Phytochemistry, Vol. 26, No. 3, pp. 645–648, 1987.
Printed in Great Britain.

0031–9422/87 $3.00 + 0.00
Pergamon Journals Ltd

THE ROLE OF INORGANIC PHOSPHATE IN THE REGULATION OF PFK ACTIVITY IN TOMATOES

J. E. ISAAC* and M. J. C. RHODES

Institute of Food Research, Norwich Laboratory, Colney Lane, Norwich, NR4 7UA, U.K.; *Department of Life Science, Bethlehem University, West Bank, Bethlehem, Via Israel

(*Revised received* 8 *August* 1986)

Key Word Index—*Lycopersicon esculentum*; Solanaceae; tomato fruit; phosphofructokinase; kinetic properties; association–dissociation.

Abstract—The effect of inorganic phosphate (Pi) on the activity of phosphofructokinase (PFK) from the tomato is dependent on the state of aggregation of the enzyme. Pi stimulates the activity of the oligomeric form of PFK but promotes its dissociation to yield a catalytically active monomeric form. Pi has an instantaneous effect of decreasing the apparent negative cooperativity between PFK and F6P while its long term effect is to completely abolish this cooperativity. Pi relieved the inhibition of both the oligomeric and monomeric forms of PFK by PEP, salts, phosphoglycerate and phosphoglycolate. Citrate inhibition of the oligomeric form was reversed by Pi but its inhibition of the monomeric form was accentuated by Pi. The significance of Pi effect on PFK activity and regulation is discussed in relation to the phosphorylation state concept.

INTRODUCTION

Phosphofructokinase (ATP: D-fructose 6-phosphate 1-phosphotransferase, EC 2.7.1.11; hereafter PFK) from animal and microbial sources is stimulated by inorganic phosphate (Pi) [1–3]. However, the effect of Pi on the activity of PFK from plants is variable [4–15]. Pi stimulated the activity of PFK from Brussels sprouts [5], pea seed [8], *Kalanchoe* sp., *Atriplex* sp. [11] and tomatoes [14] but it had no effect on PFK from potatoes [10], bananas [9] or castor bean [13]. On the other hand, PFK from corn scutellum [6] and spinach chloroplasts [7] was inhibited by Pi. However, Pi, which inhibited carrot PFK at pH 7.9, stimulated the enzyme at pH 6.6 [4, 12]. PFK from tomatoes has been purified close to homogeneity [16] and its kinetic and regulatory properties have been extensively studied [17, 18]. The tomato enzyme has been shown to exist in a stable and active oligomeric form (M_r 180000) which is liable to dissociate *in vitro* to yield an unstable but catalytically active monomeric form (M_r 35000). In this report, the effect of Pi on the activity of the different molecular forms of the enzyme from the tomato is discussed together with the interactions between Pi and other PFK modulators.

RESULTS

Figure 1 shows the instantaneous effect of Pi on the monomeric and oligomeric forms of PFK. Pi stimulated the activity of the oligomeric form at concentrations up to 20 mM. The concentration causing 50% activation ($A_{0.5}$) was 3.8 mM. However, at concentrations above 20 mM, the degree of stimulation decreased but even at 100 mM the enzyme still showed a higher activity than in the absence of Pi. On the other hand, Pi was inhibitory to the monomeric form with an inhibitor concentration causing 50% inhibition ($I_{0.5}$) of 28.7 mM. Pi stimulation of PFK oligomer was dependent on the pH with 2 mM Pi exhibiting maximal stimulation at pH 7 (125%) and decreased to 44% at pH 7.5 while at the pH optimum of PFK (pH 8), 2 mM Pi had little stimulatory effect. When the oligomeric form of PFK was incubated with Pi, the stimulatory effect of Pi on this form decreased with time. Consequently, the long term effect of Pi on the molecular form of PFK was studied by gel permeation chromatography on an Ultrogel AcA 34 column. Figure 2a shows the elution pattern of PFK on the same column using Tris–HCl buffer at pH 7.5. Using a column calibrated with proteins of known M_r, the enzyme had an estimated M_r of 180000. Figure 2b shows the elution pattern of PFK on the same column using phosphate buffer at pH 7.5. The results show that following long term exposure to phosphate, PFK eluted more slowly in a position corresponding to a M_r of 35000. In shorter

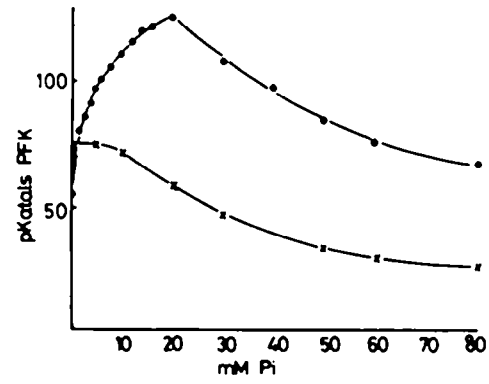

Fig. 1. The effect of inorganic phosphate on the activity of the oligomeric (○) and monomeric (×) forms of PFK.

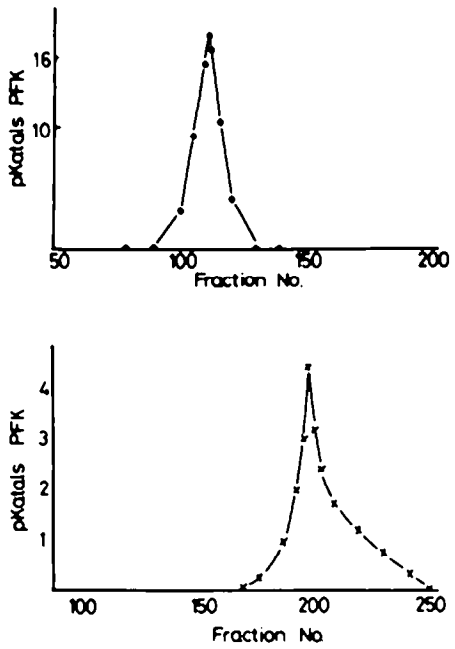

Fig. 2. Elution of PFK from Ultrogel AcA 34 column using (a) Tris-HCl and (b) K-phosphate buffer at pH 7.5. The column (34 × 2.6 diam.) was eluted at 0–4° at 15 ml/hr with the buffer and 1 ml fractions were collected.

periods of exposure to Pi, a mixed population of PFK proteins was found corresponding to the M_r 180000 and 35000 components. With time, the peak corresponding to the oligomer decreased and that of the monomer increased.

The effect of Pi was investigated further using PAGE. With the purified enzyme, there was a single major protein band on the gel which corresponded with PFK activity revealed by an *in situ* assay linked to the deposition of insoluble formazan [16]. Exposing the enzyme to Pi for 15 min resulted in the appearance of more than one protein band. There was a retardation of one protein band and a splitting of the fast moving band into two protein components. The low protein content of the purified enzyme rendered it difficult to study these protein components in detail. Nevertheless, these results confirm the role of Pi in promoting the dissociation of the oligomeric form of PFK.

Effect of Pi on the kinetics of PFK in relation to F6P

Figure 3a shows an Eadie–Hofstee plot of data for the purified enzyme at different levels of F6P in the absence and presence of 2 mM Pi. In the absence of Pi, the graph shows a major deviation from linearity indicative of negative cooperativity ($h = 0.65$). From this data, the V_{max} of the reaction and the substrate concentration for half maximal rate ($S_{0.5}$) were calculated as 1.52 pkatal/μg protein and 6.6 mM, respectively. The presence of Pi led to a decrease in the deviation from linearity ($h = 0.9$) indicating a decrease in the degree of negative cooperativity but led to an increase in the V_{max} of the reaction to 2.37 pkatal/μg protein. The presence of Pi decreased the $S_{0.5}$ value of 2.5 mM and thus caused an increase in the affinity of PFK for F6P.

The long term effect of Pi on the PFK kinetics with respect to F6P were studied using an enzyme prepared by using phosphate buffer at pH 7.5. Figure 3b shows the double reciprocal plot of the results which showed linear Michaelis–Menten kinetics ($h = 1$) with $S_{0.5}$ equal to 1.76 mM. Subsequent removal of phosphate ions using chromatography on Sephadex G-25 did not restore the cooperativity towards F6P which suggests that the dissociation of the enzyme is irreversible at least *in vitro*.

Interactions between Pi and other modulators of PFK activity

Figure 4 shows that Pi was able to relieve the inhibition by PEP of both the monomeric and oligomeric forms of PFK but did so more effectively for the oligomeric form. Analysis of these data showed that the maximum velocity for Pi stimulation of the oligomeric form did not change in the presence of 10 μM PEP but $A_{0.5}$ for the Pi effect

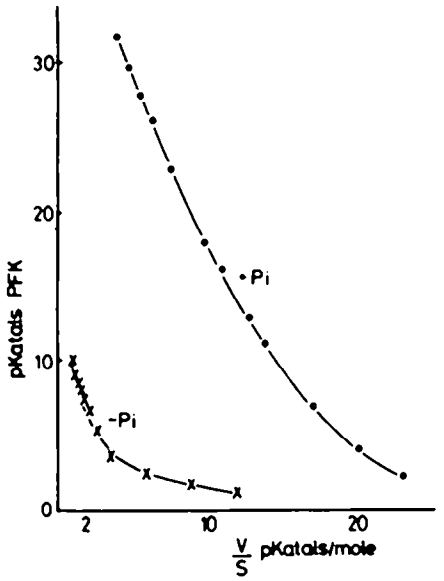

Fig. 3a. An Eadie–Hofstee plot for the kinetics of the oligomeric form of PFK with regard to F6P in the absence (×) and presence of 2 mM Pi (○).

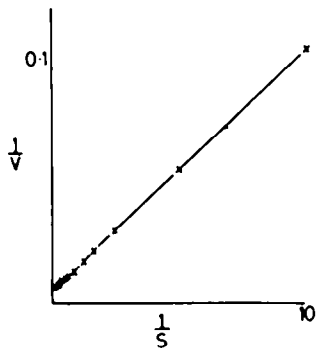

Fig. 3b. A double reciprocal plot of the kinetics of the monomeric form of PFK with respect to F6P.

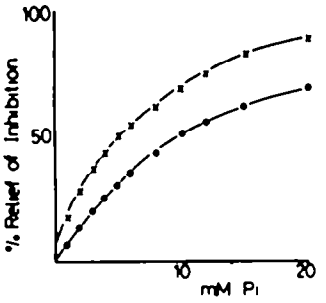

Fig. 4. The relief of the inhibitory effect of PEP on the oligomeric (×) and monomeric (○) forms of PFK by inorganic phosphate (both assays contained 10 μM PEP which initially induced 54 and 83% inhibition of the oligomeric and monomeric forms of PFK, respectively).

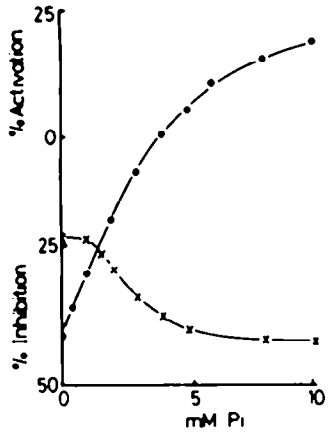

Fig. 5. Effect of inorganic phosphate on the inhibition by citrate of the oligomeric form of PFK (○) (assay contained 5 mM citrate causing 38% inhibition initially) and the monomeric form of PFK (×) (assay contained 0.25 mM citrate causing 20% inhibition initially).

increased from 3.8 mM in the absence of PEP to 8.6 mM in its presence. For the monomeric form, the inhibitory effect of Pi ($I_{0.5}$ = 28.7 mM) was changed to a stimulatory effect ($A_{0.5}$ = 11.5 mM) in the presence of 10 μM PEP. Thus, PEP decreased the apparent affinity of the two forms of the enzyme for Pi which suggests that PEP may be a competitive inhibitor for Pi. Pi also relieved the inhibition of the oligomeric form of PFK by 2-phosphoglycerate and phosphoglycolate [18].

Figure 5 shows the interaction between Pi and citrate on the activity of the oligomeric and monomeric forms of PFK. 4 mM Pi was able to overcome completely the 38% inhibition of the oligomeric form of PFK caused by 5 mM citrate. At higher concentrations of Pi, the activity of the oligomeric form was further stimulated. Citrate decreased the maximum stimulation of the oligomer by Pi but $A_{0.5}$ for the effect of Pi remained at 3.8 mM in the presence of 5 mM citrate. On the other hand, the 20% inhibition of the monomeric form of PFK by 0.25 mM citrate was not relieved by Pi but rather was accentuated.

PPi up to a concentration of 5 mM had no effect on either the oligomeric or monomeric forms of tomato PFK.

DISCUSSION

Although it is generally accepted that Pi stimulates the activity of PFK in most sources [1, 3, 5], there is little understanding of its mechanism of action. The present work indicates that an important effect of Pi is to promote dissociation of tomato PFK and this affects its kinetic and regulatory properties. While there is evidence showing that tomato PFK oligomer undergoes dissociation *in vitro* [16], there is at present no evidence that reassociation of the monomeric units of tomato PFK occurs. However, association–dissociation for PFK from animal sources is well established [19] and it may be that such a system operates in plants. In a previous publication [16], tomato PFK oligomer was shown to dissociate by conditions that are unlikely to exist *in vivo* such as elevated pH. However, here we show that tomato PFK oligomer may be promoted to dissociate by physiological concentrations of a cellular component, Pi.

In addition to its effect on the molecular form of PFK, Pi also has a direct effect on the kinetics of both the oligomeric and monomeric forms of tomato PFK. This effect may be related to the energy charge concept by which the rate of ATP regenerating systems such as PFK was postulated to decrease as the energy charge is increased [20]. It has been reported that the term (ATP)/(ADP)(Pi) (phosphorylation state) could be a more useful concept than that of the energy charge since it has an important thermodynamic significance and bears directly on free energy changes and on equilibria in enzyme systems [21]. It is likely that the oligomeric form of the enzyme is the major form of the enzyme existing *in vivo* under normal conditions [16]. Thus, when the phosphorylation state is high inside the cell, the Pi level is low and there is an excess of ATP, which stabilizes this oligomeric form of PFK. The negative cooperativity exhibited by this form with F6P and the inhibition by PEP which does not alter this cooperativity makes the PFK oligomer resistant to fluctuations in substrate levels and would tend to maintain a constant rate of activity [17]. On the other hand, if the phosphorylation state is low, and there would be an excess of Pi and ADP compared to ATP, the oligomeric form could be stimulated by relieving its inhibition by PEP and to a lesser extent by citrate. Pi may also promote its dissociation to yield the monomeric form of PFK which, unlike the oligomeric form, exhibits non-interactive kinetics with F6P [17]. The non-cooperative interactions and higher affinity of the PFK monomer to F6P ($S_{0.5}$ = 1.8 mM) compared with the negative cooperativity and lower affinity to F6P ($S_{0.5}$ = 6.6 mM) observed for the PFK oligomer would lead to an increase in PFK activity for a given substrate concentration as a result of dissociation [17]. The role of PFK during the rise in glycolytic activity associated with the climacteric rise in the ripening of tomato fruit will be discussed in a forthcoming report.

EXPERIMENTAL

Tomatoes variety Euro Cross BB were grown in the greenhouse of the Food Research Institute. PFK was extracted and

purified using a combination of blue Sepharose and hexyl ATP Sepharose chromatography [16]. For gel permeation chromatography, the PFK extract was concentrated using polyethylene glycol 4000 (BDH, Poole, U.K.) up to 65% concentration. Following centrifugation at 20000 *g* for 20 min, the precipitate was taken up in 50 mM Tris HCl or phosphate buffer at pH 7.5 containing 2 mM EDTA and 5 mM DTE. 5 ml of this enzyme preparation was applied to an Ultrogel ACA 34 (LKB, Uppsala, Sweden) column (34 × 2.6 diam.) and the column eluted with the same buffer. Full details of this procedure are described elsewhere [16].

The standard 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 GDH, 2 units TIM and 0.4 units of aldolase. The reaction rate, at 25°, was initiated by the addition of fructose-6-phosphate and was followed by measuring the decrease in the fluorescence of NADH using an Eppendorf fluorimeter [16]. For kinetic studies, Hill equation was used in the form $V = (V_0 I^h)/(Ki + I^h)$ for inhibition studies and in the form $v = (Va\, A^h)/(Ka + A^h)$ for activation studies [22] where v is the reaction rate in the presence of the modulator; V_0 is the initial velocity in the absence of modulators; I and A are the concentration of the inhibitor and activator respectively; h is the Hill coefficient, Va is the maximum velocity in the presence of an activator and Ki and Ka are constants. The kinetic data is presented as double reciprocal ($1/v$ against $1/s$) or Eadie Hofstee plots (v against v/s) and the kinetic parameters were determined using a computer iteration procedure as previously described [17].

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

REFERENCES

1. Sols, A., Castano, J. G., Aragon, J. J., Domenech, C., Lazo, P. A. and Nieto, A. (1981) in *Metabolic Interconversion of Enzymes* (Holzer, H., ed.) pp. 112–123. Springer, Berlin.
2. Hers, H. G. (1984) *Biochem. Soc. Trans.* **12**, 729.
3. Uyeda, K. (1979) *Adv. Enzymol.* **48**, 193.
4. Caldwell, R. A. and Turner, J. R. (1979) *Phytochemistry* **18**, 318.
5. Dennis, D. T. and Coultate, T. P. (1967) *Biochim. Biophys. Acta* **146**, 129.
6. Garrard, L. A. and Humphreys, T. E. (1968) *Phytochemistry* **7**, 1949.
7. Kelly, G. J. and Latzko, E. (1977) *Plant Physiol.* **60**, 295.
8. Kelly, G. J. and Turner, J. F. (1971) *Biochim. Biophys. Acta* **242**, 559.
9. Salminen, S. O. and Young, R. E. (1975) *Plant Physiol.* **55**, 45.
10. Sasaki, T., Tadokora, K. and Suzuki, S. (1973) *Phytochemistry* **12**, 2843.
11. Sutton, B. G. (1975) *Aust. J. Plant. Physiol.* **2**, 403.
12. Turner, J. F., Tomilson, J. D. and Caldwell, R. A. (1980) *Plant Physiol.* **66**, 943.
13. Garland, W. J. and Dennis, D. T. (1980) *Arch. Biochem. Biophys.* **204**, 310.
14. Chalmers, D. J. and Rowan, K. S. (1971) *Plant Physiol.* **48**, 235.
15. Turner, J. F. and Turner, D. H. (1980) in *The Biochemistry of Plants* (Davies, D. D., ed.) Vol. 2, pp. 279 316.
16. Isaac, J. E. and Rhodes, M. J. C. (1982) *Phytochemistry* **21**, 1553.
17. Isaac, J. E. and Rhodes, M. J. C. (1986) *Phytochemistry* **25**, 339.
18. Isaac, J. E. and Rhodes, M. J. C. (1986) *Phytochemistry* **25**, 345.
19. Luther, M. A., Hesterberg, L. K. and Lee, J. C. (1985) *Biochemistry* **24**, 2463.
20. Atkinson, D. E. (1968) *Biochemistry* **7**, 4030.
21. Krebs, H. A. (1973) *Soc. Exp. Biol. Symp.* **27**, 299.
22. Taketa, K. and Pogell, B. M. (1965) *J. Biol. Chem.* **240**, 651.