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COOPERATIVITY IN PEA-SEED PHOSPHOFRUCTOKINASE

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SUMMARY

1. The effects of concentration of substrates on the activity of pea-seed phosphofructokinase (ATP:D-fructose 6-phosphate 1-phosphotransferase, EC 2.7.1.11) were examined by determining substrate saturation curves and replotting these according to the Hill equation.

2. The curve of enzyme activity *versus* concentration of Fru-6-*P* contained two points of inflection and an intermediary plateau region. This curve was consistent with negative cooperative kinetics at lower Fru-6-*P* concentrations and positive cooperative kinetics at higher concentrations of Fru-6-*P*.

3. The inhibitor phosphoenolpyruvate induced a change from negative cooperative kinetics to positive cooperative kinetics for Fru-6-*P* at low concentrations of this substrate. In the presence of the stimulator P_i the positive cooperative kinetics for Fru-6-*P* at high concentrations were altered towards Michaelis–Menten kinetics.

4. The interactions between pea-seed phosphofructokinase and the substrate $MgATP^{2-}$ followed negative cooperative kinetics and were not influenced by either the stimulator P_i or the inhibitor phosphoenolpyruvate.

5. Possible explanations for these kinetic properties of pea-seed phosphofructokinase are considered and the relevance of the results to the possible regulatory role of the enzyme in plant carbohydrate metabolism is discussed.

INTRODUCTION

Sigmoid curves of enzyme activity *versus* concentration of ligand (substrate or modifier) have been found in a number of investigations on the glycolytic enzyme phosphofructokinase (ATP:D-fructose 6-phosphate 1-phosphotransferase, EC 2.7.1.11)[1–6]. Sigmoid curves are consistent with interactions between the enzyme molecule and ligand following positive cooperative kinetics[7]. Cooperative kinetics provide evidence that at least two molecules of ligand interact with the enzyme and that the interaction with one ligand molecule in some manner facilitates (positive cooperativity) or hinders (negative cooperativity) the interaction of the next ligand molecule with the same enzyme molecule (see ref. 8).

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Previous studies in this laboratory have shown positive cooperative kinetics for the inhibition of pea-seed phosphofructokinase by the glycolytic intermediates *P*-enolpyruvate[6,9], 3-*P*-glycerate and 2-*P*-glycerate[10]. The present communication describes the kinetics of the enzyme reaction with respect to substrates and the effects of an inhibitor (*P*-enolpyruvate) and a stimulator (P_i) on these kinetics. Both positive and negative cooperative kinetics for the interactions between pea-seed phosphofructokinase and the substrates were observed. Possible explanations for the kinetics are considered.

MATERIALS AND METHODS

Materials

Pea-seeds (var. Progress No. 9) were purchased from F. Cooper, Ltd, Wellington, New Zealand. The following chemicals and enzymes were purchased from the Sigma Chemical Co., St. Louis, Mo., U.S.A.: sodium salts of Fru-6-*P*, Fru-1,6-P_2, ATP, *P*-enolpyruvate and β-NADH, and $(NH_4)_2SO_4$ suspensions of Fru-1,6-P_2 aldolase, α-glycerophosphate dehydrogenase and triosephosphate isomerase. All other reagents were A.R. grade. Enzymes used in assays were dialysed against 5 mM imidazole–HCl buffer (pH 7.6) before use to remove $(NH_4)_2SO_4$.

Partial purification of pea-seed phosphofructokinase

Phosphofructokinase was purified 27-fold from defatted pea powder[11] using a combination of fractionations with ethanol and saturated $(NH_4)_2SO_4$ (ref. 9). The preparation was free from enzymes interfering in the assay system.

Assay of phosphofructokinase activity

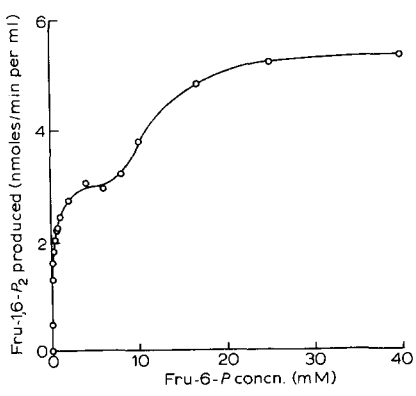
Enzyme activity was determined spectrophotometrically[9]. The standard reaction mixtures contained, in a final volume of 3.0 ml, 60 μmoles imidazole–HCl buffer (pH 7.6), 4.8 μmoles $MgCl_2$, 10.5 μmoles thioglycollate, 1.5 μmoles Fru-6-*P*, 0.75 μmole ATP, 0.20 μmole β-NADH, 50 μg aldolase, 12 μg α-glycerophosphate dehydrogenase–triosephosphate isomerase, and 50 μl (containing approx. 70 μg protein) of a 5-fold dilution of the pea-seed phosphofructokinase preparation.

Reaction mixtures were maintained at 28°. The reaction velocity was directly proportional to the amount of enzyme added with phosphofructokinase activities up to 5 times that used in the standard assay system. The reaction velocity remained constant for at least 10 min and was not increased when the quantity of coupling enzymes was increased to 5 times that employed in the standard assay.

RESULTS

Kinetics for Fru-6-P

The effect of Fru-6-*P* concentration on pea-seed phosphofructokinase activity is shown in Fig. 1. The reaction velocity increased as the Fru-6-*P* concentration was increased to 4 mM, but the velocity showed no significant change when the Fru-6-*P* concentration was increased from 4 to 8 mM. Enzyme activity was enhanced by further increase in the Fru-6-*P* concentration until a maximum velocity was attained.



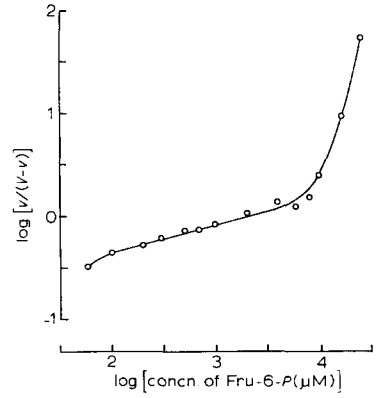


Fig. 1. Effect of Fru-6-*P* concentration on phosphofructokinase activity. The reaction mixtures were of the composition described for the standard assay, except that the concentration of Fru-6-*P* was varied as shown.
Fig. 2. Hill plot of the results shown in Fig. 1. V is the maximum velocity; v is the rate with the different concentrations of Fru-6-*P*.

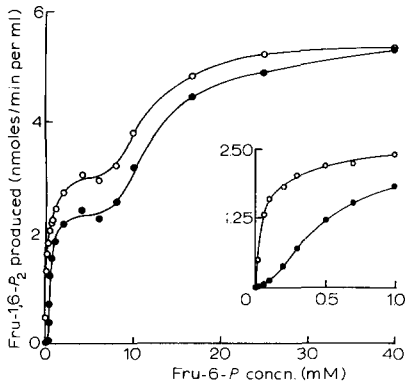
The relative insensitivity of the enzyme to changes in the Fru-6-*P* concentration between 4 and 8 mM resulted in a substrate saturation curve with two points of inflection and an intermediary plateau region. TEIPEL AND KOSHLAND[8] have shown that curves of this type are compatible with a transition from negative cooperative kinetics to positive cooperative kinetics as the concentration of ligand is increased.

The data in Fig. 1 were analysed using the equations of LINEWEAVER AND BURK[12] and HILL[13]. For concentrations of Fru-6-*P* between 0.06 and 6 mM (approx.) the Lineweaver–Burk plot gave a curve convex to the 1/(Fru-6-*P* concn.) axis[9] and a Hill plot with a slope of 0.3 (Fig. 2). LEVITZKI AND KOSHLAND[7] have proposed that convex Lineweaver–Burk plots and Hill plots with slopes of less than 1 may be expected from negative cooperative kinetics. With Fru-6-*P* concentrations between 6 and 25 mM, the slope of the Hill plot increased markedly to approx. 4, a result consistent with positive cooperativity (Fig. 2). These results suggest that the interactions between pea-seed phosphofructokinase and Fru-6-*P* followed negative cooperative kinetics at Fru-6-*P* concentrations below 6 mM, but at concentrations greater than this the interactions followed positive cooperative kinetics.

Effect of P-enolpyruvate on the Fru-6-P saturation curve

P-enolpyruvate strongly inhibits pea-seed phosphofructokinase[9]. To determine the effect of an inhibitor on the Fru-6-*P* saturation curve, the experiment described in Fig. 1 was repeated with the inclusion of *P*-enolpyruvate (4.2 nmoles).

In Fig. 3 the results are compared with those obtained in the absence of *P*-enolpyruvate. At low concentrations of Fru-6-*P*, *P*-enolpyruvate caused an alteration in the shape of the Fru-6-*P* saturation curve from hyperbolic to sigmoid. This is clearly shown in the insert in Fig. 3, and suggests that *P*-enolpyruvate induced a change from negative cooperative kinetics to positive cooperative kinetics for the interaction between the enzyme and Fru-6-*P*. The change in cooperativity is also shown in a Hill plot (Fig. 4) of the results in Fig. 3. The slope of the line obtained, with Fru-6-*P*



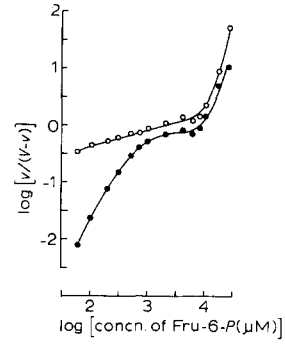
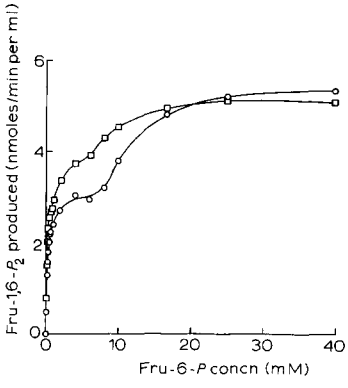


Fig. 3. Effect of *P*-enolpyruvate on the Fru-6-*P* saturation curve. The reaction mixtures were of the composition described for the standard assay, except that the concentration of Fru-6-*P* was varied as shown. ○, no *P*-enolpyruvate; ●, with 1.4 μM *P*-enolpyruvate.

Fig. 4. Hill plots of the results shown in Fig. 3. *V* is the maximum velocity; *v* is the rate with the different concentrations of Fru-6-*P*. ○, no *P*-enolpyruvate; ●, with 1.4 μM *P*-enolpyruvate.

concentrations below 0.5 mM, was greater than 1 in the presence of *P*-enolpyruvate but less than 1 in the absence of *P*-enolpyruvate. With Fru-6-*P* concentrations in excess of 1 mM, the shape of the Fru-6-*P* saturation curve was not significantly influenced by the presence of *P*-enolpyruvate. Previous work in this laboratory demonstrated that the *P*-enolpyruvate inhibition of pea-seed phosphofructokinase was relieved by increased concentrations of Fru-6-*P* (ref. 9). The results in Figs. 3 and 4 indicate that *P*-enolpyruvate induced a change from negative cooperative kinetics to positive cooperative kinetics for the substrate Fru-6-*P* only when the concentration of Fru-6-*P* was too low to relieve the inhibition by *P*-enolpyruvate.



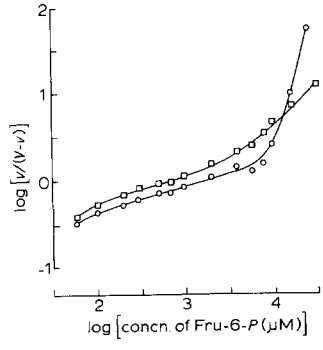


Fig. 5. Effect of P_i on the Fru-6-*P* saturation curve. The reaction mixtures were of the composition described for the standard assay, except that the concentration of Fru-6-*P* was varied as shown. ○, no P_i; □, with 0.5 mM P_i.

Fig. 6. Hill plot of the results shown in Fig. 5. *V* is the maximum velocity; *v* is the rate with the different concentrations of Fru-6-*P*. ○, no P_i; □, with 0.5 mM P_i.

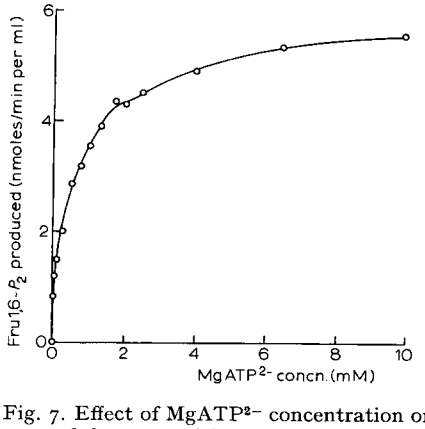
Effect of P_i on the Fru-6-P saturation curve

P_i stimulates pea-seed phosphofructokinase activity and relieves the inhibition of enzyme activity by *P*-enolpyruvate[9]. Fig. 5 shows the Fru-6-*P* saturation curve obtained in the presence and absence of 0.5 mM P_i. The addition of P_i resulted in a decrease in the prominence of the intermediary plateau region. The slope of the Hill plot of the Fru-6-*P* saturation curve was not significantly influenced by P_i at Fru-6-*P* concentrations below approx. 4 mM (Fig. 6), but at higher Fru-6-*P* concentrations the slope did not increase to the high values observed when P_i was absent. Comparison of the curves in Fig. 5 shows that P_i was most effective in increasing the affinity of the enzyme for Fru-6-*P* when the Fru-6-*P* concentration was in the vicinity of the intermediary plateau region. This may have been related to the lack of positive cooperative kinetics for Fru-6-*P* in the presence of P_i (Fig. 6).

Kinetics for $MgATP^{2-}$

The second substrate for pea-seed phosphofructokinase is $MgATP^{2-}$. Free ATP inhibits the enzyme activity[9]. In determining the substrate saturation curves for $MgATP^{2-}$, a fixed excess (2 mM) of $MgCl_2$ was added above the concentration of ATP. This technique, based on the suggestion of PAETKAU AND LARDY[14], ensured that the results were not complicated by increasing concentrations of free ATP or the effects of changing concentrations of Mg^{2+}.

Negative cooperative kinetics were found for the interactions between pea-seed phosphofructokinase and $MgATP^{2-}$ (Figs. 7 and 8). The substrate saturation curve (Fig. 7) is similar to that expected from Michaelis–Menten kinetics. However, a Hill plot of the results (Fig. 8) has a slope which gradually increased from 0.4 at low concentrations of $MgATP^{2-}$ to 1.0 at the highest concentrations tested, showing that negative cooperative kinetics were present at lower concentrations of $MgATP^{2-}$ while the kinetics approached Michaelis–Menten type at higher concentrations of this sub-



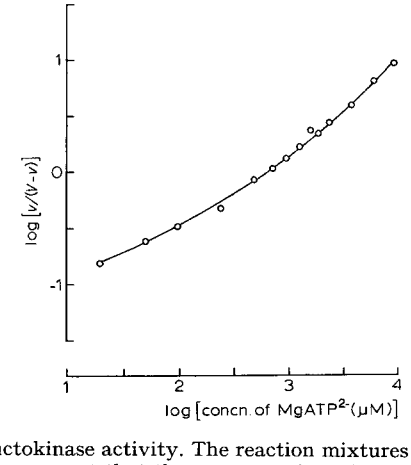


Fig. 7. Effect of $MgATP^{2-}$ concentration on phosphofructokinase activity. The reaction mixtures were of the composition described for the standard assay, except that the concentration of ATP was varied as shown and the concentration of $MgCl_2$ was kept in excess of the ATP concentration by 2 mM.

Fig. 8. Hill plot of results shown in Fig. 7. *V* is the maximum velocity; *v* is the rate with the different concentrations of $MgATP^{2-}$.

strate. The curved Hill plot may have resulted from a gradual decrease in the strength of the negative cooperative interactions between the enzyme and $MgATP^{2-}$ as the concentration of $MgATP^{2-}$ was increased towards saturating levels.

The small intermediary plateau region of the $MgATP^{2-}$ saturation curve (Fig. 7) may be significant, since the Hill plot (Fig. 8) shows a tendency for a change from negative to positive cooperativity, a feature compatible with the substrate saturation curve having an intermediary plateau[8].

The effects of modifiers on the $MgATP^{2-}$ saturation curve

No significant changes in the kinetics for $MgATP^{2-}$ were found when the experiments described in Figs. 7 and 8 were repeated with the inclusion of the inhibitor *P*-enolpyruvate (4.2 nmoles) or the stimulator P_i (1.5 μmoles) in the reaction mixtures.

DISCUSSION

Although phosphofructokinases from a number of sources have been extensively studied, the pea-seed enzyme used in this investigation appears to be the first phosphofructokinase for which negative cooperative kinetics have been reported. Results from other laboratories indicate that phosphofructokinases from other plant tissues, mammalian tissues, bacteria and yeast showed either Michaelis–Menten kinetics or positive cooperative kinetics for the substrate Fru-6-*P* (refs. 5, 15–18). Negative cooperative kinetics have been observed with a number of other enzymes including *P*-enolpyruvate carboxylase[19], glutamate dehydrogenase[20] and ADP–glucose pyrophosphorylase[21]. Substrate saturation curves for these enzymes possessed intermediary plateau regions similar to that observed with pea-seed phosphofructokinase.

Six possible explanations for negative cooperative kinetics were suggested by LEVITZKI AND KOSHLAND[7]. These authors concluded that the negative cooperative kinetics observed for the effect of one ligand on the activity of an enzyme probably result from ligand-induced conformational changes affecting subunit interactions within the enzyme if the same enzyme shows positive cooperative kinetics with respect to a second ligand. Previous experiments in this laboratory have shown positive cooperative kinetics for the interactions between pea-seed phosphofructokinase and six compounds which inhibited enzyme activity, *viz.* 6-*P*-gluconate, 3-*P*-glycerate, 2-*P*-glycerate, *P*-enolpyruvate, ADP and citrate[9,10]. With each inhibitor positive cooperativity was observed over the range of no inhibition to almost complete inhibition. Ligand-induced conformational changes affecting subunit interactions may therefore be an explanation for the substrate saturation curves of pea-seed phosphofructokinase.

Another, but less likely, explanation for the kinetic data is that the enzyme preparation contained two or more phosphofructokinases[7]. In this case, an interesting assumption would be necessary, *viz.* that pea seeds contain at least two phosphofructokinases with widely differing affinities for the substrates, but with similar regulatory properties with respect to 6-*P*-gluconate, 3-*P*-glycerate, 2-*P*-glycerate, *P*-enolpyruvate, ADP and citrate.

The kinetic properties of phosphofructokinase may be important in the regulation of plant carbohydrate metabolism[10]. Pea seeds contain approx. 15 μmoles Fru-6-*P*

per 100 g fresh weight[22]. At similar Fru-6-*P* concentrations *P*-enolpyruvate induced a change from negative cooperative kinetics to positive cooperative kinetics for the interaction between pea-seed phosphofructokinase and Fru-6-*P*. This change could form part of an *in vivo* mechanism for the regulation of enzyme activity by *P*-enolpyruvate. 2-*P*-glycerate and 3-*P*-glycerate may also be significant since the effects of these two compounds on the enzyme activity were similar to those of *P*-enolpyruvate[10].

The content of Fru-6-*P* in pea seeds[22] is equivalent to less than 4% of the concentration at which phosphofructokinase was relatively insensitive to changes in the level of this substrate (Fig. 1). Hence this insensitivity may not be important *in vivo* unless the enzyme and Fru-6-*P* are subject to compartmentation. In this connexion it is interesting to note that BARKER *et al.*[22] have suggested the existence in pea seeds of glycolytic structures associated with hexokinase and phosphofructokinase.

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