Volume 238, number 2, 405–410 FEB 06403 October 1988

A novel pyrophosphate fructose-6-phosphate 1-phosphotransferase from carrot roots

Relation to PFK from the same source

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Received 11 July 1988; revised version received 25 August 1988

A novel type of pyrophosphate fructose-6-phosphate 1-phosphotransferase (PFP) has been purified from carrot roots. The enzyme differs from its counterpart studied from other sources in subunit composition, response to regulatory metabolites and kinetic parameters. The properties of the enzyme are in accord with its function in a tissue that stores substantial quantities of sucrose. In describing the carrot root PFP, we have systematically compared its properties to those of phosphofructokinase from the same tissue. The results support the conclusion that, despite noteworthy similarities, the two enzymes are independent proteins, unlikely to undergo interconversion.

Pyrophosphate fructose-6-phosphate; 1-Phosphotransferase; Phosphofructokinase; Enzyme property; Enzyme comparison

1. INTRODUCTION

The phosphorylation of Fru-6-P to Fru-1,6-P_2, the first step committed to the glycolytic path of carbohydrate breakdown, is subject to tight regulation. Unlike animals, plants have two enzymes that catalyze this reaction, one using ATP, PFK (phosphofructokinase or ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11), and the other using PP_i, PFP (pyrophosphate:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.90) [1–7]. In contrast to PFK (eqn 1), PFP catalyzes a freely reversible reaction (eqn 2).

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Abbreviations: Fru-6-P, fructose-6-phosphate; Fru-1,6-P_2, fructose-1,6-bisphosphate; PFK, phosphofructokinase; PFP, pyrophosphate fructose-6-phosphate 1-phosphotransferase; PP_i, pyrophosphate; P_i, orthophosphate; Tricine, (*N*-tris[hydroxymethyl]methylglycine); FPLC, fast protein liquid chromatography

$$\text{Fru-6-P} + \text{ATP} \xrightarrow{\text{PFK}} \text{Fru-1,6-}P_2 + \text{ADP} \quad (1)$$

$$\text{Fru-6-P} + PP_i \rightleftharpoons[\text{PFP}] \text{Fru-1,6-}P_2 + P_i \quad (2)$$

A role for PFP in the interconversion of Fru-6-P and Fru-1,6-P_2 has significant consequences for plants. (i) The cell is able to utilize and produce PP_i in the cytosol in response to metabolic demands; (ii) the interconversion of PP_i and P_i, as well as Fru-6-P and Fru-1,6-P_2, are linked to Fru-2,6-P_2, a regulatory metabolite that strongly regulates PFP in most plant tissues; and (iii) glycolysis is potentially regulated by Fru-2,6-P_2.

As a result of our interest in PFP and PFK [8], we decided to purify and characterize these enzymes from the same source and because of the relatively greater stability of PFK in that tissue, carrot roots were selected as source material. Last year, we described pertinent properties of homogeneous cytosolic PKF from carrot roots and showed it to be distinct from PFP [9]. Here we describe the purification of PFP, also from carrot roots, and summarize evidence suggesting it to be a novel enzyme that differs from PFK as well as from other PFP's described to date.

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00145793/88/$3.50

2. MATERIALS AND METHODS

2.1. *Plant materials*

Roots were obtained from three-month-old carrot (*Daucus carota* L.) plants grown in soil in a greenhouse and stored at 4°C for 2–4 months prior to use. Results similar to these presented below were obtained with freshly harvested roots.

2.2. *Reagents*

Biochemicals, molecular mass markers, and lyophilized coupling enzymes were obtained from Sigma. DEAE (DE-52)-cellulose and phosphocellulose (P-11) were purchased from Whatman. Sephacryl S-300 and Superose 12 were obtained from Pharmacia. Other reagents were purchased from commercial sources and were of the highest quality available. Buffers were adjusted to the indicated pH at room temperature.

2.3. *Enzyme assays*

All assays were carried out at 25°C. PFP activity was assayed in the forward direction by following the formation of Fru-1,6-P_2, at pH 8.0 for monitoring purification and at pH 7.5 for determining kinetics [10]. The complete system, in 0.5 ml final volume, contained the following (mM): 50 Tris-HCl buffer, pH 8.0; 1 $MgCl_2$; 1 tetrasodium EDTA; 0.1 NADH; 1 Fru-6-P; 0.2 PP_i; and coupling enzymes: 1 unit aldolase; 1 unit α-glycerophosphate dehydrogenase; and 10 units triose phosphate isomerase. PFP was activated with 1 μM Fru-2,6-P_2, unless indicated otherwise. Reactions were initiated by addition of PP_i substrate. PFP activity in the reverse direction [11,12] was assayed by following the production of Fru-6-P in a mixture (0.5 ml final volume) that contained the following (mM): 50 Tris-HCl, pH 8.0; 5 $MgCl_2$; 1 $NADP^+$; 0.5 Fru-1,6-P_2; 1 KH_2PO_4; and coupling enzymes: 0.2 unit phosphoglucose isomerase, and 0.1 unit glucose 6-phosphate dehydrogenase. The assay was started by addition of P_i. PFK was assayed as in [9]. The complete system (1 ml final volume) contained (mM): 50 Tris-HCl buffer, pH 8.0; 5 $MgCl_2$; 1 tetrasodium EDTA; 1 Fru-6-P; 0.2 ATP; 0.1 NADH and coupling enzymes: 1 unit aldolase; 10 units triosephosphate isomerase; and 1 unit α-glycerophosphate dehydrogenase. Enzyme was added to initiate the reaction. For PFP kinetic measurements, the concentration of Fru-6-P, PP_i, Fru-1,6-P_2, P_i and $MgCl_2$ in the assay mixture was varied as indicated. The reaction was initiated by adding enzyme. Hepes-KOH buffer (50 mM) in the pH range of 5.5 to 9.0 was used for pH studies.

2.4. *Purification of PFP*

PFP was purified at 4°C from 2.0 kg of carrot roots that had been homogenized in 1.5 l of 20 mM Tricine-KOH buffer (pH 8.0) containing 10% glycerol, 14 mM 2-mercaptoethanol, 5 mM potassium phosphate, 20 mM sodium diethyldithiocarbamate, 2 mM benzamidine-HCl, 2 mM ϵ-amino-*n*-caproic acid, 0.5 mM phenylmethylsulfonyl fluoride, and 1.5% (w/v) insoluble polyvinylpolypyrrolidone. The clarified crude extract was subjected to polyethylene glycol fractionation. The 4–15% polyethylene glycol fraction, containing the bulk of the PFP activity, was dissolved in buffer A (20 mM Tricine-KOH, pH 8.0, containing 10% glycerol, 14 mM 2-mercaptoethanol and 5 mM potassium phosphate) and was further purified by sequential chromatography on columns of: (i) DE-52 (1.6 × 15 cm, developed with a linear 0–0.5 M KCl gradient in buffer A); (ii) Sephacryl S-300 (1.6 × 90 cm, eluted with buffer A containing 150 mM KCl); and (iii) phosphocellulose (1.6 × 10 cm, eluted with a linear 0–40 mM PP_i gradient in buffer A). In each case, active fractions were collected and concentrated by overnight dialysis against either solid sucrose or 50% glycerol at 4°C. The concentrated preparations could be stored for several weeks at −20°C without appreciable loss of catalytic activity.

2.5. *Protein determination*

Protein was determined by the dye-binding method of Bradford [13]. Bovine serum albumin was used as protein standard.

2.6. *Native molecular mass determinations*

Native molecular masses were determined at 25°C by gel filtration with a Pharmacia FPLC Superose 12 column (HR10/30) developed with buffer A containing 150 mM KCl. The column was calibrated with the following proteins as standards (kDa): 2000 Blue dextran; 669 thyroglobulin; 443 apoferritin; 200 β-amylase; 166 catalase; 150 alcohol dehydrogenase; 66 bovine serum albumin; 44 ovalbumin; 29 carbonic anhydrase.

2.7. *Subunit molecular mass determination*

SDS-PAGE was carried out in 7.5–15% gradient polyacrylamide gels as described by Laemmli [14]. Gels were stained with silver [15]. Molecular mass standards were used as described in [9].

2.8. *Immunological methods*

The antibody against carrot root PFK was prepared as in [9]. Antisera against the α- and β-subunits of wheat seedling PFP were a kind gift from Professor M. Tao. A previously described procedure was used for Western blot analysis of electrophoretic gels [9].

Immunoprecipitation of PFK and PFP was accomplished by modification of a previously described procedure [9]. Enzymes were incubated with either control or antisera for 15 min at 25°C, then 20 μl of insoluble protein A Affi-gel was added and incubation continued for 15 min. After centrifugation at 14000 rpm for 5 min in an Eppendorf microfuge, supernatant solutions were assayed for enzyme activity.

3. RESULTS AND DISCUSSION

3.1. *Purification of carrot root PFP*

The two enzymes phosphorylating Fru-6-P from carrot roots, PFP and PFK, were separated on a DE-52 ion-exchange column (fig.1). PFP was further purified by Sephacryl S-300 and phosphocellulose chromatography. The overall purification procedure of PFP is summarized in table 1. It is not fully clear why the specific activity of PFP dropped substantially after the Sephacryl S-300 column. The loss is likely due to instability of the enzyme during gel filtration, a relatively slow step, that helped free the enzyme of residual PFK activity as well as from a number of other

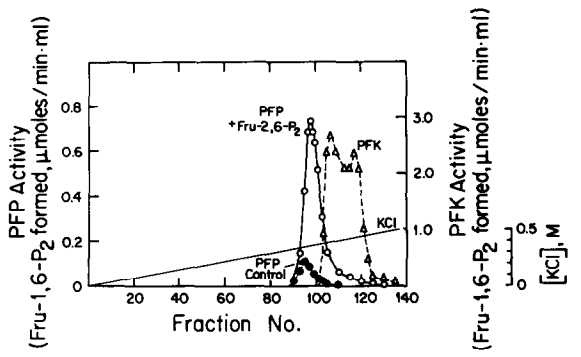

Fig.1. Elution profiles of PFP and PFK from carrot roots from a DE-52 column.

proteins. A further purification was achieved in the following phosphocellulose step in which most proteins in the preparation passed through the column and PFP was subsequently eluted as a single activity peak at 8 mM PP_i. As seen in table 1, the overall purification resulted in an enrichment in PFP activity of about 167-fold. Throughout purification the carrot root PFP retained its specificity for PP_i and showed no significant activity with ATP as phosphoryl donor.

In several preparations of carrot root PFP, we monitored activity of the reverse reaction and found it to be coincident with the forward activity throughout the purification procedure. At each step, carrot root PFP showed a high ratio of forward to reverse activity, i.e., a ratio of at least 7:1. However, as reported for castor bean PFP [12], the forward:reverse ratio for the purified enzyme was approximately 1:1 in the presence of Fru-2,6-P_2. The basis for the change during purification is not known. Similar to the enzyme from other sources [12,16], the ratio of glycolytic to gluconeogenic activity was increased by Fru-2,6-P_2, in our case more than 10-fold (cf. table 2).

3.2. *Molecular mass of carrot root PFP*

The M_r of the native carrot root PFP was estimated to be 294000 on a calibrated gel filtration column (FPLC Superose 12). The column profiles revealed no significant changes in (i) state of association/dissociation or (ii) ratio of forward to reverse activity as a result of treatment with Fru-2,6-P_2 (1 and 10 μM) and PP_i (10 mM) (not shown). The absence of such changes distinguishes the carrot root enzyme from its counterpart studied from pea seed [16], spinach leaf [8] and potato tuber [18].

3.3. *Subunit composition of carrot root PFP*

After electrophoresis in a 7.5–15% gradient SDS-polyacrylamide gel, carrot root PFP purified as in table 1 showed a major silver-staining component consisting of a doublet in the region corresponding to the subunit M_r of PFK, i.e., 60000 (fig.2), with no discernible band in the M_r region of 65000–67000. The 60 and 65–67 kDa values correspond to the molecular masses reported for the two different (β- and α-) subunits characteristically present in PFP from other plant tissues [17–20]. In preliminary immunoblotting experiments, the 60 kDa protein doublet of carrot root PFP cross-reacted with antibodies raised against the α- and β-subunits of PFP from wheat seedlings [17] (not shown). The results suggest that carrot root PFP contains two types of subunits showing similar immunological properties and

Table 1

Summary of purification of carrot root PFP

Fraction	Total protein (mg)	Total activity (units)[a]	Specific activity (units/mg)	Purification (-fold)	Recovery (%)
I. Crude extract	5590	67	0.012	1	100
II. Polyethylene glycol, 4–15%	1364	53	0.039	3	79
III. DE-52	39	25	0.65	54	37
IV. Sephacryl S-300	32	8	0.25	21	12
V. Phosphocellulose	1.3	2.6	2.0	167	4

[a] Units: μmol/min

Table 2

Kinetic constants of carrot root PFP

Parameter	Control	+ Fru 2,6-P_2
Forward direction		
K_m, Fru-6-P	1.3×10^{-4}	4.3×10^{-4}
K_m, PP_i	1.6×10^{-5}	1.9×10^{-5}
K_m, Mg^{2+}	4.8×10^{-4}	6.3×10^{-4}
V_{max}	5.9×10^{-6}	7.1×10^{-5}
Reverse direction		
K_m, Fru-1,6-P_2	1.0×10^{-3}	2.0×10^{-4}
K_m, P_i	1.7×10^{-3}	2.3×10^{-3}
K_m, Mg^{2+}	1.9×10^{-3}	1.2×10^{-3}
V_{max}	4.7×10^{-5}	4.5×10^{-5}

All K_m values are in M and V_{max} values are in $mol \cdot min^{-1} \cdot l^{-1}$

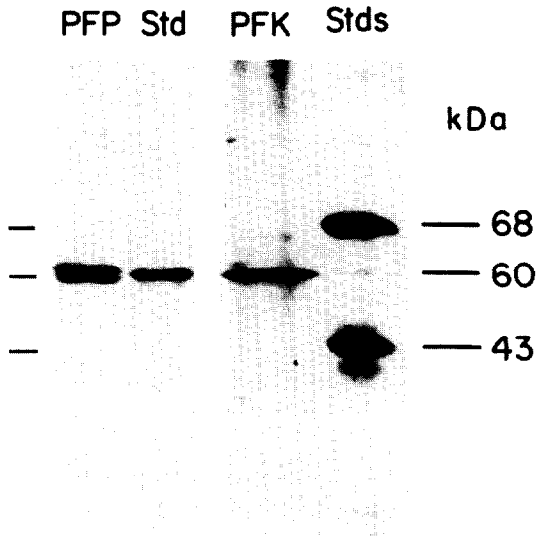

Fig.2. Photograph of SDS-PAGE of PFP and PFK from carrot roots. An SDS-gradient gel, containing 7.5–15% polyacrylamide, was developed with 5 μg each of PFP and PFK and 4–8 μg of catalase (60 kDa) and the other standards (bovine serum albumin, 68 kDa, and ovalbumin, 43 kDa). Electrophoresis was overnight at 7.5 mA. Gel was stained with silver.

slightly different molecular masses. Other properties of the subunits are yet to be determined. It should be noted that the α- and β-subunits of PFP from other sources appear to be immunologically distinct [17–20]. Furthermore, one of the forms of the wheat enzyme, containing only the β-type subunit, is specific for the corresponding antibody [17].

3.4. *Effect of anti-carrot root PFK and anti-wheat PFP antisera on PFK and PFP activities*

PFP and PFK reacted selectively with the corresponding antiserum as determined by activity measurements, thus indicating that the enzymes show significant immunological differences. An antibody against either wheat PFP-α or PFP-β inhibited carrot root PFP (75% by 10 μl) but had little effect on PFK from the same tissue. Similarly, antiserum against carrot root PFK inhibited the corresponding PFK (70% by 10 μl) but had no effect on PFP. Preimmune serum had little effect on either enzyme under the same conditions. We conclude that PFP and PFK are immunologically distinct with respect to catalysis and as, inferred from the present and other studies [14,21], are unlikely to undergo interconversion (cf. [8]). The apparent conversion of PFP to PFK observed previously [8] is concluded to be due to the metabolite-dependent interconversion of different enzyme forms now known to exist for PFK [14] that was present together with PFP in the earlier preparations. In other words, in our earlier work, we observed interconversion between the different forms of PFK rather than the presumed conversion of PFP to PFK. The basis for the apparent conversion of PFK to PFP has been clarified elsewhere [14].

3.5. *Kinetic constants of carrot root PFP*

Because of its unusual subunit composition, we determined the kinetic constants of the purified carrot root PFP (table 2). Like its counterpart from other tissues, the enzyme showed hyperbolic kinetics with regard to substrates and Mg^{2+} cofactor in both the forward and reverse directions and the formation of Fru-1,6-P_2 in the forward direction was highly activated (12-fold) by Fru-2,6-P_2 due to an increase in V_{max}. However, unlike other PFPs studied to date, Fru-2,6-P_2 increased the K_m (decreased the affinity) of carrot root PFP for Fru-6-P (3-fold) (fig.3) and, to a lesser extent, for PP_i (1.2-fold) and Mg^{2+} (1.3-fold). Nevertheless, the effect of Fru-2,6-P_2 on the breakdown of Fru-1,6-P_2 in the reverse direction was similar to other PFPs studied: i.e., no change in V_{max}, a decrease in K_m for one substrate (Fru-1,6-P_2) and cofactor (Mg^{2+}), and a small increase in K_m for the other substrate (P_i) (table 2). The kinetic properties

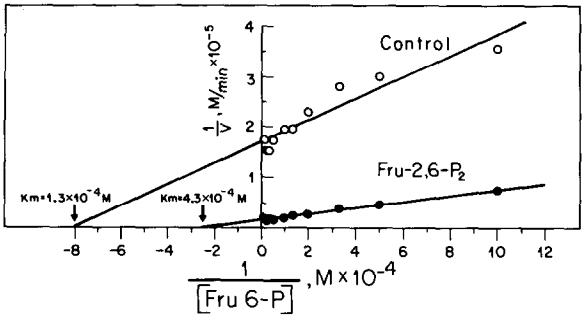

Fig.3. Lineweaver-Burk analysis of the effect of Fru-2,6-P_2 on the K_m of carrot root PFP for Fru-6-P. The x intercepts of the line for control (no Fru-2,6-P_2) indicates $K_m = 1.3 \times 10^{-4}$ M, for Fru-2,6-P_2 indicates $K_m = 4.3 \times 10^{-4}$ M.

of PFP from carrot roots are compared to those of the enzyme from other plant sources in table 3.

3.6. *Possible physiological basis for novel effects of Fru-2,6-P_2 on carrot root PFP*

While the absolute kinetic values obtained for carrot root PFP are similar to those obtained for the enzyme from other sources, there are, as noted above, significant differences with regard to the effect of Fru-2,6-P_2 on the forward reaction. Such differential effects of a regulatory metabolite are unusual for plant enzymes and, in the case of PFP, they probably stem from differences in structure, that, in turn, result from the role that the enzyme must play in a given tissue. In the case of carrot roots, a PFP showing the properties currently described would enable the tissue to store sucrose, one of its characteristic metabolic functions. Hence, because Fru-2,6-P_2 activates carrot root PFP by increasing V_{max}, with an attendant decrease in affinity for Fru-6-P substrate, the tissue could prevent excess sucrose breakdown and retain hexose for resynthesis of sucrose to a certain threshold level even when PFP is activated by enhanced Fru-2,6-P_2 concentrations. This feature helps to ensure that sucrose is retained for storage at all times. The Fru-2,6-P_2-linked decrease in K_m for Fru-1,6-P_2 in the reverse reaction (Fru-6-P formation) would also help retain the fructose and glucose precursors of sucrose synthesis. Thus, in short, the novel form of PFP present in carrot root appears to have kinetic properties which enable this tissue to maintain and retrieve substrates for sucrose synthesis, thereby making possible its sucrose storage function. It remains to be seen whether PFP from other sucrose storing tissues shows similar effects.

Table 3

Comparison of the effect of Fru-2,6-P_2 on carrot root PFP vs other plant PFPs

Parameter	Effect of Fru-2,6-P_2 on PFP	
	Carrot root	Other plants[a]
Forward reaction		
K_m, Fru-6-P	increase (3 ×)	decrease
K_m, PP_i	increase (1.2 ×)	decrease[b]
K_m, Mg^{2+}	increase (1.3 ×)	decrease
V_{max}	increase (12 ×)	increase
Reverse reaction		
K_m, Fru-1,6-P_2	decrease (5 ×)	decrease
K_m, P_i	increase (1.4 ×)	increase
K_m, Mg^{2+}	decrease (0.6 ×)	NA[c]
V_{max}	no change	no change

[a] Based on data for PFP from potato tuber [11], castor bean endosperm [12], wheat seedlings [17], pineapple leaves [19], cucumber seeds [22] and *Phaseolus vulgaris* seeds [23]
[b] In two cases, potato tubers [11] and wheat seedlings [17], there was no change in K_m for PP_i effected by Fru-2,6-P_2
[c] NA, not available

4. CONCLUDING REMARKS

PFP purified from carrot roots differs from its counterpart studied from other sources. The enzyme showed (i) a novel subunit composition, (ii) no change in state of aggregation with metabolites that alter the association state of PFP from other tissues, and (iii) changes in kinetic constants linked to activation by Fru-2,6-P_2 that have not been previously reported. The results suggest that the kinetic properties of the enzyme permit its function in a tissue that stores sucrose. It will be of interest to know whether other sucrose-storing tissues contain a PFP with similar properties. An additional question of interest is whether there is structural relatedness between PFP and cytosolic PFK: two independent phosphotransferases that contain subunits of similar size.

Acknowledgements: We wish to thank Professor Mariano Tao for the gift of rabbit antisera against wheat seedling PFP α- and β-subunits. This work was supported by a grant from the National Science Foundation.

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