

# Metabolite-mediated catalyst conversion of PFK and PFP: a mechanism of enzyme regulation in green plants

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Metabolites known to occur in the cytosol of photosynthetic leaf cells were found to mediate the reversible conversion of pyrophosphate-D-fructose-6-phosphate 1-phosphotransferase (PFP) to phosphofructokinase (PFK) in partially purified preparations from spinach leaves. Preincubation of PFP with fructose 2,6-bisphosphate, ATP or fructose 6-phosphate converted PFP to PFK. The reverse reaction (PFK → PFP) was promoted by UDP-glucose plus pyrophosphate. These conversions in catalytic capability were accompanied by changes in molecular mass and charge. The results are in accord with the view that the alterations in PFP and PFK activity, provisionally called 'metabolite-mediated catalyst conversion', represent a regulatory mechanism to direct left cytosolic carbon flux in either the biosynthetic or degradatory direction.

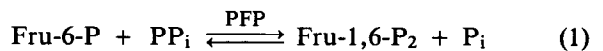
*FPLC*      *Phosphofructokinase*      *Metabolite-mediated catalyst conversion*  
*Pyrophosphate-D-fructose-6-phosphate 1-phosphotransferase*

## 1. INTRODUCTION

Enzymes of green plants are known to be post-translationally regulated by a number of mechanisms, including allosterism [1], phosphorylation [2] and reduction [3]. Each of these mechanisms results in a quantitative change in the activity of an enzyme (activation or deactivation) in response to changing metabolic needs of the cell. In addition, in two cases, the emergence of altered catalytic

activities has been reported as a result of enzyme association/dissociation [4–6]. However, to our knowledge, the induction of a qualitative change in the catalytic activity of an enzyme – i.e., a reversible shift from one type of catalytic activity to another – has not been described as a major mechanism of regulation in green plants.

We now report evidence for such a change in the cytosol of photosynthetic leaf cells. In this mechanism (designated 'metabolite-mediated catalyst conversion') an enzyme fraction catalyzing the phosphorylation of Fru-6-P is reversibly converted from a form that uses PP<sub>i</sub> as phosphoryl donor (PFP) (eq.1) to a form that uses ATP as donor (PFK) (eq.2).



In the presence of Fru-2,6-P<sub>2</sub> and either ATP or Fru-6-P, the PFP form of the enzyme is converted

*Abbreviations:* Fru-6-P, fructose 6-phosphate; PP<sub>i</sub>, inorganic pyrophosphate; PFP, pyrophosphate-D-fructose-6-phosphate 1-phosphotransferase (EC 2.7.1.90); PFK, phosphofructokinase (EC 2.7.1.11); Fru-1,6-P<sub>2</sub>, fructose 2,6-bisphosphate; Fru-2,6-P<sub>2</sub>, fructose 2,6-bisphosphate; FPLC, fast protein liquid chromatography; Glu-1,6-P<sub>2</sub>, glucose 1,6-bisphosphate; UDPG, UDP-glucose

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to PFK. UDPG and  $PP_i$  bring about a reverse of this reaction, i.e., a conversion of PFK to PFP. The evidence suggests that a reversible change in the catalytic activity of the PFP and PFK enzyme could occur *in vivo* in response to cellular needs to direct carbon flow in either the biosynthetic or degradative direction.

## 2. MATERIALS AND METHODS

### 2.1. Plant material

Spinach (*Spinacea oleracea*) leaves were obtained from a local market.

### 2.2. Reagents

Biochemicals, molecular mass markers, and lyophilized coupling enzymes were obtained from Sigma, St. Louis. Other reagents were purchased from commercial sources and were of the highest quality available. Buffers were adjusted to the indicated pH at the temperature at which they were used.

### 2.3. Enzyme assays

PFP and PFK were routinely assayed as in [7,8] except that the buffer used was 50 mM Tris-HCl (pH 7.9) without EDTA. Reactions were initiated by addition of Fru-6-P substrate.

Allosteric and kinetic properties of PFK and PFP were determined with partially purified preparations. The enzyme preparations were essentially free of fructose-1,6-bisphosphatase and other activities interfering in the assays, i.e., PFK of PFP, and PFP of PFK. As indicated, aliquots of the enzyme preparations were preincubated for 30 min at 20°C for conversion from one form to another. After preincubation, 20  $\mu$ g protein was assayed for PFP or PFK activity in 0.5 ml of the complete reaction mixture.

### 2.4. Purification of PFP and PFK

PFP and PFK were purified together at 4°C from 2.8 kg of leaves that had been homogenized in 1.5 l of 50 mM Tris-HCl buffer (pH 7.9) containing 10% glycerol and 0.1% 2-mercaptoethanol (buffer A) [9]. The polyethylene glycol fraction (5–15%) was further purified by sequential chromatography on columns of DE52 (1.6  $\times$  15 cm, developed with a linear 0–0.2 M NaCl gradient in buffer A) and Sephacryl S-300 (1.6  $\times$  90 cm, eluted

with buffer A). Active fractions were collected and concentrated by dialysis against 50 mM Tris-HCl buffer (pH 7.9) containing 80% glycerol and 0.1% 2-mercaptoethanol. The dialyzed preparations could be stored for several days at –20°C without appreciably changing catalytic activity.

### 2.5. Analytical procedure

Protein was determined as in [10].

### 2.6. Molecular mass determinations

Molecular masses were determined at 4°C by gel filtration with a Sephacryl S-300 column (1.6  $\times$  90 cm) developed with buffer A. The column was calibrated with known molecular mass markers: PFK (rabbit muscle), 320 kDa; catalase (bovine liver), 210 kDa; aldolase (rabbit muscle), 158 kDa; bovine serum albumin, 68 kDa.

### 2.7. Starch gel electrophoresis

Starch gels were prepared by a modification of [11] by mixing 26 g starch/200 ml gel buffer (6.07 mM  $K_2HPO_4$ , 1.2 mM citric acid; pH 7.0). Electrophoresis was initiated at 30 mA for 15 min and then at 40 mA for 12 h (temperature, 0°C). The running buffer was 167 mM  $K_2HPO_4$  and 27 mM citrate (pH 6.7). Gels were stained at 37°C in 50 ml of either PFP (forward) or PFK assay mixture. The PFP assay mixture contained 50 mM Tris-HCl buffer (pH 7.9); 2.5 mM  $MgCl_2$ ; 0.5 mM EDTA; 2.5 mM Fru-6-P; 2.0 mM  $PP_i$ ; 5.0 mM sodium arsenate; 0.25 mM NAD; 8 mg 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT); 0.5 mg 8-dimethylamino-2,3-benzophenoxazine (Mendola Blue); 500 units triosephosphate isomerase; 50 units glyceraldehyde-3-phosphate dehydrogenase; and 50 units aldolase (the latter 3 coupling enzymes were from rabbit muscle); 2  $\mu$ M Fru-2,6- $P_2$ . The PFK assay mixture was similar to the PFP mixture except that Fru-2,6- $P_2$  was omitted and 2.0 mM ATP replaced  $PP_i$  as phosphoryl donor.

### 2.8. Fast protein liquid chromatography

The Pharmacia FPLC system [12] for the rapid high resolution of proteins was used for demonstrating metabolite-mediated conversion of PFP to PFK and vice versa. Samples (600  $\mu$ g) of spinach leaf PFP and PFK (treated as described in text) were injected onto a pre-equilibrated Mono Q

HR 5/5 column and washed with 3 ml elution buffer [50 mM Tris-HCl (pH 7.9) containing 10% glycerol, 0.1% 2-mercaptoethanol and 5 mM  $MgCl_2$ ]. Proteins were then eluted with a linear gradient of 12 ml from 0 to 0.6 M NaCl in the same buffer. One-ml fractions were collected and assayed for enzyme activity.

### 3. RESULTS AND DISCUSSION

#### 3.1. Separation of spinach leaf cytosolic PFK and PFP activities

The cytosolic fraction of photosynthetic leaf cells has been reported to contain two enzymes catalyzing a phosphorylation of Fru-6-P – PFP [7,13,14] and PFK [15] – but so far these activities have not been followed simultaneously during purification. We, therefore, deemed it desirable to monitor the activity of both of these enzymes during fractionation of spinach leaf extracts. Fig.1 (left panel) shows that DE52 clearly resolved a large peak, containing PFP as well as the cytosolic of  $P_i$ -stimulated form of PFK, from fractions containing the plastid of  $P_i$ -inhibited form of PFK [15]. As shown in fig.1 (left panel), we observed on addition of 10 mM  $P_i$  the expected 2–3-fold increase in cytosolic PFK activity and on the addition of 20 mM  $P_i$  the expected 50% decrease in

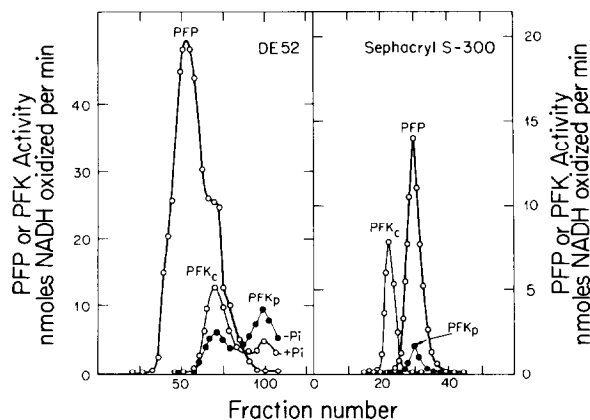


Fig.1. Separation of spinach leaf PFP and PFK [plastid (P) and cytosolic (C) forms] by DE52 and Sephacryl S-300 chromatography. As indicated, the PFK assay was carried out in the absence (●—●) or presence (10 mM, ○—○) of  $P_i$ . The DE52 profile represents a repeat chromatography of DE52 fractions enriched in PFP and cytosolic PFK.

chloroplast PFK activity. The PFP and cytosolic PFK recovered together in the DE52 peak could be separated from each other by subsequent filtration through Sephacryl S-300 (fig.1, right panel) without appreciable alteration of their kinetic properties. The cytosolic PFK (242 kDa) was eluted first, followed by the kinetically hypoactive form of PFP which eluted as a 165 kDa peak together with residual chloroplast PFK. The PFP and cytosolic PFK fractions purified and separated in this manner were used in the experiments described below.

#### 3.2. Reversible conversion of PFP from hypoactive to active form

As found previously [7], spinach leaf PFP isolated as summarized in fig.1 was strongly activated by Fru-2,6- $P_2$  (maximal activation at 70 nM). However, when added in limiting amounts (about 5 nM) so that the final rate of reaction was low and similar to that of the control, Fru-2,6- $P_2$  produced a previously unrecognized change: under these conditions, Fru-2,6- $P_2$  converted PFP from a kinetically nonlinear form showing a lag phase (here called 'hypoactive form') to a linear form ('active form') (fig.2, left panel). Glu-1,6- $P_2$ , a known activator of PFP [16], or UTP (both at 200 nM) produced the same effect, as did assay at high enzyme (>100  $\mu$ g protein/ml) concentrations (not shown). Significantly, when converted from hypoactive to active form, PFP underwent an increase in molecular mass from 165 to 242 kDa

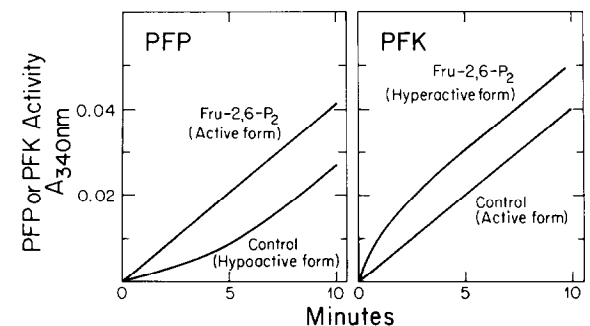


Fig.2. Effect of Fru-2,6- $P_2$  on PFP and PFK of spinach leaf cytosol. Curves represent direct recordings of the activities of partially purified PFP and PFK preparations (20  $\mu$ g) obtained after 30 min preincubation (pH 7.3) with and without Fru-2,6- $P_2$  (5 nM with PFP and 20  $\mu$ M with PFK). The reaction was started by the addition of  $PP_i$  (PFP) or ATP (PFK).

as determined by its elution from a calibrated Sephacryl S-300 column developed in the presence of  $1\ \mu\text{M}$  Fru-2,6-P<sub>2</sub> (not shown). Authors in [17] have reported an increase in the molecular mass of PFP following incubation with Fru-2,6-P<sub>2</sub>.

The conversion of PFP from hypoactive to active form was reversible. When the Fru-2,6-P<sub>2</sub>-activated (linear) form of PFP was diluted (protein  $\approx 5\ \mu\text{g}$  per ml, Fru-2,6-P<sub>2</sub>  $\approx 1\ \text{nM}$ ), it reverted to a hypoactive (nonlinear) form seemingly identical to the 165 kDa PFP initially isolated from leaves (not shown).

### 3.3. Reversible conversion of PFK from active to hyperactive form

In earlier experiments, we found that, in contrast to mammalian PFK, Fru-2,6-P<sub>2</sub> had no significant effect on the activity of the chloroplast PFK isoenzyme [7]. To our knowledge, the effect of Fru-2,6-P<sub>2</sub> on leaf cytosolic PFK has not been determined. Accordingly, in the present experiments, we conducted assays to ascertain whether Fru-2,6-P<sub>2</sub> alters the activity of the cytosolic PFK of spinach leaves. The results revealed that, like the chloroplast enzyme, Fru-2,6-P<sub>2</sub> had no effect on the final reaction rate of cytosolic PFK. However, unlike the chloroplast enzyme, Fru-2,6-P<sub>2</sub> markedly stimulated cytosolic PFK during the first minute of the reaction. As shown in fig.2 (right panel), Fru-2,6-P<sub>2</sub> converted PFK from a kinetically linear (active) form to a nonlinear form showing an initial burst of activity (here designated 'hyperactive form'). The Fru-2,6-P<sub>2</sub>-induced conversion of cytosolic PFK to a hyperactive form was accompanied by an increase in molecular mass from 242 to 320 kDa as determined by the elution pattern from a calibrated Sephacryl S-300 column developed in the presence of  $1\ \mu\text{M}$  Fru-2,6-P<sub>2</sub> (not shown).

### 3.4. Reversible conversion of PFP to PFK

The finding that leaf cytosolic PFK and PFP each exist in linear and nonlinear kinetic forms raised the possibility that these two types of catalytic activities might be related and, in fact, be interconvertible. The first indication that this might be the case arose with the finding that cytosolic PFK preparations, which initially had negligible PFP activity, gained the capability of utilizing PP<sub>i</sub> as phosphoryl donor on aging. Later experiments

showed that gel filtration and dilution had the same effect. As a result of these observations, we sought to determine whether physiological factors might also qualitatively influence the catalytic capability of fresh PFK and PFP preparations.

In surveying metabolites for an effect of PFK activity, we found that incubation with a combination of UDPG and PP<sub>i</sub> dramatically altered the active form of cytosolic PFK so that it could effectively utilize PP<sub>i</sub> as phosphoryl donor in a Fru-2,6-P<sub>2</sub>-dependent reaction (table 1). Other agents tested had no effect on conversion: 3-phosphoglycerate, oxidized and reduced glutathione, dithiothreitol, AMP, cAMP, ammonium chloride, citrate, ADP-glucose and phosphorylation with mammalian protein kinase [9]. The increase (up to 5-fold) in PFP activity brought about by incubating PFK with UDPG and PP<sub>i</sub> was accompanied by a change in molecular mass from 242 to 165 kDa as determined by Sephacryl S-300 gel filtration (not shown). Significantly, the PFP activity formed under these conditions showed a response to Fru-2,6-P<sub>2</sub>, similar to that of authentic PFP (see footnote to table 1). The results thus suggest that UDPG and PP<sub>i</sub> dissociate the active form of PFK to the lower molecular mass hypoactive PFP form, which depends on Fru-2,6-P<sub>2</sub> for maximal activity.

The finding that UDPG and PP<sub>i</sub> – metabolites known to occur in the cytosolic fraction of leaves –

Table 1

Conversion of spinach leaf cytosolic PFK to PFP by preincubation with metabolites

Preincubation treatment	% of total PFP + PFK activity	
	PFK	PFP <sup>a</sup>
(a) Control	87	13
(b) UDPG	62	38
(c) PP <sub>i</sub>	81	19
(d) UDPG + PP <sub>i</sub>	50	50

<sup>a</sup> PFP was assayed in the presence of 100 nM Fru-2,6-P<sub>2</sub>. Under the assay conditions, activity in all cases was negligible in the absence of Fru-2,6-P<sub>2</sub>.

The actual total activities, based on initial rates, were (nmol NADH oxidized/min per mg protein): (a) 176, (b) 257, (c) 186, (d) 322. The metabolite concentrations during preincubation were (mM): UDPG, 1; PP<sub>i</sub>, 1

converted PFK to PFP raised the question of whether the reverse could occur, i.e., whether metabolites could convert PFP to PFK. The results in table 2 show that this could be accomplished: on incubation with either Fru-2,6-P<sub>2</sub>, ATP or Fru-6-P (or a combination of these metabolites), PFP increased its capability of using ATP as phosphoryl donor by a factor of 3–4. In other words, these metabolites converted the isolated hypoactive form of PFP to the active form of PFK. The previously identified active form of PFP was also present in the converted sample containing Fru-2,6-P<sub>2</sub> (cf. fig.2, left panel). The conversion in catalytic capability was accompanied by a change in molecular mass from 165 kDa for the initial hypoactive PFP to 242 kDa for fractions showing the active forms of PFP and PFK (not shown).

The above results provide evidence that purified leaf cytosolic PFK and PFP are interconvertible. Thus, on the addition of appropriate metabolites, the initial enzyme form in each case gained significant levels of the opposing catalytic activity – i.e., PFP gained PFK activity and PFK gained PFP activity. The accompanying molecular mass changes suggested that these catalytic conversions are associated with an alteration in the oligomeric

state of the enzyme, and, as shown below, in charge as well.

In our initial efforts to resolve active components of metabolite-treated enzymes, we used starch gel electrophoresis with considerable success and detected the expected additional bands of enzyme activities after metabolite-mediated conversion. However, we later found that better resolution of these preparations was obtained on fractionation with FPLC. Thus, after treatment of PFP preparations with Fru-2,6-P<sub>2</sub> and Fru-6-P, FPLC profiles revealed the conversion of the hypoactive (less charged) form of PFP to an active (more highly charged) form (fig.3, left panel). Significantly, in addition to the active form of PFP, the treated enzyme preparation showed a new PFK peak. Typical of authentic cytosolic PFK, the PFK generated from PFP was activated by P<sub>i</sub> (not shown).

Experiments conducted in parallel revealed that catalyst conversion could also be carried out in the reverse direction, i.e., on incubation with UDPG and PP<sub>i</sub>, PFK was converted to PFP (fig.3, right panel). Here, PFK, which originally showed very little PFP activity, was converted to a less charged form which showed mainly PFP activity. Thus, in short, the FPLC results in fig.3 provide an independent line of evidence:

(i) That PFP and PFK are interconvertible; and

Table 2

Conversion of leaf PFP to PFK by preincubation with metabolites

Preincubation treatment	% of total PFP + PFK activity	
	PFP <sup>a</sup>	PFK
(a) Control	89	11
(b) Fru-2,6-P	79	21
(c) ATP	61	39
(d) Fru-6-P	63	37
(e) Fru-2,6-P + ATP	68	32
(f) Fru-2,6-P + Fru-6-P	60	40

The actual total activities, based on initial rates, were (nmol NADH oxidized/min per mg protein): (a) 130, (b) 422, (c) 187, (d) 228, (e) 455, (f) 440. The metabolite concentrations during preincubation were (mM): Fru-2,6-P<sub>2</sub>, 0.02; ATP, 10; Fru-6-P, 10. Similar results were obtained with the conversion metabolites added at half this concentration.

<sup>a</sup> PFP assayed in the presence of 100 nM Fru-2,6-P<sub>2</sub>

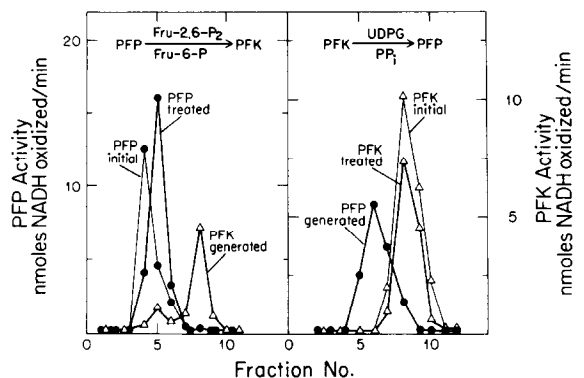


Fig.3. Demonstration of the conversion of PFP to PFK and of PFK to PFP by fractionation on FPLC. Initial and treated refer to the activity of the isolated PFP or PFK before and after treatment: generated refers to the activity developed as a result of treatment. Partially purified PFP and PFK preparations were treated as described in tables 1 and 2, respectively, and then subjected to FPLC fractionation. Activities of PFP and PFK were eluted at 0.24 and 0.32 M NaCl, respectively.

- (ii) That this conversion in catalytic capability is controlled by metabolites indigenous to the cytosol of photosynthetic leaf cells.

#### 4. CONCLUDING REMARKS

The present results have revealed a new mechanism of enzyme regulation in green plants. In this mechanism, called metabolite-mediated catalyst conversion, an enzyme is converted from a form having primarily one type of catalytic activity to a form catalyzing a different, but related, reaction. In the current case, enzyme preparations catalyzing the phosphorylation of Fru-6-P were, in the presence of appropriate leaf cytosolic metabolites, reversibly converted from PFP (which utilizes  $PP_i$  as phosphoryl donor) to PFK (which uses ATP). Accompanying this catalyst conversion, summarized schematically in fig.4, were changes in molecular mass and in charge.

Two questions arise as a result of the present findings.

- (i) Do the metabolite changes needed to mediate this conversion occur *in vivo* in accordance with the biochemical need for a particular enzyme form?  
 (ii) Do these enzyme conversions themselves occur *in vivo*?

While it is not yet possible to answer the second question, an affirmative answer can be provided to the first. Thus, under conditions when sucrose synthesis is most active (in the light), Fru-2,6-P<sub>2</sub> concentration tends to decrease [18] – an event that would promote the conversion of PFK to the hypoactive form of PFP, thereby likely decreasing the rate of glycolysis. This conversion to hypoactive PFP would be enhanced by UDPG, which was found recently to be somewhat more abundant in leaves in the light than in the dark (M. Stitt, personal communication). Conversely, when sucrose synthesis abates (in the dark), the reverse catalyst

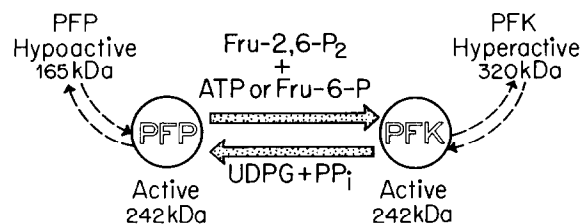


Fig.4. Factors promoting the interconversion of PFP and PFK in spinach leaf cytosol.

conversion would occur, i.e., PFP would be converted to PFK as a result of the increase in Fru-2,6-P<sub>2</sub> and the decrease in UDPG. Such a conversion to PFK would likely be accompanied by an acceleration of glycolysis. In this series of events, Fru-2,6-P<sub>2</sub> would thus accelerate glycolysis not only by increasing the catalytic activity of PFP as recognized previously [7], but also by converting PFP to PFK. Future experiments will be designed to determine if this is, in fact, the case.

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