Phytochemistry, Vol. 25, No. 2, pp. 339–343, 1986.
Printed in Great Britain.

0031-9422/86 $3.00 + 0.00
Pergamon Press Ltd.

PHOSPHOFRUCTOKINASE FROM *LYCOPERSICON ESCULENTUM* FRUITS—I. KINETIC PROPERTIES IN RELATION TO ITS SUBSTRATES

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(*Revised received* 31 *July* 1985)

Key Word Index—*Lycopersicon esculentum*; Solanaceae; tomato fruit; phosphofructokinase; kinetic properties; allosteric interactions.

Abstract—The kinetic properties of phosphofructokinase with regard to its substrates are discussed. Free ATP is inhibitory to the enzyme while the Mg-ATP complex at a concentration up to 5 mM is not. The kinetics with respect to Mg-ATP follow simple Michaelis–Menten kinetics and this pattern is not affected by changes in concentration of the second substrate fructose-6-phosphate (F6P). The kinetics with respect to F6P showed apparent negative co-operative interactions in the presence of saturating levels of Mg^{2+} relative to ATP. In the presence of inhibitory levels of free ATP, the kinetics showed positive co-operative interactions. The relationship between the nature of the kinetics of the enzyme with F6P and the various molecular forms of PFK are discussed.

INTRODUCTION

Phosphofructokinase (ATP: D-fructose-6-phosphate 1-phosphotransferase; EC 2.7.1.11, hereafter PFK) is considered to be an allosteric enzyme which plays a key role in the regulation of the glycolytic flux [1, 2]. In recent years, our knowledge of the regulatory properties of PFK has taken a new approach with the discovery of fructose-2,6-bisphosphate as the most potent activator for PFK from animal and fungal sources [3]. This metabolite, however, was found to have no effect on PFK from plant and bacterial sources [4–9]. In plants, a PPi phosphofructokinase (pyrophosphate: fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.90) has been isolated from several sources [6, 7, 9, 10]. However, the actual role of this enzyme in glycolysis is not clear [3]. A recent report investigating the relative importance of PPi-PFK and ATP-PFK in plant respiration shows that PPi-PFK does not contribute appreciably to control of glycolysis [10].

The kinetic and regulatory properties of PFK from animal and microbial sources have been extensively studied [1–3]. However, our knowledge of PFK in plants is fragmentary [11, 12]. The published work on PFK in plants not only shows apparent differences between the properties of this enzyme in various plant tissues, but even considerable differences between PFK preparations from the same tissue [12–14]. The reports on the existence of a plastid PFK in addition to the cytosolic enzyme possessing different properties has further complicated comparison of kinetic properties from various plant sources [15–17]. In addition, most work on PFK in plants has been made on crude extracts, except for few recent reports in which the enzyme has been successfully purified [15, 16, 18, 19].

In the tomato, PFK has been purified 260-fold and analysis of the purified fraction on PAGE revealed one major protein band which corresponded to PFK activity [20]. The enzyme at pH 7.5 existed in an oligomeric form which has an M_r of 180000. Increasing the pH to 8 favoured the dissociation of the enzyme and yielded a catalytically active monomeric form which had an M_r of 35000 [20]. The aim of this work is to study in detail the kinetic properties of the different molecular forms of the enzyme with respect to the two substrates of the enzyme, F6P and Mg-ATP. This paper describes, for the first time, kinetic studies on a plant PFK which has been purified almost to homogeneity.

RESULTS

PFK kinetics with respect to Mg^{2+} *and ATP*

Figure 1a shows the saturation plot of PFK activity at pH 7.5 against magnesium chloride concentration in the presence of 1 mM ATP and 2 mM F6P. The figure shows that under these conditions, the optimum concentration of Mg^{2+} is 5 mM. Figure 1b shows the saturation plot of PFK activity against ATP concentration in the presence of various concentrations of Mg^{2+} while F6P concentration was maintained at 2 mM. The results indicate that concentrations of ATP above 0.5 mM can be inhibitory at 2 mM Mg^{2+} and that this inhibition can be relieved by increasing Mg^{2+}. The ratio between Mg^{2+} and ATP plays a major role in determining the activity of this enzyme and 5 mM Mg^{2+} relative to 1 mM ATP is the concentration for exhibiting optimum catalytic activity. Analysis of the data of Figs 1 and 2 revealed that the enzyme kinetics at high [Mg^{2+}] relative to ATP followed simple Michaelis–Menten kinetics (Hill coefficient, $h = 1$) with K_m of 0.46 mM for ATP and 0.84 mM for Mg^{2+}. The optimal ratio of Mg:ATP as revealed by the K_m values is 2:1 respectively. However the presence of 2 mM EDTA in the assay can explain why a higher ratio (5:1) with respect to Mg:ATP was optimal under our assay conditions.

Figure 2 shows a double reciprocal plot of PFK kinetics

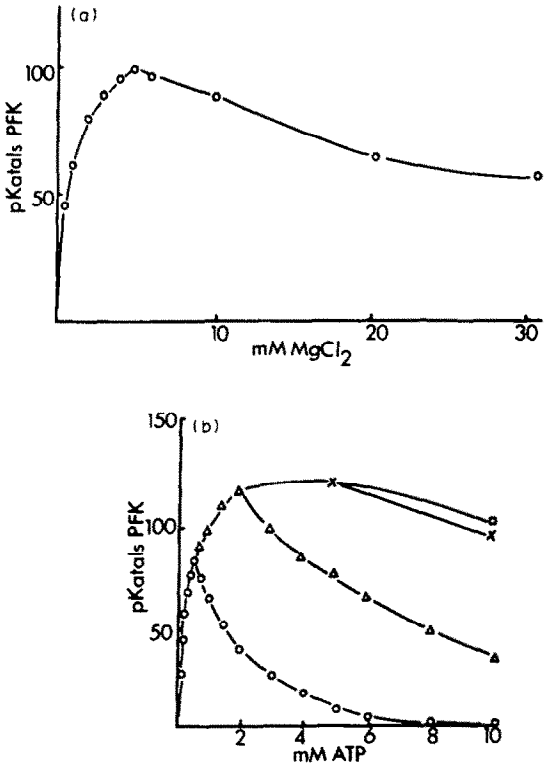

Fig. 1. (a) Effect of Mg^{2+} on PFK activity. (b) Effect of ATP on PFK activity in the presence of 2 (○), 5 (△), 10 (×) and 20 (□) mM Mg^{2+}.

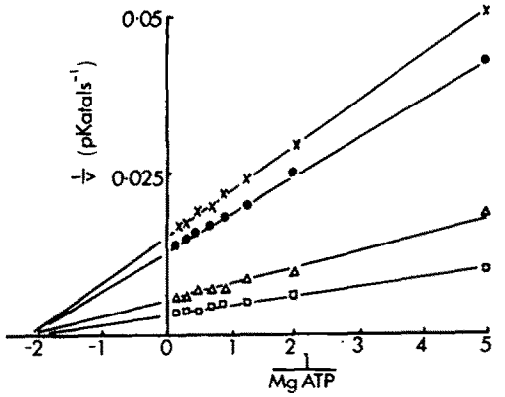

Fig. 2. A double reciprocal plot of the kinetics with respect to Mg-ATP for PFK at pH 8 (●) and pH 7.5 (△), in the presence of 10 mM F6P (□) and in the presence of 20 mM ammonium sulphate (×).

with regard to Mg-ATP under different conditions. The results show that promoting dissociation by raising the pH to 8, increasing F6P concentration to 10 mM or introducing ammonium sulphate, an inhibitor of PFK, had no effect on the nature of the kinetics [20]. In all these cases, the kinetics followed the sample Michaelis–Menten pattern and while the various conditions affected V_{max}, they had no effect on K_m.

Phosphoryl donor capacity

Table 1 shows that ATP can be replaced as phosphoryl donor respectively by GTP, UTP, CTP and ITP with decreasing effectiveness as measured by the concentration required for 50% maximal activity ($S_{0.5}$). GTP is almost as effective as ATP but the V_{max} is lower. In all cases these phosphoryl donors showed simple Michaelis–Menten kinetics ($h = 1$) in the presence of excess Mg^{2+}.

When supplied alone, at concentrations above 1 mM all four nucleotides were inhibitory and inhibition was relieved, as is the case of ATP, by addition of Mg^{2+}. This is illustrated for GTP in Fig. 3.

PFK kinetics with respect to F6P

In the study of the kinetics of PFK with regard to F6P, the concentration of Mg^{2+} was maintained at 5 mM and that of ATP at 1 mM. Figure 4a shows an Eadie–Hofstee plot of the reaction kinetics for the effect of F6P on a highly purified fraction in which the enzyme was maintained in the oligomeric form.

Analysis of these results showed that V_{max}, h and $S_{0.5}$ under these conditions were 62.3 pkat/μg protein, 0.72 and 5.6 mM respectively. This shows that the oligomeric form of PFK exhibits negatively cooperative interactions (i.e. $h < 1$) with F6P which suggests that the binding of one molecule of F6P to the active site of PFK hinders the binding of further substrate molecules. The kinetics with respect to F6P for PFK at various stages of the purification procedure [20] were repeated and the results are shown in Table 2. Those results indicate that all these PFK preparations exhibited negative cooperativity in relation to F6P with minor deviations in kinetic para-

Table 1. The kinetic properties of PFK in relation to some nucleotide triphosphates supplied in the presence of excess Mg^{2+} (5:1)

Trinucleotide	V_{max} (pkat/g protein)	h	$S_{0.5}$ (mM)
ATP	7.8	1	0.46
GTP	6.46	1	0.47
ITP	4.88	1	1.27
UTP	4.66	1	0.93
CTP	3	1	1.1

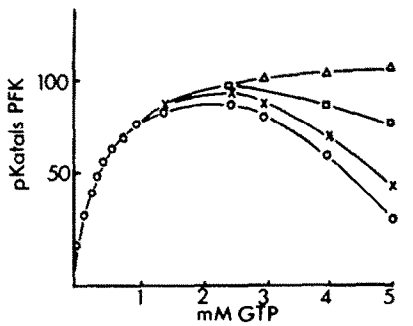

Fig. 3. Effect of GTP on PFK activity in the presence of 5 (○), 10 (×), 15 (□) and 25 (Δ) mM Mg^{2+}.

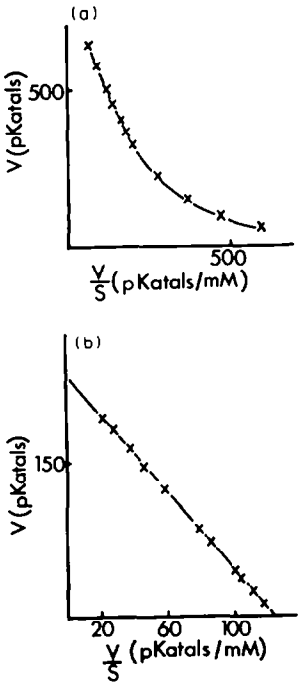

Fig. 4. Eadie–Hofstee plots for the kinetics with regard to F6P for (a) the oligomeric and (b) monomeric forms of PFK.

meters. Since the enzyme was shown to exist as a single peak on Ultrogel AcA 34 columns at pH 7.5 [20] and this peak of PFK revealed negative cooperativity with regard to F6P, it would appear that negative cooperativity is a feature of the oligomeric form of PFK.

Subsequently, the kinetics of the monomeric form of PFK with F6P were studied and an Eadie–Hofstee plot of the results is shown in Fig. 4b. Analysis of this data showed that V_{max}, h and $S_{0.5}$ under these conditions were 11.3 pkat/μg protein, 1 and 1.8 mM respectively. Thus, the monomeric form exhibited simple Michaelis–Menten kinetics with regard to F6P. The monomeric form has a higher affinity for the substrate F6P compared to the oligomeric form ($S_{0.5}$ of 1.8 and 5.6 mM for the monomeric and oligomeric forms respectively). These kinetic studies were performed on either the oligomeric or monomeric forms of PFK. Kinetic studies on the dissociating system when both forms of PFK were present do not give a clear picture.

The effect of changing Mg^{2+} and ATP concentration on the kinetics of the oligomeric form of PFK with respect to F6P was studied. Figure 5 shows the saturation plots of PFK activity against F6P concentration in the presence of various concentrations of Mg^{2+} and ATP. The results, summarized in Table 3, show that as the Mg^{2+}/ATP is decreased the Hill coefficient rises from 0.7 to above 2.0. At Mg^{2+}/ATP ratios of 2.0, or below, the saturation plots reveal highly sigmoidal curves and significantly lower values for V_{max}.

DISCUSSION

PFK is an allosteric enzyme which is affected by a large number of modulators and its behaviour towards its two substrates, F6P and Mg-ATP, is complex [1, 2]. In general, PFK is inhibited by free ATP while Mg^{2+} relieves this inhibition and the kinetics with Mg-ATP follow the simple Michaelis–Menten pattern [1, 2, 12, 17, 21–24]. The one exception to this is PFK from pea seedlings which showed negative cooperativity at low concentrations of Mg-ATP while the kinetics approached Michaelis–Menten type at higher concentrations of Mg-ATP [25]. However, these workers were using Mg^{2+} at a fixed concentration of 2 mM above that of ATP which may explain the variation in kinetics over the range of ATP levels studied. The non-cooperative binding of Mg-ATP to tomato PFK is not affected either by factors that

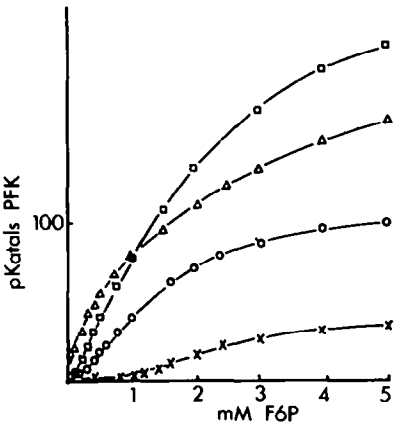

Fig. 5. Effect of Mg^{2+} and ATP on the kinetics of the oligomeric form of PFK with regard to F6P. The concentrations of Mg^{2+} and ATP (mM) were respectively: 10, 4 (□); 5, 1 (△); 2, 1 (○); 1, 1 (×).

Table 2. PFK kinetics with regard to F6P at various stages of the purification procedure according to ref. [20].

Preparation	h*	$S_{0.5}$*	No. of replicates
Dialysed crude extract	0.73 ± 0.08	6.65 ± 1.25	5
ATP Sepharose peak	0.71 ± 0.01	5.8 ± 0.27	3
Blue Sepharose peak	0.72 ± 0.04	5.7 ± 0.4	4
Ultrogel oligomeric peak	0.65	7.3	2

*These values are expressed as means for different runs ± standard deviation of the mean.

Table 3. Effect of Mg:ATP ratio on PFK kinetics with F6P at pH 7.5

mM Mg^{2+}	mM ATP	V_{max} (pkat/g protein)	h	$S_{0.5}$(mM)
5	1	10.43	0.72	5.6
2	1	6.42	1.6	1.5
1	1	2.19	2.6	2.3
10	4	12.58	1.2	2.6

promote dissociation of the oligomer or by variations in the level of the second substrate, F6P. In agreement with other workers, other nucleotide triphosphates can replace ATP but they are generally less efficient [23, 26–28]. However, in all cases when Mg^{2+} was supplied in excess, the kinetics with nucleotide triphosphates showed non-cooperative interactions.

The main controversy in PFK kinetics relates to its interactions with its second substrate, F6P. PFK from most sources has been shown to exhibit positive cooperativity with F6P [1, 2, 26, 27, 29–31] and the degree of cooperativity was reduced by raising the pH or increasing Mg^{2+} [31–35]. The present work shows that the nature of PFK kinetics with F6P depends on the levels of Mg^{2+} relative to ATP and on the molecular form of the enzyme. The most significant finding in this work is the negative cooperativity, which the PFK oligomer exhibited with respect to F6P in the presence of excess Mg^{2+} relative to ATP. As far as we know, this is one of the first reports in which negative cooperativity with F6P has been shown for PFK from plants [25, 28] and it is the first example where a PFK purified almost to homogeneity shows negative cooperativity with F6P. The pea seed enzyme exhibited a biphasic saturation plot with F6P which at low F6P showed negative cooperativity with a Hill coefficient of 0.3–0.7 [25]. These workers were using Mg^{2+} and ATP at a concentration of 4.8 and 0.75 mM respectively [25]. The partially purified banana PFK showed negative cooperativity with F6P ($h = 0.68$ and $S_{0.5} = 5.6$ mM) [28] and this is in agreement with our findings.

At low levels of Mg^{2+} relative to ATP, the oligomeric form of tomato PFK shows positive cooperativity and this agrees with that shown by many other workers [1, 2, 26, 27, 29–35]. In contrast, the monomeric form shows non-cooperative interactions towards F6P and it could be that the examples in the literature in which simple Michaelis–Menten kinetics with respect to F6P have been observed, reflects the presence of dissociated enzyme [5, 7, 15, 18].

The physiological significance of the negative cooperativity is that it tends to insulate the tomato oligomeric PFK from fluctuations in F6P concentrations and it prevents the total inhibition of the enzyme by a particular inhibitor [36, 37]. It also means that the enzyme would be operating under 'constraint' far below its potential activity [37]. These characteristics would help in maintaining a constant rate of activity for the PFK oligomer *in vivo*. The levels of Mg^{2+} relative to ATP could play a major role in regulating the activity of tomato PFK *in vitro*. At low concentrations of Mg^{2+} relative to ATP, PFK is inhibited, and yet it exhibits positive cooperativity showing a higher affinity to F6P ($S_{0.5} = 1.5$ mM) than when Mg^{2+} is in excess ($S_{0.5} = 5.6$ mM). Nevertheless, the physiological significance of this finding is not clear since although the concentrations of Mg^{2+} in the tomato are quite adequate to complex all the cellular ATP [38, 39], the level of Mg^{2+} in the compartment housing PFK is unknown.

The monomeric form of PFK shows simple Michaelis–Menten kinetics and a change in configuration of the enzyme from the constrained oligomer to the monomer leads to a significant increase in the affinity of the enzyme for F6P ($S_{0.5}$ from 5.6 to 1.8 mM) and to a considerable increase in activity at a given substrate concentration. *In vitro* studies have demonstrated the dissociation of PFK oligomer to catalytically active subunits [20]. However, there is no evidence, at present, for the reassociation of such monomers and thus, the physiological significance of association-dissociation reactions in the regulation of tomato PFK *in vivo* remains unclear.

The effect of other modulators on PFK activity and on the negative cooperativity with F6P will be discussed in the subsequent paper.

EXPERIMENTAL

Tomatoes (*Lycopersicon esculentum* var. Eurocross BB) were grown in the Food Research Institute greenhouse. All the work was done on fresh fruits obtained at the well-defined breaker stage when the enzyme exists solely in the oligomeric form [20]. The extraction medium consisted of 0.2 M Tris–HCl at pH 7.5 containing 2 mM EDTA and 5 mM DTE and it was used in the ratio of 2 ml of the extraction medium/g of tissue in addition to PolyClar AT (50 mg/g tissue). The enzyme was purified to homogeneity using a combination of Blue Sepharose and ATP-Sepharose chromatography. Full details of the materials, extraction procedures and purification for PFK activity have been described in ref. [20]. Unless stated otherwise, the standard reaction mixture for PFK assay contained the following in a 1 ml cuvette: 16 μmol NADH, 5 μmol $MgCl_2$, 1 μmol ATP, 2 μmol F6P, 0.4 units of α-glycerolphosphate dehydrogenase, 2 units of triosephosphate isomerase and 0.4 units of aldolase. The rate of the reaction at 25°, initiated by F6P, was followed fluorimetrically using an Eppendorf fluorimeter. The purified PFK preparations were free from interfering enzymes such as phosphoglucoisomerase, fructose 1,6-bisphosphatase, NADH oxidoreductase, phosphoglucomutase and ATPase. The kinetic data was analysed using Eadie–Hofstee plots (v against v/s) which yields a straight line for simple Michaelis–Menten kinetics with a slope of $-K_m$ and y-axis intercept as V_{max}. The Hill equation was used in the form

$$v = \frac{V_{max}[S]^h}{S_{0.5} + [S]^h}$$

where v = initial velocity, V_{max} = maximal velocity, [S] = substrate concentration, K_m = substrate concentration giving half maximal velocity and h = Hill coefficient. A computer program based on an iteration procedure was used for estimating maximum velocity (V_{max}), h (Hill coefficient) and $S_{0.5}$ (the substrate concentration giving half maximal rate) in relation to each other from a set of values of reaction rates and substrate concentrations.

Acknowledgement—The authors would like to thank the British Council for the scholarship awarded to J.E. Isaac during this work.

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