Characterization of phosphofructokinase II and regulation of fructose 2,6-bisphosphate levels in *Trichoderma reesei*

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Phosphofructokinase II (PFK II) from *Trichoderma reesei* was partially purified (247-fold). The calculated K_m values for fructose 6-phosphate and ATP were 0.7 mM and 40 μ M, respectively. Upon incubation in the presence of [γ - 32 P]ATP, the enzyme formed a radioactive phosphoprotein with molecular mass of 67 kDa in autoradiography analysis after SDS-PAGE. Upon incubation in the presence of ATP-Mg and the catalytic subunit of cAMP-dependent protein kinase, its activity was not modified. The same result was obtained when a cell-free extract of *T. reesei* was incubated with ATP-Mg and cAMP. 2,4-Dinitrophenol caused a transient rise in cAMP levels in the fungal cell. These results provide evidence that the fructose 2,6-bisphosphate level in *T. reesei* is independent of cAMP concentrations and not related to a cAMP-dependent mechanism, but to the availability of substrate fructose 6-phosphate.

Introduction

Fructose 2,6-bisphosphate (Fru-2,6-P₂) controls glycolysis and gluconeogenesis by activating fructose 6-phosphate 1-kinase (PFK I; EC 2.7.1.11) and inhibiting fructose 1,6-bisphosphatase (EC 3.1.3.11). Fru-2,6-P₂ plays a central regulatory role in carbohydrate metabolism in eukaryotic organisms. Since its discovery (Van Schaftingen & Hers, 1980), Fru-2,6-P₂ has been found in plants (Larondelle *et al.*, 1986), animals (Van Schaftingen, 1987) and fungi (Van Laere *et al.*, 1983; Vandercammen *et al.*, 1990). An additional regulatory role of Fru-2,6-P₂ in regulation of the 'futile cycle' (fructose 6-phosphate/fructose 1,6-bisphosphate cycle) has been proposed (Leite *et al.*, 1988).

Fru-2,6-P₂ is formed from ATP and Fru-6-P in a reaction catalysed by 6-phosphofructo-2-kinase (PFK II) (El-Maghrabi *et al.*, 1981; Furuya & Uyeda, 1981; Hue *et al.*, 1981; Van Schaftingen & Hers, 1981). The hydrolysis of Fru-2,6-P₂ to Fru-6-P and P_i is catalysed,

in liver and lower eukaryotes, by fructose-2,6-bisphosphatase (FBPase II; EC 3.1.3.46). In liver, muscle and higher plants, PFK II and FBPase II are associated in a bifunctional protein (Pilkis *et al.*, 1984; Bazan *et al.*, 1989). In yeast FBPase II and PFK II can be separated (Kretschmer *et al.*, 1987; François *et al.*, 1988).

Phosphorylation of the bifunctional enzyme from liver, by a cAMP-dependent protein kinase, causes PFK II inactivation and FBPase II activation (Van Schaftingen, 1987). In *Saccharomyces cerevisiae*, on the other hand, cAMP-dependent phosphorylation of PFK II activates the enzyme (François *et al.*, 1984).

The filamentous fungus *Trichoderma reesei* secretes a very efficient cellulolytic system that has synergistic action on cellulose degradation to glucose (Montenecourt & Eveleigh, 1979; Béguin, 1990). This system is dependent on substrates that can induce the extracellular enzymes (El-Gogary *et al.*, 1989).

The purpose of this work was to characterize PFK II of *T. reesei* and to investigate the control of its activity in relation to the production of Fru-2,6-P₂.

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Abbreviations: PFK I, ATP:D-fructose-6-phosphate 1-phosphotransferase; PFK II, ATP:D-fructose-6-phosphate 2-phosphotransferase; Fru-6-P, fructose 6-phosphate; Fru-2,6-P₂, fructose 2,6-bisphosphate; Glu-6-P, glucose 6-phosphate; DNP, 2,4-dinitrophenol; FBPase II, fructose-2,6-bisphosphatase.

Methods

Materials. Blue trisacryl was from IBF Biotechnics; dithiothreitol, phenylmethylsulphonyl fluoride (PMSF), catalytic subunit of cyclic-AMP-dependent protein kinase, aldolase, triosephosphate isomerase

and α -glycerophosphate dehydrogenase were from Sigma; SDS-PAGE molecular mass standards, Superose-6B, DEAE-Sepharose and Blue-Sepharose were from Pharmacia LKB Biotechnology. Other chemicals were from Merck and were of analytical grade. PFK II of *Saccharomyces cerevisiae* (François *et al.*, 1988), potato tuber pyrophosphate:fructose-6-phosphate 1-phosphotransferase (Van Schaftingen *et al.*, 1982b), PFK phosphofructokinase of *T. reesei* (Leite *et al.*, 1988), [2-³²P]Fru-2,6-P₂ (Van Schaftingen *et al.*, 1982a) were prepared as described previously.

Inoculum and culture conditions. Cultures of *T. reesei* were maintained on potato dextrose agar slants at 4 °C. Inocula were prepared by harvesting spores from 7 d-old cultures in 0.9% NaCl and filtering through glass wool. Culture medium contained 0.08% glycerol, 0.2% Bacto-peptone (Difco), 0.21% (NH₄)₂SO₄, 0.03% urea, 0.03% MgCl₂·7H₂O, 0.03% CaCl₂ and 0.1% trace metal solution (0.2% CoCl₂, 0.5% FeSO₄·7H₂O, 0.15% MnSO₄·H₂O and 0.17% ZnCl₂ in 28 mM-HCl) in 100 mM-potassium phosphate buffer (pH 6.0). Cultures from spore inocula (final concentration, 10⁶ spores ml⁻¹) were incubated on a rotatory shaker for 14 h to yield a physiologically active inoculum.

Germinated spores (5 ml) were added to the above culture medium containing the appropriate carbon source for study. All cultures were incubated on a rotary shaker (200 r.p.m.) at 28 °C.

Determination of enzyme activities. PFK II was assayed by determining the production of Fru-2,6-P₂ in a mixture containing 50 mM-HEPES, pH 6.9, 10 mM-MgCl₂, 100 mM-KCl, 1 mM-DTT, 1 mg bovine serum albumin ml⁻¹, 1 mM-P_i, 5 mM-Fru-6-P, 17.5 mM-Glu-6-P and 5 mM-ATP. At various times, samples (0.1 ml) were mixed with 1 vol. 0.1 M-NaOH, incubated for 10 min and used for determination of Fru-2,6-P₂ (Van Schaftingen *et al.*, 1982b).

PFK I activity was determined by following the rate of NADH oxidation at 340 nm in a reaction mixture containing 50 mM-HEPES, pH 7.6, 10 mM-NH₄Cl, 5 mM-MgCl₂, 2.5 mM-ATP, 5 mM-Fru-6-P, 2 mM-DTT, 0.15 mM-NADH, aldolase 1.0 IU, triosephosphate isomerase 5.0 IU and α -glycerophosphate dehydrogenase 0.5 IU.

All enzymic assays were performed at 30 °C. One unit is the amount of enzyme that catalyses the conversion of 1 μ mol substrate in 1 min under the conditions of the assay.

Measurement of cyclic AMP and DNA. Samples of cultures were collected by filtration and the residue was scraped off and frozen in liquid nitrogen within less than 15 s after collection of the sample. The frozen pellet was weighed, mixed with 1 ml 20% (v/v) perchloric acid and freeze-thawed four times. The extract was centrifuged (3000 g for 2 min) and the supernatant divided into two samples. One sample was neutralized (2 M-KOH and 0.5 M-triethanolamine) and, after centrifugation, was used for determination of cAMP (kit supplied by Amersham). The other sample was used for determination of DNA as described by Abraham *et al.* (1972).

Purification of the PFK II of rat liver. Livers were isolated from fed rats killed by concussion and used immediately. The tissue was homogenized for 1 min in a Waring blender with 3 vols cold 25 mM-HEPES, pH 7.5, 1 mM-DTT, 1 mM-PMSF, 100 mM-KCl and 0.25 M-sucrose. The purification was carried out essentially as described by Van Schaftingen & Hers (1986).

Purification of the PFK II of *T. reesei*. The cells, cultivated in 1% (w/v) glucose, were harvested in the stationary phase 10 h after glucose exhaustion. Cells (100 g) were homogenized for five 30 s periods in a cell homogenizer (Marconi T-120) in 300 ml ice-cold solution containing 20 mM-potassium phosphate, pH 7.5, 1 mM-DTT, 1 mM-PMSF, 5 mM-EDTA and 0.5 M-KCl. The homogenate was centrifuged for

10 min at 35000 g and filtered through glass wool. The purification was performed as described by François *et al.* (1988).

Phosphorylation of PFK II of liver and *T. reesei*. The partially purified enzymes (20 μ g) were incubated with the catalytic subunit of cAMP-dependent protein kinase (20 picomolar units ml⁻¹; 1 picomolar unit transfers 1 pmol phosphate from [γ -³²P]ATP to hydrolysed, partially dephosphorylated casein per min at pH 6.5 and 30 °C) at 30 °C in a final volume of 0.1 ml containing 10 mM-potassium phosphate, pH 7.5, 2 mM-ATP and 1 mM-DTT. After incubation for various times, samples were collected and mixed with 10 vols 10 mM-potassium phosphate, pH 7.5, 1 mM-DTT, 5 mM-EDTA and 20 mM-NaF. The PFK II activity was determined as described above.

Protein determination. The concentration of protein was determined by the method of Bradford (1976) with bovine serum albumin as a standard.

Results

Purification of PFK II

In view of the differences in the catalytic and regulatory properties of PFK II in higher and lower eukaryotes, we undertook the simultaneous purification of the enzymes from *T. reesei*, *S. cerevisiae* and rat liver PFK II.

The purification of PFK II of *T. reesei* involved extraction, affinity chromatography on blue trisacryl and gel filtration on Superose-6B. The 247-fold purified PFK II was obtained with a yield of 13% (Table 1). The elution profile of this purification (Fig. 1) showed differences from previous results obtained with *S. cerevisiae* (François *et al.*, 1988).

The purification procedure for rat liver PFK II resulted in 6500-fold purified enzyme with a specific activity of 286 mU mg⁻¹ and a recovery of 24% (Table 2).

Effect of the catalytic subunit of cAMP-dependent protein kinase on the activity of purified PFK II of *T. reesei* and rat liver

Incubation of purified *T. reesei* PFK II with the catalytic subunit of cAMP-dependent protein kinase caused no change in the enzymic activity, whereas under the same conditions rat liver PFK II was inhibited (Table 3).

Experiment with a cell-free system

In order to verify a possible phosphorylation by a cell-specific cAMP-dependent protein kinase present in the homogenate, the extract of *T. reesei* was incubated with cAMP and ATP-Mg (François *et al.*, 1984). A portion of extract (200 μ l) was incubated (30 °C) with 5 mM-MgSO₄, 2 mM-ATP and 10 μ M-cAMP, and at various times samples were collected and mixed with 5 vols 10 mM-potassium phosphate, pH 7.5, 1 mM-DTT, 5 mM-EDTA and 20 mM-NaF. The PFK II activity was determined in

Table 1. Purification of PFK II from *T. reesei*

Purification step	Vol. (ml)	Protein (mg)	Total activity (mU)	Specific activity [mU (mg protein) ⁻¹]	Purification (-fold)	Yield (%)
Crude extract	200	800	131	0.164	1	100
Blue-trisacryl	40	3.5	69.5	19.9	121	53
Superose-6B	20	0.42	17	40.5	247	13

Table 2. Purification of PFK II from rat liver

Purification step	Vol. (ml)	Protein (mg)	Total activity (mU)	Specific activity [mU (mg protein) ⁻¹]	Purification (-fold)	Yield (%)
Crude extract	350	17000	748	0.044	1	100
PEG 22%	30	5625	394	0.070	1.6	53
DEAE-Sepharose	50	85	264	3.1	70.4	35
Blue-Sepharose	20	0.63	181	286	6500	24

Fig. 1. (a) Elution profile of PFK II of *T. reesei* from the blue-trisacryl column. The crude extract (200 ml) containing 131.2 mU of PFK II was applied to the gel previously equilibrated with 20 mM-potassium phosphate, pH 7.5, 1 mM-DTT, 1 mM-PMSF, 5 mM-EDTA and 0.5 M-KCl. The column was washed with the same buffer and then eluted with a linear gradient of KCl (0.5–3 M in 2 × 200 ml of same buffer) at a rate of 30 ml h⁻¹. Fractions of 5 ml were collected and PFK II activity was determined as described in Methods. ●, A_{280} ; ○, Fru-2,6-P₂; —, KCl. (b) Elution profile of PFK II of *T. reesei* from the Superose-6B column. The activity fractions obtained by elution of the column of blue-trisacryl were pooled and concentrated to 2 ml by ultrafiltration on Amicon YM-100 filters. The concentrate was applied to a column of Superose-6B previously washed with 20 mM-HEPES, pH 7.5, 0.3 M-KCl, 1 mM-DTT, 0.4 mM-PMSF and then eluted with the same buffer. Fractions of 5 ml were collected and PFK II activity was determined as described in Methods. ●, A_{280} ; ○, Fru-2,6-P₂.

Table 3. Phosphorylation of PFK II of *T. reesei* (A) and rat liver (B)

The partially purified enzymes (20 µg) were incubated at 30 °C with 20 picomolar units (see Methods) of the catalytic subunit ml⁻¹ in 10 mM-potassium phosphate, pH 7.5, 2 mM-ATP and 1 mM-DTT. Samples were collected at the times indicated and mixed with 10 vols 10 mM-potassium phosphate, pH 7.5, 1 mM-DTT, 5 mM-EDTA and 20 mM-NaF. The PFK II activity was determined as described in Methods

Time (min)	Fru-2,6-P ₂ produced [nmol min ⁻¹ (mg protein) ⁻¹]			
	Phosphorylated sample		Control	
	A	B	A	B
1	34	250	33	280
5	33	180	34	280
10	32	100	33	250
20	34	100	33	250
30	33	100	32	250

a mixture containing 50 mM-Tris/acetate, pH 7.5, 1 mg bovine serum albumin ml⁻¹, 10 mM-magnesium acetate, 2 mM-P_i, 8 mM-Glu-6-P, 2 mM-Fru-6-P, 7 mM-ATP and 1 mM-DTT. The Fru-2,6-P₂ produced was assayed (Van Schaftingen *et al.*, 1982b). No change in the enzyme activity was detected by this procedure, in good agreement with the results obtained *in vitro* (see above).

Complexation of PFK II of *T. reesei* with [γ -³²P]ATP

SDS-PAGE analysis of the fractions eluted from the Superose-6B purification of PFK II of *T. reesei* showed the presence of many bands. In order to identify the

Fig. 2. Complexation of PFK II of rat liver and *T. reesei* with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. The partially purified enzymes (10 μg) were incubated at 30 °C with 10 μM - $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (12000 c.p.m. pmol^{-1}) in 20 mM-HEPES, pH 7.5, 0.1 mM-DTT, 100 mM-KCl, 0.1 mM-EDTA, 5 mM- MgCl_2 . Samples (10 μl) were collected at the times indicated, mixed with 1 vol. loading buffer, heated in a boiling water bath for 7 min, and subjected to SDS-PAGE. For autoradiography the gel was dried and exposed at -80 °C to X-ray film with an intensifying screen.

Fig. 3. Saturation curve of PFK II of *T. reesei* for Fru-6-P. PFK II was assayed in the presence of 50 mM-HEPES, pH 6.9, 100 mM-KCl, 5 mM- MgCl_2 , 1 mM-DTT, 1 mM- P_i , 5 mM-ATP and 1 mg bovine serum albumin ml^{-1} . The inset shows a plot of $[\text{Fru-6-P}]/v$ against $[\text{Fru-6-P}]$.

peptide band corresponding to the subunit of this enzyme, advantage was taken of experimental data suggesting that the mechanism of the (rat liver) PFK II

Fig. 4. Saturation curve of PFK II of *T. reesei* for ATP. PFK II was assayed in the presence of 50 mM-HEPES, pH 6.9, 100 mM-KCl, 5 mM- MgCl_2 , 1 mM-DTT, 1 mM- P_i , 5 mM-Fru-6-P and 1 mg bovine serum albumin ml^{-1} . The inset shows a plot of $[\text{ATP}]/v$ against $[\text{ATP}]$.

catalysis involves the formation of a phosphoryl enzyme intermediate (E-P_i) from ATP and Fru-2,6- P_2 (Pilkis *et al.*, 1984). Incubation of purified PFK II of *T. reesei* and rat liver with 10 μM - $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and 2 μM - $[\text{Fru-2,6-P}_2]$ allowed the identification of the protein band corresponding to PFK II of *T. reesei* (Fig. 2).

Kinetic properties of purified PFK II of *T. reesei*

The partially purified enzyme displayed typical Michaelis-Menten kinetics, with a K_m of about 0.7 mM for Fru-6-P (Fig. 3) and 40 μM for ATP (Fig. 4).

Stimulation of PFK I of *T. reesei* by Fru-2,6- P_2

The general rule that Fru-2,6- P_2 is a positive effector also applies to *T. reesei* PFK I. Addition of Fru-2,6- P_2 changed the saturation curve for Fru-6-P from sigmoidal to hyperbolic and decreased the K_m for Fru-6-P (Fig. 5).

Effect of glucose and 2,4-dinitrophenol (DNP) on the concentration of cAMP

In order to verify whether addition of glucose and DNP to a suspension of *T. reesei* in stationary phase caused a transient increase in concentration of cAMP, as described previously for the enzymes from *S. cerevisiae* (François *et al.*, 1984) and *Neurospora crassa* (Ann Dumbrava & Pall, 1987), the culture of *T. reesei* was

Fig. 5. Effect of Fru-2,6-P₂ on the saturation curve of *T. reesei* PFK I for Fru-6-P. PFK I was assayed in the presence of 50 mM-HEPES, pH 7.6, 10 mM-NH₄Cl, 5 mM-MgCl₂, 2.5 mM-ATP, 2 mM-DTT, 0.15 mM-NADH, 1 IU aldolase, 5 IU triosephosphate isomerase, 0.5 IU α -glycerophosphate dehydrogenase. The concentrations of Fru-2,6-P₂ are shown next to the curves.

Table 4. Effect of addition of glucose and 2,4-dinitrophenol (DNP) on the concentration of cAMP

T. reesei and *S. cerevisiae* were cultivated under the same conditions as used for purification of PFK II until the homogenization step (see Methods). Glucose was added to the culture of *S. cerevisiae* at a final concentration of 0.1 M and to the culture of *T. reesei* at 0.03 M and 0.1 M. DNP was added to the culture of *T. reesei* at a final concentration of 1 mM. At the indicated times, samples of cultures were collected by filtration, frozen in liquid nitrogen and the concentration of cyclic AMP was determined (see Methods).

Time (min)	cAMP concn [pmol (μ g DNA) ⁻¹]			
	<i>S. cerevisiae</i>		<i>T. reesei</i>	
	+ Glucose (0.1 M)	+ Glucose		+ DNP (1 mM)
		(0.03 M)	(0.1 M)	
0	3.0	0.9	0.9	0.9
1	3.5	0.6	0.4	1.0
2	9.0	0.6	0.4	3.0
3	4.0	0.4	0.4	2.0
5	4.5	0.6	0.2	1.5

submitted to two concentrations of glucose and to 1 mM-DNP. The same procedure was also performed for *S. cerevisiae* in the presence of 0.1 M glucose alone (Table 4).

Discussion

As shown in Tables 1 and 2, the purification of PFK II of rat liver gave a purification factor much greater (6500-fold) than that of the enzyme of *T. reesei*. In order to separate PFK II of *T. reesei* from cAMP-dependent and

cAMP-independent protein kinases, the suggestions of François *et al.* (1988) were followed. Thus we avoided procedures of purification involving, as a first step, fractionation with polyethylene glycol or chromatography on DEAE-trisacryl or blue-Sepharose which could cause the possible activation of PFK II either during the purification or in the assay mixture. This activation, on the other hand, did not occur in the enzyme from liver, either in this work or in others (Van Schaftingen & Hers, 1986) (Table 2).

T. reesei PFK II was strongly retained on blue trisacryl and eluted only at a high salt concentration. In this respect the behaviour of the *T. reesei* enzyme is comparable to that of the yeast PFK II (François *et al.*, 1988). The elution from blue trisacryl yields a purification of 100-fold. The active fractions (Fig. 1a) were pooled and concentrated by ultrafiltration and applied to the column of Superose-6B. This step was less efficient in terms of purification; however, it is worth noting that the elution profile of the enzyme was strikingly different from that of the preparation obtained with *S. cerevisiae* (François *et al.*, 1988) (Fig. 1b).

A further, and more fundamental difference between the *T. reesei* enzyme and those of *S. cerevisiae* (François *et al.*, 1984) and rat liver is the lack of cAMP-dependent regulation in the former enzyme (Table 3).

Catalysis by rat liver PFK II involves a double displacement with the transient formation of a phosphoryl enzyme intermediate (E-P_i) (Pilkis *et al.*, 1984). Analysis of [³²P]phosphoprotein formed upon incubation of the purified enzymes with [γ -³²P]ATP revealed a band with molecular mass of about 55 kDa for liver enzyme (El-Maghrabi *et al.*, 1982; Furuya *et al.*, 1982) and one with a molecular mass of about 67 kDa for the *T. reesei* enzyme (Fig. 2). On the other hand, it was not possible to obtain [³²P]phosphoprotein upon incubation of *T. reesei* enzyme with [³²P]Fru-2,6-P₂, indicating that this enzyme is not bifunctional. The purified enzyme (PFK II) of *T. reesei* did not catalyse the phosphorylation of histones, casein and albumin (not shown).

From the saturation curves of PFK II from *T. reesei* for Fru-6-P and ATP (Figs 3 and 4) K_m values of about 0.7 mM and 40 μ M, respectively, were obtained. The K_m for Fru-6-P of PFK II from *S. cerevisiae* decreased from 1.3 mM to 0.6 mM after phosphorylation (François *et al.*, 1984), values close to those obtained for the *T. reesei* enzyme. On the other hand, the K_m of PFK II from *S. cerevisiae* for ATP (0.5 mM) is not affected by phosphorylation. Thus the enzyme of *T. reesei* is about tenfold more sensitive for ATP than its counterpart. In addition, the relatively high K_m values for PFK II from *S. cerevisiae* and *T. reesei* indicate that these enzymes are highly dependent on the Fru-6-P concentration.

The general effect of Fru-2,6-P₂ as a potent stimulator of glycolysis also holds for *T. reesei*, since the activity of PFK I in this fungus is greatly increased by this effector (Fig. 5).

The effect of Fru-2,6-P₂ contrasts with that of cAMP in the regulation of carbohydrate metabolism. While Fru-2,6-P₂ is known as the most positive effector of PFK I, it acts solely by increasing the glycolytic rate and decreasing gluconeogenesis by inhibition of fructose 1,6-bisphosphatase; cAMP shows a diverse action in eukaryotic cells, that may even be antagonistic. Thus, in liver, cAMP inhibits glycolysis, whereas it has the reverse effect in heart and muscle (François *et al.*, 1988; Kitamura *et al.*, 1989; Pall, 1984; Van Schaftingen & Hers, 1986; Van Schaftingen, 1987).

François *et al.* (1984) added DNP to cultures of *S. cerevisiae* and obtained data that suggested a close relationship between levels of Fru-2,6-P₂ and a transient rise of cAMP. This relation led to the proposal of a secondary activation of PFK II by a cAMP-dependent phosphorylation. It should be noted, however, that the transient rise of cAMP in lower eukaryotes treated with uncoupler agents, or even with antibiotics which induce pore formation, is a general response and should not be used for establishing specific correlations (Jaynes *et al.*, 1982; Pall *et al.*, 1981; Trevillyan & Pall, 1979; Uno & Ishikawa, 1981). For example, the addition of glucose to a suspension of *S. cerevisiae* leads to a transient increase in the concentration of cyclic AMP, but addition to a culture of *T. reesei*, had the opposite effect. Furthermore, the addition of DNP to the *T. reesei* caused a transient rise of cAMP (Table 4) and a twofold increase in Fru-2,6-P₂ after 30 min (not shown).

Results shown in this work indicate that, unlike *S. cerevisiae*, levels of Fru-2,6-P₂ in *T. reesei* are independent of levels of cAMP, as in *Blastocladiella emersonii* (Vandercammen *et al.*, 1990). *Dictyostelium discoideum* (Aragón *et al.*, 1986) and *N. crassa* (Ann Dumbraiva & Pall, 1987). This may indicate that for these organisms the level of Fru-2,6-P₂ is controlled by the availability of the Fru-6-P.

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