Purification and Characterization of Pyrophosphate-Dependent Phosphofructokinase from Phosphate-Starved Brassica nigra Suspension Cells

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Previously, we reported that inorganic phosphate (Pi) deprivation of Brassica nigra suspension cells or seedlings leads to a progressive increase in the α:β-subunit ratio of the inorganic pyrophosphate (PPI)-dependent phosphofructokinase (PFP) and that this coincides with a marked enhancement in the enzyme's activity and sensitivity to its allosteric activator, fructose-2,6-bisphosphate (Fru-2,6-P₂). To further investigate the effect of Pi nutrition on B. nigra PFP, the enzyme was purified and characterized from Pi-starved B. nigra suspension cell cultures. Polyacrylamide gel electrophoresis, immunoblot, and gel-filtration analyses of the final preparation indicated that this enzyme exists as a heterooctamer of approximately 500 kD and is composed of a 1:1 ratio of immunologically distinct α (66 kD) and β (60 kD) subunits. The enzyme’s α subunit was susceptible to partial proteolysis during purification, but this was prevented by the presence of chymostatin and leupeptin. In the presence and absence of 5 μM Fru-2,6-P₂, the forward activity of PFP displayed pH optima of pH 6.8 and 7.6, respectively. Maximal activation of the forward and reverse reactions by Fru-2,6-P₂ occurred at pH 6.8. The potent inhibition of the forward activity by Pi (concentration of inhibitor producing 50% inhibition of enzyme activity, I₅₀ = 1.3 mM) was attributed to a marked Pi-dependent reduction in Fru-2,6-P₂ binding. The reverse reaction was substrate-inhibited by Pi (I₅₀ = 13 mM) and product-inhibited by PPI (I₅₀ = 0.9 mM). The kinetic data are consistent with the hypothesis that PFP may function to bypass the ATP-dependent PFP in Pi-starved B. nigra. The importance of the Pi nutritional status to the regulation and predicted physiological function of PFP is emphasized.

PFP catalyzes the reversible phosphorylation of Fru-6-P to Fru-1,6-P₂, using PPI as a phosphoryl donor in the glycolytic (forward) direction and Pi as a phosphoryl acceptor in the gluconeogenic (reverse) direction. Although the enzyme has been purified and characterized from a wide variety of plant sources, an incontrovertable physiological role for PFP has yet to emerge (Kombrink et al., 1984; Yan and Tao, 1984; Botha et al., 1986, 1987; Kruger and Dennis, 1987; Mahajan and Singh, 1989; Stitt, 1989, 1990; Wong et al., 1990; Botha and Botha, 1991a, 1991b; Moorhead and Plaxton, 1991; Nielsen, 1994). A growing body of evidence suggests that PFP is an adaptive enzyme with kinetic and molecular properties that are responsive to a variety of environmental, developmental, and tissue-specific cues (Plaxton, 1996). The present study focuses on the influence of Pi nutrition as one of a number of determinants that may contribute to the modulation of PFP properties in vivo.

The most notable form of allosteric control of PFP is its potent activation by low concentrations of the regulatory metabolite Fru-2,6-P₂ (Stitt, 1990; Plaxton, 1996). The affinity of PFP for Fru-2,6-P₂ appears to depend mainly on catalytic conditions and the enzyme's subunit composition (Yan and Tao, 1984; Stitt, 1990; Wong et al., 1990; Botha and Botha, 1991a, 1991b; Theodorou et al., 1992; Podestá and Plaxton, 1994). The affinity for Fru-2,6-P₂ is increased by Fru-6-P and Fru-1,6-P₂, whereas it is decreased by Pi, various phosphorylated metabolites, and (in)organic anions (Kombrink and Kruger, 1984; Stitt, 1989, 1990). Pi is a potent mixed-type inhibitor of the forward reaction of potato and wheat PFP (Mahajan and Singh, 1989; Stitt, 1989, 1990). However, the significance of Pi as an allosteric effector of PFP has been eclipsed in the literature by the attention that has been devoted to Fru-2,6-P₂.

Another potentially important mechanism for modulating the activity and regulation of PFP in vivo is the differential expression of the enzyme’s α and β subunits. Various evidence indicates that the β subunit contains the catalytic site, and that the α subunit is involved in the regulation of catalytic activity by Fru-2,6-P₂ (Yan and Tao, 1984; Carlisle et al., 1990; Cheng and Tao, 1990; Stitt, 1990; Botha and Botha, 1991a, 1991b; Theodorou et al., 1992; Montavon and Kruger, 1993; Podestá and Plaxton, 1994; Theodorou and Plaxton, 1994, 1995). Generally, the α subunit-containing forms of the enzyme appear to display greater activity and sensitivity to activation by various evidence indicates that the β subunit contains the catalytic site, and that the α subunit is involved in the regulation of catalytic activity by Fru-2,6-P₂ (Yan and Tao, 1984; Carlisle et al., 1990; Cheng and Tao, 1990; Stitt, 1990; Botha and Botha, 1991a, 1991b; Theodorou et al., 1992; Montavon and Kruger, 1993; Podestá and Plaxton, 1994; Theodorou and Plaxton, 1994, 1995). Generally, the α subunit-containing forms of the enzyme appear to display greater activity and sensitivity to activation by

Abbreviations: FPLC, fast protein liquid chromatography; Fru-1,6-P₂, Fru-1,6-bisphosphate; Fru-2,6-P₂, Fru-2,6-bisphosphate; I₅₀, concentration of inhibitor producing 50% inhibition; Kᵥ, concentration of activator producing 50% activation of enzyme activity; MS, Murashige-Skoog; PFK, ATP:Fru-6-P 1-phosphotransferase (EC 2.7.1.11); PFP, PPI:Fru-6-P 1-phosphotransferase (EC 2.7.1.90).

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Fru-2,6-P₂, whereas forms enriched with the β subunit appear to be less sensitive to Fru-2,6-P₂ (Yan and Tao, 1984; Cheng and Tao, 1990; Wong et al., 1990; Theodorou et al., 1992; Podestá and Plaxton, 1994; Theodorou and Plaxton, 1994, 1995).

Studies involving PFP and Pi deprivation are relevant not only because Pi limitation is common to many natural environments, but also since Pi may be intimately involved in dictating the physiological function of PFP in plants. We have previously shown that Pi starvation of heterotrophic *Brassica nigra* (black mustard) suspension cell cultures results in a 19-fold elevation in the forward (glycolytic) activity of PFP, whereas the activity of PFK was unaffected (Duff et al., 1989b). This large induction of PFP activity appears to be based on de novo synthesis of the enzyme’s α subunit, leading to a significant enhancement in activation by Fru-2,6-P₂ (Theodorou et al., 1992). Similarly, the large reduction in PFP activity and sensitivity to Fru-2,6-P₂ that occurs within 24 h of Pi re-supply to Pi-starved *B. nigra* cells arises from inhibition of synthesis and/or enhanced degradation of the α subunit. By contrast, the β subunit of *B. nigra* PFP is constitutively expressed under all nutrient regimes (Theodorou et al., 1992). Pi starvation of *B. nigra* suspension cells is also accompanied by dramatic reductions in the intracellular concentrations of adenylates and Pi, whereas levels of Pi and Fru-2,6-P₂ are largely unaffected (Duff et al., 1989b). These findings led us to postulate that the PFP of Pi-deprived *B. nigra* plays a pivotal role in the catalysis of one step of a series of Pi starvation-inducible glycolytic “bypasses” (Duff et al., 1989a, 1989b; Theodorou and Plaxton, 1995). Subsequent whole-plant studies have demonstrated that Pi starvation-inducible PFP activity is not unique to *B. nigra* suspension cells but also occurs in leaves and roots of *B. nigra* seedlings (Theodorou and Plaxton, 1994), as well as in a diverse variety of crucifers that are phylogenetically related to *B. nigra* (V.R. Murray and W.C. Plaxton, unpublished data).

The aim of the present study was to characterize the physical, immunological, and kinetic properties of PFP isolated from Pi-starved *B. nigra* suspension cells. Our results are consistent with a glycolytic function for PFP in Pi-starved *B. nigra* and emphasize the importance of Pi as an effector of PFP activity and as a critical determinant of PFP’s role in vivo.

**MATERIALS AND METHODS**

Mineral nutrients, vitamins, and plant hormones for MS media and protein standards used for native PAGE were purchased from Sigma. Suc used for cell culture was of analytical grade and obtained from British Drug House (Toronto, Ontario, Canada), whereas Ribi adjuvant (product code R730) was from Ribi Immunochemical Research (Hamilton, MT). Homogeneous potato tuber PFP and rabbit anti-(native potato tuber PFP) immune serum were obtained as previously described (Moorhead and Plaxton, 1991). All other biochemicals and reagents were obtained as previously described (Theodorou et al., 1992; Theodorou and Plaxton, 1994).

Heterotrophic suspension cells of *Brassica nigra* (line L/T) were isolated as previously reported (Duff et al., 1989a). Cells (0.02–0.06 g/mL) were grown in MS medium (Murashige and Skoog, 1962) containing 1.25 mM Pi and 6% (w/v) Suc. Cultures were maintained on a rotational shaker (120 rpm) at 25°C, with subculturing every 7 d. Cells used in PFP purification were cultured for 7 d at 1.25 mM Pi and inoculated (approximately 15 g in 30 mL) into 470 mL of MS medium containing 6% (w/v) Suc and 0 mM Pi. After 7 d of subculture, the Pi-deficient cells were harvested as described previously (Duff et al., 1989a), quick-frozen in liquid N₂, and stored at −80°C.

**Enzyme Assay and Kinetic Studies**

All cuvettes and glassware used for enzyme assays and kinetic studies were treated overnight with 6 n HCl and rinsed with deionized water prior to use. Fru-6-P and Fru-1,6-P₂ were also acid-treated (titrated to pH 3.0 with HCl, incubated for 1 h, and then neutralized with NaOH) to hydrolyze any contaminating traces of Fru-2,6-P₂. PFP was assayed at 25°C in a 1-mL final volume by following the reduction of NAD⁺ or oxidation of NADH at 340 nm using a recording spectrophotometer (Gilford Instruments, Oberlin, OH). Forward PFP activity was routinely assayed in 50 mM Hepes-NaOH (pH 6.8) containing 5 mM Fru-6-P, 0.4 mM Pi, 5 μM Fru-2,6-P₂, 0.15 mM NADH, 5 mM MgCl₂, 1 unit of aldolase, 10 units of triose-P isomerase, and 1 unit of glyceraldehyde-3-P dehydrogenase. Reverse PFP activity was assayed in 50 mM Hepes-NaOH (pH 6.8) containing 0.5 mM Fru-1,6-P₂, 5 mM Pi, 5 μM Fru-2,6-P₂, 5 mM MgCl₂, 0.5 mM NAD⁺, 2 units of phosphoglucone isomerase, and 1 unit of Leuconostoc mesenteroides Glc-6-P dehydrogenase. Coupling enzymes were desalted before use. One unit of enzyme activity was defined as the amount of PFP resulting in the production of 1 μmol of product/min at 25°C. All assays were linear with respect to time and the concentration of enzyme assayed.

Apparent *Kₘ* values were calculated from the Michaelis-Menten equation, whereas *Sₐₐ* and *h* (Hill coefficient) values were calculated from the Hill equations. Both equations were fitted to a nonlinear least-squares regression computer kinetics program (Brooks, 1992). *Kₘ* and *Iₜₚ* values were determined using the previously mentioned kinetics software. All kinetic parameters are the means of at least two determinations.

**Buffers Used in PFP Purification**

All buffers were degassed, filtered through a 0.2-μm membrane, adjusted to their respective pH values at 25°C, and cooled to 4°C prior to use. Buffer A consisted of 50 mM Hepes-NaOH (pH 7.5) containing 1 mM EGTA, 1 mM EDTA, 2 mM MgCl₂, 150 mM KCl, 20% (v/v) glycerol, 2% (w/v) PEG, 0.05% (v/v) Nonidet-P-40, 1% (w/v) insoluble PVP, 1% (w/v) soluble PVP, 2 mM DTT, 0.5 mM Fru-6-P, 2 mM PMSF, 10 μg/mL chymostatin, and 10 μg/mL leupeptin. Buffer B consisted of 20 mM imidazole-HCl (pH 6.6) containing 1 mM EDTA, 2 mM DTT and 2 mM MgCl₂.
Buffer C consisted of 20 mM Tris-HCl (pH 8.2) containing 20 mM KCl, 2 mM DTT, 1 mM EDTA, and 2 mM MgCl₂. Buffer D consisted of 25 mM Heps-NaOH (pH 7.2) containing 1 mM EDTA, 0.2 mM EGTA, 1 mM DTT, 2 mM MgCl₂, and 30% (saturation) (NH₄)₂SO₄. Buffer E consisted of 25 mM Heps-NaOH (pH 7.2) containing 1 mM EDTA, 0.2 mM EGTA, 1 mM DTT, 2 mM MgCl₂, and 20% (v/v) ethylene glycol.

Enzyme Purification

All procedures were carried out at 4°C.

Crude Extract

Quick-frozen cells (300 g) were broken up into approximately 0.5-g pieces in liquid N₂ using a mortar and pestle and homogenized in buffer A (1.2, w/v) using a Polytron (Kinematica, Luzern, Switzerland) at maximum speed. The homogenate was centrifuged for 25 min at 14,300g. The supernatant was filtered through two layers of Miracloth (Calbiochem).

PEG Fractionation

PEG (average molecular mass 8 kD; 50% [w/v] dissolved in 50 mM Heps-NaOH [pH 7.5] containing 1 mM EDTA) was added to the clarified homogenate to a final concentration of 21% (w/v). The solution was stirred for 15 min and centrifuged as above. The pellets were immediately resuspended in 300 mL of buffer B to which chymostatin (25 μg/mL), leupeptin (25 μg/mL), and PMSF (2 mM) were added. The resuspension was clarified by centrifugation at 27,000g for 15 min.

Phosphocellulose Chromatography

The supernatant was adsorbed at 1 mL/min onto a column of phosphocellulose P11 (2.1 × 26 cm) that had been connected to an FPLC system (Pharmacia) and preequilibrated with buffer B. The column was washed with buffer B until the A₂₈₀ decreased to baseline and PFP eluted with a step of 0 to 40 mM Na₂PPi in buffer B (fraction size = 20 mL). Peak activity fractions (320 mL) were pooled and concentrated to 60 mL using an ultrafiltration system (Minitan, Millipore) equipped with six cellulose plates having a molecular mass cutoff of approximately 100 kD. Chymostatin (25 μg/mL), leupeptin (25 μg/mL), and PMSF (2 mM) were added to the concentrate, and this solution was clarified by centrifugation at 27,000g for 10 min. The supernatant was further concentrated to 30 mL with an ultrafilter (YM-30, Amicon, Beverly, MA).

Protein Pak Q-8HR Anion-Exchange FPLC

The concentrated phosphocellulose fractions were filtered through a 0.2-μm membrane and adsorbed at 1 mL/min onto an anion-exchange column (1.0 × 10 cm; Protein Pak Q-8HR, Waters) that had been connected to an FPLC system and preequilibrated with buffer C. The column was washed with buffer C until the A₂₈₀ decreased to baseline and the enzyme eluted with 100 mL of a linear 20 to 400 mM KCl gradient in buffer C (fraction size = 3 mL). PFP activity began to elute in a single peak at about 180 mM KCl. Peak activity fractions (21 mL) were pooled and concentrated to 0.9 mL using an ultrafilter (YM-30, Amicon).

Phenyl Superose FPLC

The concentrated anion-exchange fractions were adjusted to 30% (saturation) (NH₄)₂SO₄ by the addition of solid (NH₄)₂SO₄. The solution was stirred for 20 min, centrifuged at 27,000g for 10 min, filtered through a 0.2-μm membrane, and adsorbed at 0.25 mL/min onto a phenyl superose HR 5/5 column (Pharmacia) preequilibrated with buffer D. The enzyme was eluted in a stepwise fashion with decreasing concentrations of buffer D and simultaneously increasing concentrations of buffer E (fraction size = 2 mL). PFP eluted following the step from 80 to 100% buffer E (20 to 0% buffer D). The pooled peak activity fractions (15 mL) were concentrated to 0.9 mL using an (Amicon) ultrafilter. The concentrated enzyme was adjusted to 50% (v/v) glycerol and 25 μg/mL each of chymostatin and leupeptin, frozen in liquid N₂, and stored at −80°C. After 8 months of storage, the enzyme retained 65% of its original activity.

Electrophoresis and Immunoblotting

SDS-PAGE, subunit M₁ estimations, and immunoblotting were performed using a mini-gel apparatus (Bio-Rad) as described previously (Theodorou and Plaxton, 1994). Antigenic polypeptides were detected using an alkaline-phosphatase-conjugated secondary antibody (Theodorou and Plaxton, 1994). Immunological specificities were confirmed by immunoblots in which rabbit preimmune serum was substituted for various immune sera. Silver staining of SDS gels was performed according to the method of Hochstrasser and Merrill (1988). Nondenaturing PAGE was performed with a mini-gel apparatus (Bio-Rad) using the following modifications of the Laemmli (1970) SDS-PAGE system: (a) SDS was omitted from gels and running buffer, and (b) 10% (v/v) ethylene glycol and 20% (v/v) glycerol were added to the stacking and separating gels. The final acrylamide monomer concentration in the 0.75-mm-thick slab gels was 6% (w/v) for the separating gel and 3% (w/v) for the stacking gel. Samples were diluted 1:1 in a sample buffer (50 mM Heps-NaOH [pH 7.5] containing 20% [v/v] glycerol and 0.005% [w/v] bromphenol blue), and gels were run at a constant 150 V for 3 h. Gels and buffers were cooled to 4°C prior to use and were maintained at this temperature during electrophoresis. Gels were either stained for protein using Coomassie blue R-250 or incubated in a PFP activity stain. To detect PFP activity, a lane was incubated for 1 h at 30°C in 200 mM Tris-HCl (pH 7.5) containing 5 mM MgCl₂, 5 mM Fru-6-P, 2 mM Pi, 2 μM Fru-2,6-P₂, 1 mM NADP, 1 unit/mL Glc-6-P dehydrogenase, 1 unit/mL phosphoglucone isomerase, 0.1 mg/mL nitroblue tetrazolium, and 5 μg/mL phenazine methosulfate. PFP activity gels were fixed in 5% (v/v) acetic acid. For an estimation of native molecular mass via nondenaturing PAGE a plot of relative mobility versus log molec-
ular mass was constructed using the following protein standards: urease (545,272 kD), thyroglobulin (669 kD), ferritin (440 kD), catalase (232 kD), and β-amylase (200 kD). For second-dimension PAGE, Coomassie blue-stained PFP was excised from a native gel and incubated in 150 μL of 50 mM Tris-HCl (pH 6.8) containing 5% (v/v) methanol, 20% (v/v) glycerol, and 2% (w/v) SDS for 3 h at 30°C. The gel slice was subjected to SDS-PAGE, and denatured PFP polypeptides were detected by immunoblotting as described above.

Subunit-Specific Antibody Production

The α and β subunits of homogeneous potato tuber PFP (1.0 mg) were separated by SDS-PAGE as described above, except that the gels were 1.5-mm thick. Gels were Coomassie blue stained, and the separated α and β subunits of potato PFP (Moorhead and Plaxton, 1991) were excised and electroeluted overnight at a 9-mA constant current using an electroeluter (Bio-Rad). The electrode buffer was the same as that used for SDS-PAGE. The electroeluted proteins were dialyzed exhaustively against PBS (40 mM NaH₂PO₄, pH 7.4, containing 150 mM NaCl) at room temperature, filtered through a 0.2-μm membrane, and emulsified in Ribi adjuvant (0.7 mL total volume). After the collection of preimmune serum, 0.25 mg of each subunit was separately injected (0.4 mL intramuscularly, 0.3 mL subcutaneously) into two 2-kg New Zealand rabbits. Booster injections (0.15 mg each) of each subunit emulsified in Ribi adjuvant were given at 4 and 5 weeks. One week after the final injection, blood was collected by cardiac puncture. After incubation overnight at 4°C, the clotted blood cells were removed by centrifugation at 1500g for 10 min. The crude immune sera were adjusted to 0.04% (w/v) NaN₃, frozen in liquid N₂, and stored at -80°C.

Other Methods

The native molecular mass of PFP was also estimated by gel-filtration FPLC on a calibrated Superose-6 Prep Grade column (Pharmacia LKB Biotechnology, Montreal, Quebec, Canada) as previously described (Moorhead and Plaxton, 1991). Protein concentrations were determined by the Coo-FPLC gel-filtration method of Bollag and Edelstein (1991) using bovine γ-globulin as the standard.

RESULTS

Purification, Physical, and Immunological Properties

Maximal activity of PFP (forward reaction) in the Pi-starved suspension cells was about 2.25 units g⁻¹ fresh weight. As shown in Table I, PFP was purified 184-fold to a final specific activity of 26 units mg⁻¹ and an overall recovery of 4%. Denaturation followed by SDS-PAGE of the final preparation resolved two major silver-staining polypeptides of 66 and 60 kD, which co-migrated with the α and β subunits of pure potato tuber PFP, and three less prominent polypeptides of 62, 59, and 57 kD (Fig. 1A). An immunoblot of the final preparation was probed with anti-native potato tuber PFP immune serum and revealed two equal intensity staining antigenic polypeptides of 66 and 60 kD that co-migrated with the α and β subunits of homogeneous potato tuber PFP (Fig. 1B, lanes 1 and 2). Antibodies against the separated α and β subunits of potato tuber PFP cross-reacted specifically with the corresponding subunits of both B. nigra and potato tuber PFP (Fig. 1, C and D). Nondenaturing PAGE of the final preparation resolved a single protein staining band of about 520 kD that co-migrated with PFP activity (Fig. 2A, lanes 1 and 2). When the 520-kD protein staining band present following nondenaturing PAGE was excised, equilibrated with SDS, and subjected to SDS-PAGE and immunoblotting, antigenic polypeptides of 66- and 60-kD polypeptides were resolved and stained with similar intensities (Fig. 2B).

Immunoblotting revealed that an approximately 3-kD polypeptide was cleaved from PFP's α subunit during the enzyme's purification in the absence of added chymotatin and leupeptin (Fig. 1B, lane 3). The inclusion of 1 to 20 mM Pi in the crude extract did not hasten proteolysis (results not shown); however, the repeated inclusion of 25 µg/mL chymotatin and 25 µg/mL leupeptin at various stages of the purification prevented partial degradation of the enzyme's α subunit (Fig. 1B, lane 1).

Estimation of Native Molecular Mass by Gel Filtration

The native molecular mass of nondegraded B. nigra PFP, as estimated by Superose-6 gel-filtration FPLC, was 460 kD.

Kinetic Properties

The final preparation of nondegraded PFP was used for all kinetic studies and was free of contaminating PFK and Fru-1,6-P₂ 1-phosphohydrolase activities.

Table I. Purification of PFP from 300 g of Pi-starved B. nigra suspension cells

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (mL)</th>
<th>Activity (units)</th>
<th>Protein (mg)</th>
<th>Specific Activity (units mg⁻¹)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>6.20</td>
<td>718</td>
<td>5084</td>
<td>0.14</td>
<td>0</td>
<td>-1</td>
</tr>
<tr>
<td>PEG fractionation</td>
<td>500</td>
<td>564</td>
<td>1440</td>
<td>0.39</td>
<td>3</td>
<td>78</td>
</tr>
<tr>
<td>Phosphocellulose</td>
<td>60</td>
<td>180</td>
<td>102</td>
<td>1.76</td>
<td>12</td>
<td>25</td>
</tr>
<tr>
<td>Protein Pak Q-8HRb</td>
<td>0.9</td>
<td>75</td>
<td>6</td>
<td>11.9</td>
<td>84</td>
<td>10</td>
</tr>
<tr>
<td>Phenyl Superoseb</td>
<td>0.9</td>
<td>26</td>
<td>1</td>
<td>26</td>
<td>184</td>
<td>4</td>
</tr>
</tbody>
</table>

* PFP activity was measured in the forward direction at pH 6.8.  
  ** Concentrated pooled fractions.
In the presence of 5 μM Fru-2,6-P₂, B. nigra PFP displayed a broad pH activity profile for the forward reaction, with maximum activity occurring at pH 6.8 (Fig. 3A). In the absence of Fru-2,6-P₂, a narrower pH activity profile was observed, with maximum activity at pH 7.6. Below pH 6.8, negligible PFP activity was detected in the absence of Fru-2,6-P₂ (Fig. 3A). In the reverse reaction, maximal activity occurred at pH 7.6 in both the presence and absence of Fru-2,6-P₂ (Fig. 3B). However, the pH activity profile for the reverse reaction was very broad, with 96% of maximum activity observed at pH 6.8. Also, as in the forward reaction, the reverse PFP activity displayed greater sensitivity to Fru-2,6-P₂ at pH 6.8 than it did at pH 7.6. The V_max in the reverse direction was equivalent to that of the forward direction in the presence of 5 μM Fru-2,6-P₂ at pH 6.8. All subsequent kinetic studies of both the forward and reverse PFP activities were carried out at pH 6.8.

Substrate Saturation Kinetics and Effect of Fru-2,6-P₂

V_max, apparent Kₘ or S₀.₅, and Hill coefficient (h) values obtained for PFP's substrates in the presence and absence of 5 μM Fru-2,6-P₂ are summarized in Table II. PFP displayed hyperbolic kinetics with respect to all of its substrates with the exception of PPI, which displayed a sigmoidal saturation curve in the absence of Fru-2,6-P₂ (Table II). The enzyme's affinity for Fru-6-P was increased 60-fold by the addition of 5 μM Fru-2,6-P₂ (Table II). Fru-2,6-P₂ also increased PFP's affinities for PPI and Pi and the enzyme was a potent inhibitor of the forward activity and a less effective inhibitor of the reverse reaction (Fig. 4; Table III). Pi inhibition of the forward reaction is mainly attributed to a dramatic decline in PFP's affinity for Fru-2,6-P₂ in the presence of Pi. At saturating levels of the forward reaction substrates, the enzyme's Kₘ(Fru-2,6-P₂) was increased more than 50-fold by the addition of 5 mM Pi (Table III). In physiological concentrations of Fru-6-P and PPI, the addition of 0.2 and 5 mM Pi resulted in approximately 130- and 320-fold increases in Kₘ(Fru-2,6-P₂), respectively (Table III). In the reverse direction, Pi binding shifted from normal Michaelis-Menten kinetics at concentrations higher than 1 mM, owing to substrate inhibition occurring above this value (Fig. 4B). The Iₐ₀ values for Pi however, were 1 order of magnitude higher for the reverse reaction than the corresponding values obtained for the forward reaction (Table III). PPI was a far more effective inhibitor of the reverse reaction, relative to Pi (Table III).

Effects of Other Metabolites

A variety of compounds were tested as possible effectors of B. nigra PFP forward activity in the presence of 5 μM Fru-2,6-P₂. As shown in Table II, the addition of 5 μM Fru-2,6-P₂ stimulated PFP activity 15 nM at saturating concentrations for Fru-6-P and PPI, but this value was increased by about 3-fold under subsaturating conditions (Table III).

Inhibition by Pi and PPI

Pi was a potent inhibitor of the forward activity and a less effective inhibitor of the reverse reaction (Fig. 4; Table III). Pi inhibition of the forward reaction is mainly attributed to a dramatic decline in PFP's affinity for Fru-2,6-P₂ in the presence of Pi. At saturating levels of the forward reaction substrates, the enzyme's Kₘ(Fru-2,6-P₂) was increased more than 50-fold by the addition of 5 mM Pi (Table III). In physiological concentrations of Fru-6-P and PPI, the addition of 0.2 and 5 mM Pi resulted in approximately 130- and 320-fold increases in Kₘ(Fru-2,6-P₂), respectively (Table III). In the reverse direction, Pi binding shifted from normal Michaelis-Menten kinetics at concentrations higher than 1 mM, owing to substrate inhibition occurring above this value (Fig. 4B). The Iₐ₀ values for Pi however, were 1 order of magnitude higher for the reverse reaction than the corresponding values obtained for the forward reaction (Table III). PPI was a far more effective inhibitor of the reverse reaction, relative to Pi (Table III).
Fru-2,6-P₂ and saturating substrate concentrations. The following substances, tested at a concentration of up to 20 mM, had no effect (±20% control velocity) on enzyme activity: MgAMP, Glc-6-P, 2-phosphoglycerate, 3-phosphoglycerate, and citrate. MgATP, MgADP, and PEP were inhibitors of the forward and reverse activities of PFP. Iₜ₀ values for these compounds were uniformly in excess of 2 mM (Table III). The most effective inhibitors (other than Pi and PPI) were MgADP and PEP; however, no activators other than Fru-2,6-P₂ were found.

Table II. Effect of Fru-2,6-P₂ on Vₘₐₓ, and Kₘ, or S₀₅ values for substrates of B. nigra PFP

<table>
<thead>
<tr>
<th>Parameter</th>
<th>- Fru-2,6-P₂</th>
<th>+5 mM Fru-2,6-P₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward reaction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vₘₐₓ (units mg⁻¹ protein)</td>
<td>2.6</td>
<td>26</td>
</tr>
<tr>
<td>Kₘ (Fru-6-P) (mM)</td>
<td>3.00</td>
<td>0.05</td>
</tr>
<tr>
<td>Kₘ (PPI) (μM)</td>
<td>50 (4.0)</td>
<td>15</td>
</tr>
<tr>
<td>Reverse reaction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vₘₐₓ (units mg⁻¹ protein)</td>
<td>8</td>
<td>24</td>
</tr>
<tr>
<td>Kₘ (Fru-1,6-P₂) (μM)</td>
<td>47</td>
<td>9</td>
</tr>
<tr>
<td>Kₘ (Pi) (mM)</td>
<td>0.25</td>
<td>0.25</td>
</tr>
</tbody>
</table>

* This value represents an S₀₅(PPI) as h > 1.0.

Figure 3. Activity of B. nigra PFP as a function of pH. PFP was assayed in the forward (A) and reverse (B) directions in the presence (●) and absence (O) of 5 μM Fru-2,6-P₂. The following buffers (50 mM each) were used: Mes-NaOH from pH 5.8 to 6.8, Hepes-NaOH from pH 6.8 to 8.0, and Tris-HCl from pH 8.0 to 9.0.

DISCUSSION

Purification of PFP from Pi-Starved B. nigra

B. nigra PFP was purified 184-fold to a final specific activity of 26 units mg⁻¹ protein (Table I), a value similar to that reported for homogeneous PFPs from various plant sources (Botha et al., 1987; Kruger and Dennis, 1987; Mahajan and Singh, 1989; Moorhead and Plaxton, 1991; Enomoto et al., 1992; Nielsen, 1994). During the initial trials of enzyme purification, the α subunit of PFP was found to be susceptible to partial proteolysis (Fig. 1B, lane 3). Proteolysis was prevented by the repeated inclusion of chymostatin and leupeptin at various stages of the purification, suggesting the presence and co-purification of a protease specific to the PFP α subunit. Very similar partial proteolysis of the α subunit also has been reported to occur during storage at -20°C, but not -80°C, of potato tuber PFP that had been purified to apparent homogeneity (Podestá et al., 1994). The rapid degradation in vivo of the B. nigra PFP α subunit within 24 h of Pi refeeding of Pi-starved B. nigra suspension cells suggests that a tightly regulated system for turnover of the α subunit must be in place within the Pi-starved cells (Theodorou et al., 1992). Extraction of the...
Table III. Kinetic constants for several effectors of B. nigra PFP

<table>
<thead>
<tr>
<th>Effector</th>
<th>$K_a$ (mM)</th>
<th>$I_{50}$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fru-2,6-P$_2$</td>
<td>15</td>
<td>-</td>
</tr>
<tr>
<td>MgADP</td>
<td>-</td>
<td>1.0</td>
</tr>
<tr>
<td>MgATP</td>
<td>-</td>
<td>19</td>
</tr>
<tr>
<td>PEP</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>Reverse reaction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fru-2,6-P$_2$</td>
<td>49</td>
<td>-</td>
</tr>
<tr>
<td>PPI</td>
<td>-</td>
<td>0.9</td>
</tr>
<tr>
<td>Pi</td>
<td>-</td>
<td>13</td>
</tr>
<tr>
<td>MgADP</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>MgATP</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>PEP</td>
<td>-</td>
<td>11</td>
</tr>
</tbody>
</table>

a. No data. b. Assayed with 0.1 mM Fru-6-P and 0.05 mM PPI. c. Assayed with 5 mM Fru-6-P and 0.4 mM PPI in the presence of 5 mM Pi. d. Assayed with 1 mM Fru-6-P and 0.2 mM PPI in the presence of 0.2 mM Pi. e. Assayed with 1 mM Fru-6-P and 0.2 mM PPI in the presence of 5 mM Pi.

Pi-starved cells may disrupt regulation of this system and result in partial degradation of the $\alpha$ subunit.

Physical and Immunological Properties

SDS-PAGE of the final preparation revealed two major protein staining bands of 66 and 60 kD and three less prominent bands of 62, 59, and 57 kD (Fig. 1A). Immuno-blots probed with antibodies raised against native potato tuber PFP revealed two equal-intensity-staining immuno-reactive polypeptides of 66 and 60 kD (Fig. 1B), suggesting that the 62-, 59-, and 57-kD polypeptides were contaminants of the purified PFP preparation. Since the 66-kD polypeptide silver stained about twice as intensely as the 60-kD polypeptide (Fig. 1A, lane 1), the 66-kD protein staining band seems to be a mixture of PFP’s $\alpha$ subunit and a contaminating polypeptide having a similar molecular mass. Nondenaturing PAGE of the final preparation revealed a well-resolved major protein-staining band of approximately 520 kD that co-migrated with PFP activity (Fig. 2A). This native molecular mass is similar to the value of 560 kD determined by gel-filtration FPLC of the final preparation on a calibrated Superose-6 column. The single band from the native gel still produced anti- (native potato PFP) immune serum antigenic 66- and 60-kD polypeptides upon SDS-PAGE (Fig. 2B). Therefore, similar to potato tuber PFP (Podestá et al., 1994), the enzyme from Pi-starved B. nigra suspension cells appears to exist as an $\alpha_4\beta_4$ hetero-octamer. The results also demonstrate that, as is the case for the potato tuber enzyme (Kruger and Dennis, 1987; Carlisle et al., 1990), the $\alpha$ and $\beta$ subunits of the B. nigra PFP are unlikely to share extensive amino acid homology since they are immunologically distinct (Fig. 2, C and D).

Kinetic and Regulatory Properties

A unique characteristic of Pi-starved B. nigra PFP was the effect of Fru-2,6-P$_2$ on the pH optimum of the forward reaction. Fru-2,6-P$_2$ shifted the enzyme’s pH optimum for forward activity from 7.6 to 6.8 (Fig. 3A). Fru-2,6-P$_2$, however, generally does not affect the response of other PFPs to pH (Kombrink et al., 1984; Botha et al., 1986, 1987; Stitt, 1990). Also, pH 6.8 is a somewhat more acidic pH optimum than that reported for other PFPs, which typically display pH optima from pH 7.2 to 7.8 in the forward direction (Kombrink et al., 1984; Botha et al., 1986, 1987; Botha and Small, 1987; Mahajan and Singh, 1989; Wong et al., 1990; Enomoto et al., 1992; Nielsens, 1994). By contrast, Fru-2,6-P$_2$ exerted a less prominent effect on the pH optimum of the reverse activity (Fig. 3B). This result suggests a glycolytic role for B. nigra PFP during anoxia. Anaerobiosis generally results in a 0.4- to 0.8-unit decrease in the cytosolic pH of plant cells (Dancer and ap Rees, 1989). It is interesting that the only report of a similar effect of Fru-2,6-P$_2$ on PFP’s pH optimum was for the purified enzyme from rice seedling (Enomoto et al., 1992). Fru-2,6-P$_2$ was found to shift the pH optimum of rice seedling PFP toward a more acidic value for both the forward (decreased from pH 8.1 to 7.5) and reverse reactions (decreased from pH 7.9 to 7.4). It has been suggested that rice seedling PFP may function during anaerobiosis as a glycolytic bypass to PFK (Mertens et al., 1990; Mertens, 1991; Enomoto et al., 1992).

The forward activity of the purified B. nigra PFP was stimulated 10-fold by Fru-2,6-P$_2$ (Fig. 3; Table II). However, Fru-2,6-P$_2$ had a less pronounced effect on the $V_{max}$ of the reverse reaction (Fig. 3; Table II). This has been attributed to the partial activation of the reverse reaction by Fru-1,6-P$_2$, as described by others (Kombrink et al., 1984; Botha et al., 1986; Stitt, 1990; Nielsen, 1994, 1995). Fru-2,6-P$_2$ also caused a large increase in the enzyme’s affinity for Fru-6-P and to a lesser extent increased the affinities for both PPI and Fru-1,6-P$_2$ but had no effect on $K_a$(Pi) (Table II). These results are similar to those reported for other plant PFPs (Van Schaftingen et al., 1982; Kombrink et al., 1984; Botha et al., 1986, 1987; Mahajan and Singh, 1989; Wong et al., 1990; Nielsen, 1994). By contrast, the forward activity of the apparent $\beta$ subunit form of PFP from Pi-sufficient B. nigra suspension cells is relatively unresponsive to Fru-6-P$_2$ and to a lesser extent increased the affinities for both PPI and Fru-1,6-P$_2$ but had no effect on $K_a$(Pi) (Table II). These results are similar to those reported for other plant PFPs (Van Schaftingen et al., 1982; Kombrink et al., 1984; Botha et al., 1986, 1987; Mahajan and Singh, 1989; Wong et al., 1990; Nielsen, 1994).
order of magnitude beyond their probable in vivo concentrations, they probably are not important effectors of the enzyme in vivo.

**Regulation by Pi: Physiological Relevance**

Similar to other plant PFPs (Kombrink et al., 1984; Mahajan and Singh, 1989; Stitt, 1989, 1990; Wong et al., 1990), the forward activity of the Pi-starved *B. nigra* PFP demonstrated potent product inhibition by Pi (Table III; Fig. 4A). The reverse activity was far less susceptible to substrate inhibition by Pi (Table III; Fig. 4B). The intracellular Pi concentration of Pi-fed and Pi-starved *B. nigra* suspension cells has been estimated to be 17.4 and 0.4 mM, respectively (Duff et al., 1989b). Based on these estimates and the observed sensitivity of *B. nigra* PFP to Pi inhibition, one would predict that the forward activity of PFP would be almost completely inhibited in Pi-fed cells and that this inhibition would be almost completely relieved in Pi-starved cells (Fig. 4A). Also, under catalytic conditions mimicking the predicted Fru-6-P, PPI, and Pi levels in the cytosol of Pi-fed *B. nigra* suspension cells, the $K_p$ of PFP for Fru-2,6-P$_2$ was 2 μM (Table III), a value that is 4-fold greater than the estimated cytosolic level of Fru-2,6-P$_2$ in Pi-fed *B. nigra* cells (Duff et al., 1989b). This suggests that in vivo PFP activity would be largely unresponsive to activation by Fru-2,6-P$_2$ in Pi-sufficient *B. nigra*. The profound decrease in PFP's affinity for Fru-2,6-P$_2$ with increasing Pi is consistent with findings obtained with PFPs isolated from germinating castor and bean seeds (Kombrink and Kruger, 1984; Botha et al., 1987), potato tubers (Stitt, 1990), wheat endosperm (Mahajan and Singh, 1989), and spinach leaves (M.E. Theodorou and N.J. Kruger, unpublished data). Another important consideration is the PPI/Pi concentration ratio. This ratio increases approximately 43-fold following Pi starvation of *B. nigra* suspension cells (Duff et al., 1989b) and should promote the glycolytic activity of PFP during Pi stress.

Many of the previously mentioned anticipated effects of Pi on *B. nigra* PFP rely on the assumption that the same αβ$_4$ oligomeric form is expressed in Pi-starved and Pi-fed *B. nigra* suspension cells. Immunoblotting studies, however, have previously indicated that the *B. nigra* PFP from Pi-fed cells is made up solely of the β subunit, whereas a 1:1 ratio of αβ subunits is only achieved after 18 d of Pi starvation (Theodorou et al., 1992). Thus, Pi inhibition of the heteromeric PFP present in Pi-starved *B. nigra* cells may be relevant only during transitions from Pi sufficiency to Pi deficiency and vice versa. Following subculture of Pi-sufficient cells into Pi-free media, as cytosolic Pi pools become depleted, one would predict steadily increasing relief of Pi inhibition of the accumulating α and β subunit containing (i.e. Pi-starved) forms of PFP. Intracellular Pi and adenylate pools markedly increase within minutes of subculture of Pi-starved plant suspension cells into Pi-sufficient media (Theodorou and Plaxton, 1995). Thus, immediately following Pi resupply to Pi-starved *B. nigra* cells, the rapid increase in cytosolic Pi levels should potently inhibit the glycolytic activity of the heteromeric PFP present in the Pi-starved cells, while promoting glycolytic flux through the Pi-activated and ATP-dependent PFK. Further studies are required to evaluate the effect of Pi on the kinetic properties of the apparent β subunit form of PFP expressed in Pi-fed *B. nigra* suspension cells. However, inhibition by Pi may not play as significant role in regulating the forward activity of the Pi-fed PFP, since the $V_{max}$ of this enzyme in the glycolytic direction is already extremely low (Theodorou et al., 1992).

**CONCLUSIONS**

That PFP plays an important role in the metabolism of Pi-starved *B. nigra* suspension cells was previously indicated by the large (20-fold) induction of its activity that occurs during the transition from Pi sufficiency to Pi deficiency and the reversal of this process within 24 h of Pi resupply to Pi-starved cells (Duff et al., 1989b; Theodorou et al., 1992). The kinetic properties of purified *B. nigra* PFP are consistent with a glycolytic function for this enzyme in the Pi-starved cells. Although PFP from Pi-deficient *B. nigra* suspension cells is capable of catalyzing the forward and reverse reactions at almost equal maximal rates under optimal catalytic conditions in vitro, one would predict a net glycolytic flux in the Pi-starved cells in vivo based primarily on the differential regulation of the forward and reverse activities by Pi. Future studies are required to evaluate the physical and kinetic properties of the apparent β subunit form of PFP purified from Pi-sufficient *B. nigra* suspension cells and to relate these properties to the findings of the present study. However, it is tempting to speculate on a possible gluconeogenic function for the Pi-fed PFP, since its $V_{max}$ in the reverse direction is at least 1 order of magnitude greater than its $V_{max}$ in the forward direction (Theodorou et al., 1992).

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**LITERATURE CITED**


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