Phosphate Starvation-inducible Synthesis of the α-Subunit of the Pyrophosphate-dependent Phosphofructokinase in Black Mustard Suspension Cells*

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PPi-dependent phosphofructokinase (PFP) activity, measured in the forward direction, increased approximately 19-fold when suspension cell cultures of black mustard (Brassica nigra) were subjected to 18 days of P, deprivation. Fructose 2,6-bisphosphate (2 μM) elicited a 10-fold activation of PFP from P,-deficient cells, compared to only a 2-fold activation of the enzyme from nutrient-sufficient cells. Also, PFP from P,-starved cells exhibited a greater affinity for the activator (K, = 0.09 μM) than the enzyme from nutrient-sufficient cells (K, = 0.32 μM). Western blots of extracts from P,-deficient cells were probed with rabbit anti-(potato tuber PFP) immune serum and revealed equal intensity staining immunoreactive polypeptides of M, 66,000 (α-subunit) and 60,000 (β-subunit) that co-migrated with the α- and β-subunits of homogeneous potato tuber PFP. By contrast, only the M, 60,000 β-subunit was observed on immunoblots of extracts prepared from nutrient-sufficient cells. Quantification of immunoblots indicated that in black mustard cells experiencing transition from P, deficiency to sufficiency or vice versa, the relative amount of immunoreactive α-subunit correlated with the degree of activation of PFP by fructose 2,6-bisphosphate. These observations provide additional evidence that (i) plant PFP is an adaptive enzyme that may function in glycolysis during P, deprivation, and (ii) the α-subunit acts as a regulatory protein in controlling the catalytic activity of the β-subunit and its regulation by fructose 2,6-bisphosphate.

In animals, the direction of carbon flow through Fru-6-P and Fru-1,6-P2 is coordinated by the opposing activities of ATP: d-fructose-6-phosphate 1-phosphotransferase (EC 2.7.1.11) and d-fructose-1,6-bisphosphate 1-phosphohydrolase (EC 3.1.3.11). These enzymes catalyze thermodynamically irreversible reactions in vivo and are allosterically regulated by Fru-2,6-P2, which activates ATP: d-fructose-6-phosphate 1-phosphotransferase and inhibits d-fructose-1,6-

bisphosphate 1-phosphohydrolase (1–3). One of the most significant deviations from animal glycolysis that occurs in higher plants and some microorganisms is the existence of a cytosolic PFP (PPi: d-fructose-6-phosphate 1-phosphotransferase) that catalyzes the reversible conversion of Fru-6-P and PPi to Fru-1,6-P2 and P, while plant ATP: d-fructose-6-phosphate 1-phosphotransferase is insensitive to Fru-2,6-P2. Most plant PFPs display potent activation by nanomolar concentrations of this regulatory metabolite (4).

The PFP of many plants consists of two pairs of different subunits of approximately M, 66,000 (α-subunit) and 60,000 (β-subunit) (4–7). Several recent reports, however, have clearly demonstrated that a variety of molecular forms of PFP can exist within and between different plant tissues (8–10). Although there is an approximate 60% homology between the deduced amino acid sequence of the α- and β-subunits of PFP from potato tubers and castor oil seeds (11), the two subunits are immunologically distinct (6, 7). The functions of the α- and β-subunits of PFP have not been fully resolved. However, various evidence indicates that the β-subunit contains the catalytic site, while the α-subunit is involved in the regulation of catalytic activity by Fru-2,6-P2 (7, 9, 11, 12).

Although the molecular and kinetic properties of plant PFP have been studied in some detail (4–15), the physiological role of the enzyme is still in question. It has been proposed that PFP functions as a glycogenolytic enzyme (16–19), a gluconeogenic enzyme (9, 15, 17, 18), or as a source of PPi for the sucrose synthase pathway of sucrose degradation (19–21). Others have provided evidence that PFP catalyzes a major equilibrium reaction in vivo (22–24) and therefore could operate to equilibrate hexose and triose phosphate pools by rapid substrate cycling (25) and/or to maintain appreciable and precisely regulated concentrations of PPi in the plant cytosol (20, 26–28).

We have previously shown that P, starvation of heterotrophic suspension cells of Brassica nigra (black mustard) results in a large elevation in the activity of PFP, as well as dramatic reductions in the intracellular concentrations of adenylates and P, (29). By contrast, P, levels remain selectively high in P,-deficient cells of B. nigra (29). These findings led us to postulate that the PFP of P,-deprived cells of B. nigra plays a pivotal role in the catalysis of one step of a series of P, starvation-inducible glycolytic "bypasses." It was suggested (29) that these alternative glycolytic reactions circumvent the adenylate- and P,-dependent reactions of glycolysis, thus allowing glycolysis to proceed in P,-deprived cells. Two of these bypass reactions, PFP and phosphoenolpyruvate phosphatase, may also fulfill an additional role as a P,-recycling system that converts esterified phosphate to P, that would be rapidly reassimilated by the P,-deficient cells. Potent feedback inhibition of both PFP (forward direction) and

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1 The abbreviations used are: Fru-6-P, fructose 6-phosphate; PFP, PPi: d-fructose-6-phosphate 1-phosphotransferase (EC 2.7.1.90); Fru-1,6-P2, fructose 1,6-bisphosphate; Fru-2,6-P2, fructose 2,6-bisphosphate.
phosphate 1-phosphohydrolase activity was recorded as containing 10 mM Pi, with subsequent culturing as described above.

- Fructose-1,6-bisphosphate saturation kinetics for PFP from P₃-starved and nutrient-sufficient B. nigra. All assays were performed with desalted extracts prepared from suspension cells cultured for 18 days in media containing no P₃ (-P₃) or 10 mM P₃ (+P₃). All data points represent means ± S.E. of three separate experiments.

- Immuonprecipitation of PFPs by rabbit anti-(potato tuber PFP) immune serum. Immunoremovelation was performed on homogenous potato tuber PFP (31) and the PFP in extracts of P₃-deficient suspension cells of B. nigra.

- mM, pH 7.5) was then added, and the resultant activity was recorded as D. Fru-2,6-P₂-stimulated reverse PFP activity was determined as (D - C).

- Coupling enzymes were desalted prior to use. All assays were conducted in duplicate; optimized with respect to pH, substrate, and cofactor concentrations; and corrected for any contaminating NADH oxidase or NAD₃ reduction activity. Activity in all assays was proportional to the amount of extract added and remained linear with respect to time. One unit of enzyme activity is defined as the amount of enzyme required to catalyze the formation of 1 µmol of product min⁻¹. Activation constants (Kₐ values) for Fru-2,6-P₂ were calculated by the Michaelis-Menten equation fitted to a nonlinear least squares regression computer program.

- Electrophoresis, Western Blotting, and Immunounquantification—SDS-polyacrylamide gel electrophoresis was performed as previously reported (33) using a Bio-Rad minigel apparatus and the discontinuous system of Doucet and Trifaro (34). The final acrylamide monomer concentration in the separating gel was 10% (w/v). Electrophoretic coomassie blue tetrazolium, and polyvinylpolypyrrolidone were purchased from Sigma. All other reagents were of analytical grade and were obtained from British Drug House. Homogenous potato tuber PFP and rabbit anti-(potato tuber PFP) immune serum were obtained as described previously (31).

- Enzyme Extraction—All procedures were carried out at 4 °C. Frozen cells (0.5-1.5 g) were ground (1:1, w/v) for 5 min using a pestle and a mortar containing a small scoop of acid-washed sand. The homogenate was centrifuged for 15 min at 16,000 g using an Eppendorf microcentrifuge. With the exception of pH 7.5 instead of 6.9, the extraction buffer was as previously described (29). For example, aliquots of clarified homogenates were desalted at 0.5 ml min⁻¹ on a column (1.0 cm X 14 cm) of Sephadex G-25 that had been pre-equilibrated in extraction buffer minus polyvinylpolypyrrolidone.

- Enzyme Assays and Kinetic Studies—All cuvettes and glassware were used for enzyme assays were treated overnight with 6 N HCl and rinsed with deionized water prior to use. Frui-6-P and Frui-1,6-P₂ were also acid treated (titrated to pH 3 with HCl, incubated for 15 min, and then neutralized with NaOH) to hydrolyze any contaminating traces of Frui-2,6-P₂. Enzymes were assayed at 25 °C in a 1.0 ml final volume by following the reduction of NADP⁺ or oxidation of NADH at 340 nm using a Gilford recording spectrophotometer. PFP was assayed in the forward direction in 50 mM Tris-HCl (pH 7.5) containing 5 mM Frui-6-P, 0.4 mM Pi, 0.15 mM NADH, 5 mM MgCl₂, 1 unit of aldolase, 10 units of triose-phosphate isomerase, and 1 unit of glyceraldehyde phosphate dehydrogenase. Assays were initiated by the addition of Frui-6-P and NADH, and PFP activity was monitored for approximately 3 min (Frui-2,6-P₂) (2 µM unless otherwise indicated) was then added, and the Frui-2,6-P₂-stimulated PFP activity was recorded.

- d-Fructose-1,6-bisphosphate 1-phosphohydrolase and reverse PFP activities were assayed consecutively in the same cuvette using the following initial reaction mixture: 50 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 0.5 mM Frui-1,6-P₂, 1 mM NAD⁺, 2 units of phosphoglucoisomerase, and 1 unit of glucose-6-phosphate dehydrogenase. Assays were initiated by the addition of desalted extract, and d-fructose-1,6-bisphosphate 1-phosphohydrolase activity was recorded as A. NaPi (5 mM, pH 7.5) was then added, and the resultant activity was recorded as B. Reverse PFP activity was calculated as (B - A).

- In the preparation of the d-fructose-1,6-bisphosphate 1-phosphohydrolase activity was recorded as C. NaPi (5 mM, pH 7.5) was then added, and the resultant activity was recorded as D. Reverse PFP activity was calculated as (D - C).

- Electrotransfer to nitrocellulose. Blots were probed with 1000-fold diluted rabbit anti-(potato tuber PFP) immune serum, and immunoreactive polypeptides were visualized as in Ref. 33. Immunological specificity was confirmed by performing Western blots in which rabbit pre-immune serum was substituted for the rabbit anti-(potato tuber PFP) immune serum. A Pharmacia Ultros XL enhanced laser densitometer was used to scan Western blots. Densitometric data were analyzed and M values were estimated using the Pharmacia Gelscan XL software.
the amount of \( B. \ nigra \) extract applied to Western blots. The \( M_r \) values of the immunoreactive polypeptides were estimated by comparing the mobility of the various bands with that of the following standard proteins: myosin (205 kDa), phosphorylase \( b \) (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), and trypsin inhibitor (20.1 kDa).

Other Methods—Immunotitration of PFP activity was performed as described by Moorhead and Plaxton (31) with the exception that 0.088 unit of homogeneous potato tuber PFP and 0.068 unit of PFP from extracts of \( \Pi \)-deficient \( B. \ nigra \) were incubated with various amounts of immune or pre-immune sera. Protein concentrations were determined by the method of Bradford (35) using bovine \( \gamma \)-globulin as standard.

RESULTS

When suspension cells of \( B. \ nigra \) were subjected to 18 days of \( \Pi \) deprivation, the extractable activity of the PFP was increased approximately 4- and 19-fold when assayed in the forward direction in the absence and presence, respectively, of 2 \( \mu \)M Fru-2,6-P\(_2\) (Fig. 1). Moreover, the enzyme from \( \Pi \)-deprived cells exhibited a more than 3-fold greater affinity for the activator than the PFP from nutrient-sufficient cells (Fig. 1) did. Thus, a significant portion of the \( \Pi \) starvation-dependent induction of PFP activity can be attributed to a marked increase in sensitivity of the enzyme to Fru-2,6-P\(_2\).

The extractable activity of the Fru-2,6-P\(_2\)-stimulated PFP assayed in the reverse direction increased from 0.025 to 0.038 unit-\( \text{mg}^{-1} \) when suspension cells of \( B. \ nigra \) were subjected to 18 days of \( \Pi \) deprivation. This change could be attributed to a small increase in sensitivity of the enzyme’s reverse activity to Fru-2,6-P\(_2\) since activities in the reverse direction measured in the absence of the activator were 0.016 and 0.017 unit-\( \text{mg}^{-1} \) for the PFP from \( \Pi \)-fed and \( \Pi \)-starved cells, respectively. As \( \Pi \) starvation caused a much greater elevation of PFP activity in the forward direction, the ratio of Fru-2,6-P\(_2\)-stimulated forward-reverse activities was increased about 13-fold following \( \Pi \) deprivation.
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FIG. 4. Correlation between PFP subunit ratio (α/β) and relative PFP activity and -fold activation by Fru-2,6-P$_2$. PFP activity was determined in the forward direction with saturating Fru-2,6-P$_2$ and is expressed relative to the maximal specific activity (0.062 unit/mg protein$^{-1}$) that was observed in P$_1$-starved cells. -Fold activation by Fru-2,6-P$_2$ was calculated as the -fold increase in PFP activity elicited by the addition of 2 μM Fru-2,6-P$_2$. Data were fitted using a computer statistics program (Statview Graphics), and in both cases the line of best fit was a third-order polynomial.

Combining activities of PFP in the forward direction and d-fructose-1,6-bisphosphate 1-phosphohydrolase could function as a cytosolic pyrophosphatase, which makes both phosphates of P$_1P$, available to the P$_1$-deprived cells.

Immunological studies using rabbit anti-(potato tuber PFP) immune serum (31) were initiated to examine the molecular properties of the PFP from P$_1$-fed and -starved B. nigra cells. Increasing amounts of the anti-(PFP) immune serum immunoprecipitated 100% of the activities of PFP from potato tubers or P$_1$-starved suspension cells of B. nigra (Fig. 2). The amount of antiserum required for 50% immunoprecipitation was about 15 and 45 μl/unit$^{-1}$ for the PFP from potato tubers and B. nigra, respectively.

Western blots of extracts prepared from P$_1$-starved B. nigra revealed immunoreactive polypeptides of 66 (α-subunit) and 60 kDa (β-subunit) staining in a 1:1 ratio that co-migrated with the α- and β-subunits of homogeneous potato tuber PFP (Figs. 3 and 5). However, only the 60-kDa polypeptide was observed on immunoblots of extracts prepared from P$_1$-sufficient cells (Figs. 3 and 5). The correlation between PFP subunit ratio (α/β) and activity profile of the PFP in suspension cells during the transition from P$_1$ sufficiency to deficiency. Induction of PFP activity and the appearance of the α-subunit began approximately 6 days after the transfer of P$_1$-fed cells to media lacking P$_1$ and was complete by day 18 when an approximate 1:1 ratio of α-subunit:β-subunit was achieved (Fig. 3). Hence, the apparent P$_1$ starvation-induced de novo synthesis of the α-subunit was coincident with an increase in the sensitivity of the enzyme to Fru-2,6-P$_2$. No further increase in enzyme activity or the amount of the α-subunit occurred when the time course was extended to 22 days (data not shown). The subunit ratio (α:β) correlated well with Fru-2,6-P$_2$-stimulated PFP activity and -fold activation of the enzyme by Fru-2,6-P$_2$ (Fig. 4).

Fig. 5. Immunological detection of PFP from cells of B. nigra becoming P$_1$-sufficient. Cells were cultured for 18 days in 0 mM P$_1$ (P$_1$-starved) and then transferred to media containing 10 mM P$_1$. Samples were subjected to Western blotting as described in Fig. 3A. The first lane contains 20 ng of purified potato tuber PFP (31); all other lanes contain 60 μg of protein from extracts prepared from cells at days 0, 1, and 14 as indicated. O, origin; TD, tracker dye front.

DISCUSSION

The findings of the present investigation indicate that the amount of the α-subunit of PFP is tightly regulated in suspension cells of B. nigra and that this regulation is dependent on cellular P$_1$ status. In contrast, the β-subunit of PFP from B. nigra is constitutively expressed under all nutrient regimes. The induction of PFP activity by P$_1$ starvation of B. nigra appears to be based upon de novo synthesis of the enzyme’s α-subunit, leading to a significant enhancement in sensitivity of the enzyme to its activator Fru-2,6-P$_2$ (Fig. 3). Similarly, the large reduction in PFP activity that occurs when P$_1$-deprived cells become P$_1$-sufficient arises from an inhibition of synthesis and/or enhanced degradation of the α-subunit (Fig. 5). It is evident that some form of proteolytic specificity toward the α-subunit must exist to facilitate the selective disappearance of this polypeptide upon P$_1$ refeeding. Overall, these results provide additional evidence that the α-subunit may function as a regulatory protein in controlling the catalytic activity of the β-subunit and its regulation by Fru-2,6-P$_2$. The concentration of Fru-2,6-P$_2$ in the cytosol of P$_1$-deprived B. nigra was previously estimated to be about 0.5 μM (29), a level that almost fully saturates the PFP from the P$_1$-starved cells (Fig. 1).

Recent gel filtration chromatography studies have revealed...
that comparable to the PFP of many plants (4–7), the native enzyme from P₃-deprived B. nigra probably exists as an α₂β₂ heterotetramer having a molecular mass of approximately 260 kDa. Further investigations involving the purified enzyme from nutrient-sufficient and P₃-deficient cells are required to fully resolve the structure-function relationships of the PFP isoforms of B. nigra. The results outlined above, however, are in accord with our previous suggestion (29) that the PFP of B. nigra is an adaptive enzyme that functions as a P₃ starvation-inducible glycolytic bypass to ATP:ß-fructose-6-phosphate 1-phosphotransferase when intracellular pools of ATP and P₃, but not PPi, are greatly depleted. It is of interest to note that the PFP of several heterotrophic plant tissues has also been proposed to operate as a glycolytic bypass to ATP:ß-fructose-6-phosphate 1-phosphotransferase during periods of anaerobiosis (37). The use of PPi, rather than ATP could confer a significant energetic advantage to plants subjected to environmental stresses such as P₃ deprivation or anoxia. Phosphate starvation-inducible synthesis of the 66-kDa α-subunit of PFP in suspension cells of B. nigra is coincident with de novo synthesis of cytosolic phosphoenolpyruvate carboxylase, vacuolar phosphoenolpyruvate phosphatase, and a cell wall-localized nonspecific acid phosphatase (38). Parallel induction of these enzymes with a simultaneous enhancement of cellular αP₃ absorption capacity (39) points to the existence of a plant “P₃ stimulus” (i.e., a set of genes that are co-regulated by P₃) as has been demonstrated in a variety of microorganisms (40).

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