slab gels (0.8-mm thick) using the gel system described by Bollag and Edelstein (1991). Gels were pre-electrophoresed for 30 min before applying the sample, and the pH gradient established at 600 V for 3 h.

Native Molecular Mass Estimation

Gel-filtration chromatography was performed at 0.25 mL ml^{-1} using a Sephacryl S300 HR column (1.1 × 24 cm) connected to an FPLC system (Pharmacia). The standard running buffer consisted of 50 mM Tris-HCl, pH 7.5, and 2 mM 2-ME. Runs were also performed adding 10% (v/v) glycerol plus or minus 50 mM KCl to the standard buffer. The column was calibrated using the following standards: bovine thyroglobulin (669 kD), horse spleen apoferritin (443 kD), sweet potato β-amylase (200 kD), yeast alcohol dehydrogenase (150 kD), BSA (66 kD), and carbonic anhydrase (25 kD). The void volume (V_o) was determined with blue dextran. Distribution coefficients (K_D) were calculated using the following equation:

$$K_D = \frac{V_e - V_o}{V_t - V_o}$$

where V_e is the elution volume and V_t is the total column volume. K_D values were plotted against the log of molecular masses of the standards, and the size of the PFP samples was calculated from the regression line thus obtained.

RESULTS

PFP Purification

The activity of PFP in illuminated pineapple leaf crude extracts was 2.7 ± 0.3 units g^{-1} fresh weight. PFP was purified about 40-fold, with a recovery of 11%, from this source (Table I) using a combination of $(NH_4)_2SO_4$ fractionation, strong anion-exchange chromatography on Q-Sepharose, and hydrophobic interaction on phenyl Superose. The final preparation was devoid of ATP-dependent PFK or FBPase activities, and showed a specific activity of 43.8 and 8.9 units mg^{-1} of protein for the forward and reverse reactions, respectively, in the presence of 2 μM Fru-2,6-P_2-saturating substrates and a pH of 7.7.

Subunit and Native Structure

Purified PFP migrated as a single band of 61.5 kD after SDS-PAGE (Fig. 1). No other polypeptides were visualized after silver-staining of the gel. Immunolocalization on western blots using anti-potato PFP IgG (polyclonal antibodies against both potato PFP subunit types) revealed a

Table I. *Pineapple PFP purification*

Step	Protein	Activity[a]	Specific Activity[a]	Yield	Purification
	mg	*units*	*units mg^{-1}*	*%*	*-fold*
Crude extract	238	284	1.2	100	1
35–60% $(NH_4)_2SO_4$ + dialysis	69.6	142	2.0	50	1.7
Q-Sepharose	17.1	122	7.1	43	5.9
Phenyl Superose	0.73	32	43.8	11	36.5

[a] Activity determinations of the forward reaction were performed at pH 7.7 in the presence of 2 μM Fru-2,6-P_2.

single band with both the purified PFP and with a crude leaf extract obtained under denaturing conditions (Fig. 2A, lanes 2 and 3, respectively). The identity of the single subunit was assessed using specific antibodies raised against potato PFP α- or β-type subunits. Purified pineapple PFP showed reactivity with anti-β but not anti-α immunoglobulins (Fig. 2B, lanes 3 and 4, respectively). The same pattern was evident in western blots in which crude leaf extracts obtained under denaturing conditions were probed (Fig. 2B, lanes 5 and 6). Overloaded blots (1 μg of pineapple PFP protein per well) probed with the anti-α antiserum were negative as well (not shown). A single-protein band was also visualized after denaturing IEF (pI = 6.25) or activity staining following nondenaturing PAGE or IEF (pI = 6.25) (results not shown). The theoretical pIs for the potato tuber α and β subunits, calculated from sequence data obtained from Carlisle et al. (1990), were 6.9 and 6.2, respectively. Thus, pineapple PFP appears to be composed of a β-like subunit. The native molecular mass was calculated to be 97.2 ± 1.6 kD (n = 3) according to gel-filtration chromatography on Sephacryl S300 HR at pH 7.5. No differences in M_r were observed upon addition of 10% (v/v) glycerol plus or minus 50 mM KCl to the standard buffer. These data indicate that pineapple leaf PFP likely possesses a homodimeric (β-type) quaternary structure. However, because the native molecular mass is not exactly twice the subunit molecular mass, it is possible that under the conditions in which the chromatographic separations were performed, a dimer-monomer mixture might exist.

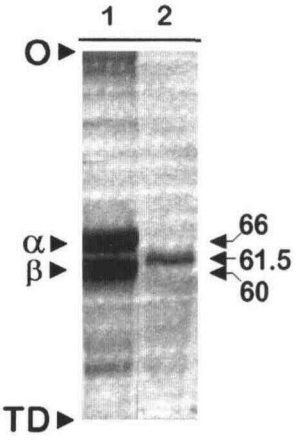

Figure 1. Silver-stained SDS-PAGE analysis of purified pineapple PFP. Lane 1, 100 ng of purified potato tuber PFP. Lane 2, 50 ng of purified pineapple leaf PFP. Numbers indicate molecular mass in kD; the symbols α and β denote the migration of the respective subunits of potato tuber PFP (66 and 60 kD, respectively). O, Origin; TD, tracker dye.

Kinetic Properties

Assays of activity with homogeneous PFP in both directions were linear with respect to the amount of enzyme added. Activity was strictly dependent on the presence of PPi and Fru-6-P in the glycolytic direction, on Pi and Fru-1,6-P_2 in the gluconeogenic direction, and on Mg^{2+} in both directions. Mn^{2+} could substitute for Mg^{2+}, but Co^{2+} (5 mM), Ca^{2+} (5 mM), Cu^{2+} (0.4 mM), Be^{2+} (2.5 mM), or Ni^{2+} (2.5 mM) were not effective as cofactors. A strong hysteresis, particularly evident at low PPi or Pi concentrations, was observed at pH values lower than 7.3, and increased in span with decreasing pH. This hysteresis consisted of a lag (0.25–0.5 min) to achieve maximum and constant activity. A similar lag is also evident at the optimum pH and saturating substrates when crude extracts are assayed.

Purified pineapple PFP gluconeogenic and glycolytic activities, measured in the absence of Fru-2,6-P_2, responded to assay pH in a different manner. The rate of dephosphorylation of Fru-1,6-P_2 was rather constant in the pH range of 6.7 to 8.0, whereas Fru-6-P phosphorylation showed a marked dependency on pH, displaying maximum velocity when assayed at pH 7.9 (Fig. 3A).

Activation by 2 μM Fru-2,6-P_2 was also dependent on pH. Fru-2,6-P_2 did not markedly change the optimum pH in either direction in terms of absolute rates (Fig. 3A), but the extent of activation of the forward reaction was about 5-fold greater at pH 6.7 compared with pH 7.7 (Fig. 3B). For the reverse reaction, maximal activation occurred in the pH range of 7.9 to 8.6. The activation measured reflects a response of V_{max}, because the substrate concentrations were maintained as saturating in the entire pH range tested. The magnitude of the activation by Fru-2,6-P_2 at any pH value was always higher for the glycolytic reaction. Moreover, a slight inhibition of activity by Fru-2,6-P_2 occurred in the reverse reaction at pH values lower than 7.0 when Fru-1,6-P_2 concentration was subsaturating (results not shown). Activation by Fru-2,6-P_2 followed hyperbolic saturation kinetics. Apparent affinity for Fru-2,6-P_2 (K_a) was higher at pH 7.7 (26.3 ± 3.0 nM) than at pH 6.7 (43.5 ± 6.3 nM) in the glycolytic direction, although maximal activation was observed at the lower pH (Fig. 3B). Affinity for the effector in the gluconeogenic direction was considerably higher than for the forward reaction, and remained similar at both pH values (2.4 ± 0 nM [pH 6.7] and 2.7 ± 0.8 nM [pH 7.7]).

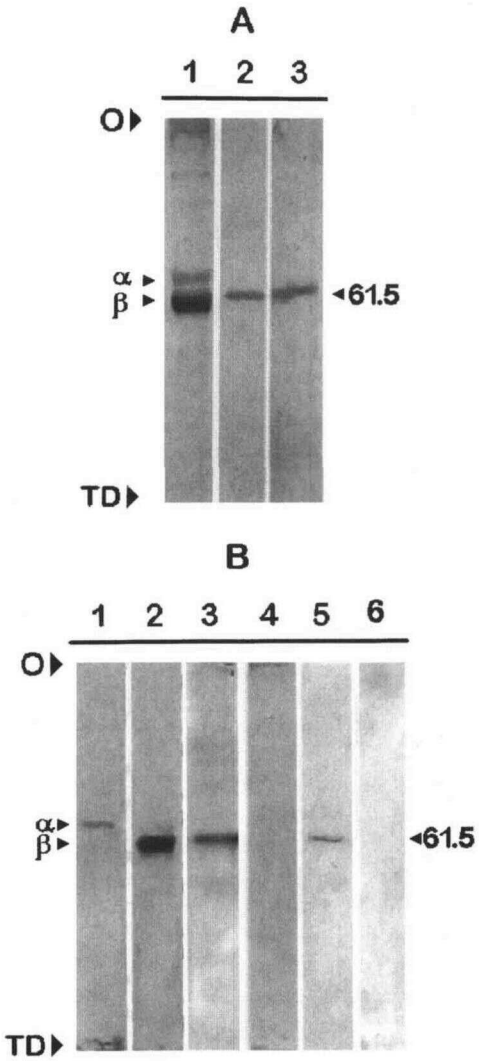

Figure 2. Western blot analysis of pineapple and potato PFPs. A, Lane 1, 50 ng of homogeneous potato tuber PFP; lane 2, 100 ng of homogeneous pineapple leaf PPF; lane 3, 1.5 μg of a pineapple leaf crude extract obtained under denaturing conditions (Plaxton, 1989). Transferred membranes were probed with a 1/1,000 dilution of anti-potato tuber PFP antiserum reactive against both α and β subunits. B, Lanes 1 and 2, 50 ng of homogeneous potato tuber PFP; lanes 3 and 4, 100 ng of homogeneous pineapple leaf PFP; lanes 5 and 6, pineapple leaf crude extract obtained under denaturing conditions (Plaxton, 1989). Lanes 1, 4, and 6 were probed with a 1/1,000 dilution of anti-potato tuber PFP antiserum raised against the α subunit; lanes 2, 3, and 5 were probed with a 1/10,000 dilution of anti-potato tuber β-subunit. Numbers indicate molecular mass in kD; α and β indicate the position of the potato tuber PFP subunits. O, Origin; TD, tracker dye.

The kinetic parameters for the forward and reverse reactions were determined at pH 6.7 and 7.7. As a first step, the nature of the true reactants of both reactions was addressed. For this, forward or reverse activities were measured at increasing Mg^{2+} concentrations and several fixed concentrations of either PPi or Pi, respectively. In both cases an apparent inhibition was observed at high Mg^{2+} at both high and low PPi or Pi (not shown), as has been reported before (Bertagnolli and Cook, 1984; Montavon and Kruger, 1992). Varying Pi or PPi concentration at fixed Mg^{2+} also resulted in inhibition at high anion concentration (not shown). If the free, uncomplexed species are the true substrates, inhibition can result as a consequence of sequestration of the free substrate forms by Mg^{2+}-complexing. Additionally, in the case of PPi, inhibition at high Mg^{2+} can result even if MgPPi is the reactive species, due to formation of the nonreactive Mg_2PPi complex (Montavon and Kruger, 1992). Previous work has determined that in all PFPs studied to date the complex MgPPi is the substrate in the forward reaction (Bertagnolli and Cook, 1984; Montavon and Kruger, 1992). This is functionally advantageous, because at physiological pH and PPi and Mg^{2+} concentrations, most of the anion (>98%) is chelated.

Our results show a similar behavior for pineapple PFP. Varying Mg^{2+} concentration at constant levels of combinations of the free or complexed forms of PPi or Fru-6-P yielded a straight line only when MgPPi and free Fru-6-P were maintained constant (Fig. 4A). In this experiment, total PPi concentration was maintained low (0.1 mM), while Mg^{2+} concentration never surpassed 1 mM, to avoid the formation of high levels of the potentially interfering Mg_2PPi complex (Montavon and Kruger, 1992). Phillips and Li (1995) argued that the *Giardia lamblia* PFP accepts the Pi-Mg complex as substrate for the reverse reaction, although the free anion has been reported to be the substrate for other plant and nonplant PFPs (Bertagnolli and Cook, 1984; Montavon and Kruger, 1992). This subject was addressed by measuring PFP activity at fixed concentrations of combinations of the free or complexed forms of the reverse reaction substrates and varying concentrations of Mg^{2+}. The results of the experiment are depicted in Figure 4B, which shows that free Pi and Fru-1,6-P_2 are the

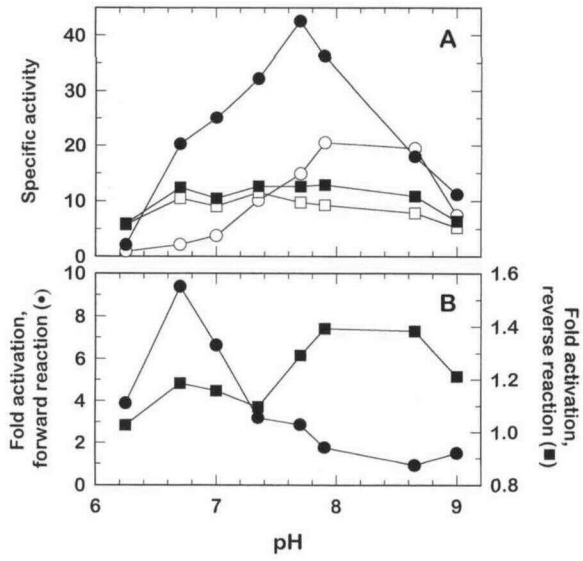

Figure 3. Dependence of pineapple leaf PFP activity on pH. A, Glycolytic (○, ●) and gluconeogenic (□, ■) activities were measured at different pH values in the absence (open symbols) or presence (closed symbols) of 2 μM Fru-2,6-P_2. B, Activation of pineapple PFP as a function of pH. Data were recalculated from A.

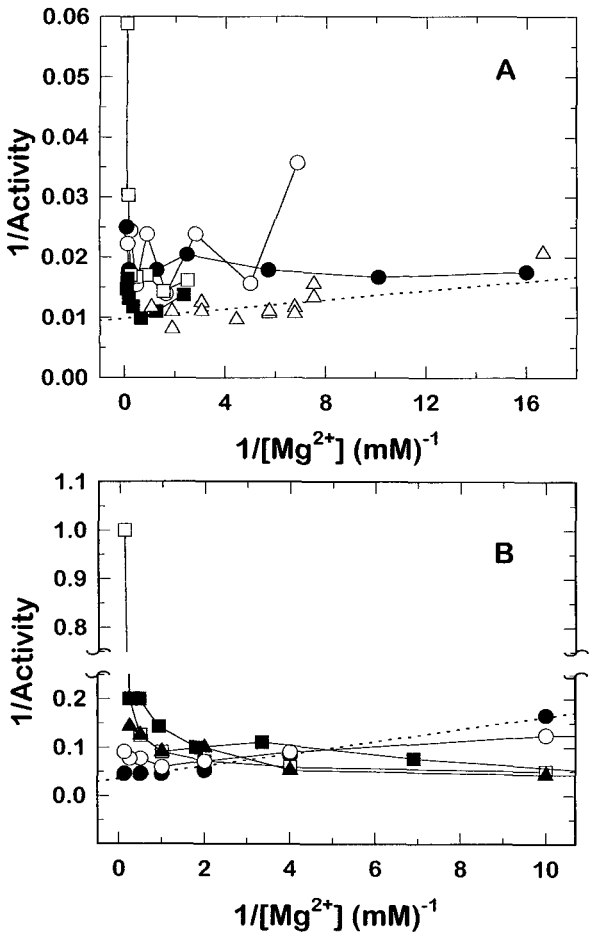

Figure 4. Determination of the true substrates for the pineapple PFP forward and reverse reactions. PFP activity was measured at varying concentrations of Mg^{2+} and several fixed concentrations of the different substrates. A, (Forward) 0.1 mM total PPi and 0.5 mM total Fru-6-P (○); 0.5 mM free Fru-6-P and 0.001 mM free PPi (●); 0.05 mM Mg-Fru-6-P and 0.001 mM MgPPi (□); 0.05 mM Mg-Fru-6-P and 0.1 mM free PPi (■); 0.5 mM free Fru-6-P and 0.001 MgPPi (Δ). B, (Reverse) 0.1 mM total Pi and 0.05 mM total Fru-1,6-P_2 (○); 0.1 mM free Pi and 0.05 mM free Fru-1,6-P_2 (●); 0.02 mM MgPi and 0.01 mM Mg-Fru-1,6-P_2 (□); 0.1 mM free Pi and 0.01 mM Mg-Fru-1,6-P_2 (■); 0.02 mM MgPi and 0.05 mM free Fru-1,6-P_2 (▲). Dashed lines represent the result of a linear regression fit to the data points (data marked with Δ in A and ● in B).

catalytically active substrate forms of pineapple PFP, which therefore resembles potato PFP (Montavon and Kruger, 1992) and microbial PFP (Bertagnolli and Cook, 1984) in that it uses free Fru-6-P and PPi-Mg in the forward direction and free Pi, free Fru-1,6-P_2, and Mg^{2+} for the reverse reaction.

Table II shows the observed values of apparent K_m for the substrates of both reactions catalyzed by PFP at pH 6.7 and 7.7. Observed values for V_{max} in the presence of 2 μM Fru-2,6-P_2 were 41.1 ± 0.3 and 19.6 ± 0.1 units mg^{-1} of protein for the forward reaction at pH 7.7 and 6.7, respectively, and 15.0 ± 0.2 units mg^{-1} of protein at both pH values for the reverse reaction.

Effect of Temperature on PFP Activity

Arrhenius plots were obtained for forward and reverse reactions of PFP in the presence or absence of Fru-2,6-P_2, in the temperature range between 2 and 40°C at pH 7.7 (data not shown). Plots were linear in all cases. Activation energies were 1763 J mol^{-1} in the glycolytic direction and 1217 J mol^{-1} in the gluconeogenic direction. These values were reduced to 673 and 1032 J mol^{-1}, respectively, by Fru-2,6-P_2. To allow comparison with earlier results with potato PFP (Trevanion and Kruger, 1991), Q_{10} values were also calculated in the range of 15 to 25°C and 2 to 15°C. In the forward direction, Fru-2,6-P_2 (2 μM) decreased Q_{10} from 2.02 to 1.12, and from 3.16 to 1.43 for the higher and lower temperature ranges, respectively. In the reverse direction, Q_{10} values were 1.32 and 1.41 for the 15 to 25°C and 2 to 15°C ranges, respectively, and were not altered significantly by inclusion of Fru-2,6-P_2 in the assay medium.

DISCUSSION

PFP was purified to electrophoretic homogeneity from illuminated pineapple leaves, with an overall yield of 11%, and possessed a specific activity of 44 units mg^{-1}. Structural characterization of the homogeneous pineapple PFP by SDS-PAGE, IEF, immunoblotting, and gel filtration revealed a single subunit of 61.5 kD, immunologically related to the β subunit but not the α subunit of potato PFP, which assembles as a β_2 homodimer to form the native, active enzyme. Western blots carried out with crude pineapple leaf extracts prepared under denaturing conditions showed: (a) the absence of cross-reaction when probed with anti-potato PFP α-subunit antibodies, thus ruling out the loss of this polypeptide during purification, and (b) a single immunoreactive band at 61.5 kD when probed with anti-potato PFP β-subunit antibodies, suggesting that pineapple leaf PFP is not subject to partial proteolytic degrada-

Table II. *Kinetic parameters of homogeneous pineapple PFP*

Substrate	K_m	
	pH 6.7	pH 7.7
	mM	
Forward reaction[a]		
Fru-6-P	2.51 ± 0.08	1.62 ± 0.06
Fru-6-P + Fru-2,6-P_2	0.75 ± 0.06	0.89 ± 0.07
PPi-Mg^{2+}	0.085 ± 0.031	0.009 ± 0.002
PPi-Mg^{2+} + Fru-2,6-P_2	0.014 ± 0.003	0.011 ± 0.002
Reverse reaction[a]		
Fru-1,6-P_2	0.067 ± 0.007	0.088 ± 0.015
Fru-1,6-P_2 + Fru-2,6-P_2	0.105 ± 0.015	0.094 ± 0.010
Pi	0.215 ± 0.057	0.088 ± 0.053
Pi + Fru-2,6-P_2	0.128 ± 0.028	0.149 ± 0.035
Mg^{2+}	Not determined	0.63 ± 0.06

[a] Activity determinations were performed at pH 6.7 and 7.7 in the presence or absence of 2 μM Fru-2,6-P_2. Invariant cosubstrate concentrations were 5 mM Fru-6-P, 1 mM PPi, 2 mM Fru-1,6-P_2, and 2 mM Pi. V_{max} values remained unaltered by Fru-2,6-P_2 in all cases (see text).

tion during purification. A dimeric PFP consisting of 60-kD (β) subunits has been previously reported to coexist with more abundant $\alpha_2\beta_2$ tetramers in wheat seedlings (Yan and Tao, 1984). Mung bean PFP has also been reported to contain only a 60-kD polypeptide (Cheng and Tao, 1990), but a tetrameric arrangement has been suggested for this enzyme (Bertagnolli et al., 1986). Actually, mung bean PFP may be composed of SDS-PAGE-comigrating α- and β-subunits (Cheng and Tao, 1990). The other monocot-leaf PFP purified to date, that from barley seedlings (Nielsen, 1994), possesses a heterotetrameric arrangement. The peculiar structure of pineapple PFP raises some interesting questions regarding its regulation. It has been conjectured that in heteromeric PFPs, the α subunit is responsible for conferring on the enzyme an increased Fru-2,6-P_2 sensitivity and basal activity, whereas the β subunit is involved in catalysis. In pineapple PFP, on the other hand, the β subunit alone is sufficient to achieve a highly active, yet Fru-2,6-P_2-sensitive enzyme. Kinetic data related to the response to Fru-2,6-P_2 by this enzyme indicate a high (on the nM scale) affinity toward Fru-2,6-P_2, comparable to that found in heteromeric PFPs (Yan and Tao, 1984; Nielsen, 1994).

The pH dependence of several PFPs has yielded similar results, all with an optimum situated near pH 7.7. The response of pineapple PFP to H^+ concentration shows a similar trend (Fig. 3A). The glycolytic reaction's response to Fru-2,6-P_2 shifts considerably as pH decreases. In fact, although specific activity falls with pH, activation by Fru-2,6-P_2 increases in this direction, reaching a maximum of 10-fold at pH 6.7 (Fig. 3). This characteristic may represent an effective mechanism of maintaining a high glycolytic PFP activity at the physiological pH inside the cell, especially during night acidification in CAM plants, because a cytosolic pH decrease, which would otherwise curtail PFP activity, would be compensated for by a simultaneous increase in the enzyme's sensitivity to Fru-2,6-P_2 and in the concentration of this signal metabolite in the darkened pineapple leaf (Fahrendorf et al., 1987).

Conversely, the gluconeogenic activity of PFP is not as markedly affected by pH in the range of 6.7 to 8.0. It should be noted that the reverse reaction is not as sensitive to Fru-2,6-P_2 as the forward reaction (Fig. 3). It has recently been pointed out that this property could arise from a masking effect caused by activation of PFP by Fru-1,6-P_2 (Nielsen, 1995). In fact, when gluconeogenic activity is measured under subsaturating Fru-1,6-P_2 levels, the addition of 2 μM Fru-2,6-P_2 results in a slight inhibition of PFP activity, probably because of a decrease in the affinity of PFP toward Fru-1,6-P_2 (not shown). This could originate from competition between Fru-2,6-P_2 and Fru-1,6-P_2 for the activator site, for which Fru-2,6-P_2 has a higher affinity (about 2.5 nM) but a lower activating effect compared with Fru-1,6-P_2. The above-described kinetic properties could have some influence on the regulation of cytosolic carbon flow in pineapple leaves. Activation of the reverse reaction by a high concentration of gluconeogenic precursors (and, hence, Fru-1,6-P_2, a PFP activator) would shift carbon flow toward gluconeogenesis, even if Fru-2,6-P_2 concentration drops (as is expected in illuminated pineapple leaves; see Fahrendorf et al. [1987]).

Most PFPs studied so far in plant tissues or microorganisms use MgPPi and Fru-6-P in the forward reaction and free Pi, Fru-1,6-P_2, and Mg^{2+} in the reverse direction, with the probable exception of the *Giardia lamblia* enzyme, which has been reported to use MgPi instead of the free forms of these substrates (Phillips and Li, 1995). Pineapple PFP substrate requirements fall within those found for the first group, and thus the reaction catalyzed by this enzyme is:

$$\text{Fru-6-P} + \text{MgPPi} \rightleftharpoons \text{Fru-1,6-P}_2 + P_i + Mg^{2+}$$

As noted by Carnal and Black (1989), low night temperatures play a pivotal role in promoting tissue acidification during CAM. Thus, studying the thermal response of pineapple PFP may shed light on important aspects regarding its regulation. Also, because the temperature effects have been studied in some detail in potato tuber PFP by Trevanion and Kruger (1991), comparison with this heteromeric enzyme may be of interest. The observed Q_{10} values are similar to those obtained by Carnal and Black (1989) in desalted pineapple leaf extracts, but quite different from those observed for potato tuber PFP in the absence of Fru-2,6-P_2; the latter enzyme's activation by Fru-2,6-P_2 increases sharply (i.e. 10-fold) in the range of 25° to 2°C. On the other hand, pineapple PFP Q_{10} values are only modestly decreased in the presence of Fru-2,6-P_2. In fact, activation of the forward reaction by Fru-2,6-P_2 increases from 2-fold at 25°C to 2.5-fold at 2°C, a modest 25% increase. It is possible that this difference may arise as a consequence of the contrasting structural composition of both enzymes.

In summary, the kinetic, regulatory, and structural properties of pineapple leaf PFP render this enzyme particularly well suited for channeling the majority of the carbon derived from sugars into glycolytic PEP that will serve as the substrate for nocturnal CO_2 fixation in this CAM plant. Its response to pH and Fru-2,6-P_2 concentration in vitro makes it tempting to hypothesize that changes in these two factors may influence PFP activity in vivo. It is also evident from this work that a β-type subunit alone is sufficient to confer on pineapple PFP all of the traits related to regulation and high activity observed in heteromeric PFPs. It would be interesting to see whether PFPs with different subunit compositions exhibit the same response to pH as pineapple PFP. Further comparative work with the enzyme from different sources is probably necessary to determine how and why the subunit structure of PFP affects its biological function.

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