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Biological action of phosphonate analogs of fructose 2,6-bisphosphate on enzymes from higher plants

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Two analogs of fructose 2,6-bisphosphate have been prepared, in which the phosphoryl group on the second carbon is replaced by a methylphosphonoyl or a phosphonoyl group. Both inhibited spinach leaf cytosolic fructose-1,6-bisphosphatase and activated potato tuber pyrophosphate:fructose-6-phosphate phosphotransferase. Their relative effectiveness depended on the enzyme, revealing differences in the fructose 2,6-bisphosphate binding sites on the two enzymes. Their relative effectiveness and their binding constants were the same when pyrophosphate:fructose-6-phosphate phosphotransferase was assayed in the forward and in the reverse direction. It is concluded that pyrophosphate:fructose-6-phosphate phosphotransferase possesses one type of fructose 2,6-bisphosphate binding site, and catalysis is modified in parallel in both directions as the activator concentration is altered. These results led to the reinterpretation of the role of pyrophosphate:fructose-6-phosphate phosphotransferase and fructose 2,6-bisphosphate in higher plants.

Fructose 2,6-bisphosphate; Fructose-1,6-bisphosphatase; Pyrophosphate:fructose-6-phosphate phosphotransferase; Regulation; (Phosphonoyl analog)

1. INTRODUCTION

The cytosolic FBPase from higher plants resembles its mammalian counterpart, in being inhibited by $Fru2,6P_2$ [1]. However, ATP-phosphofructokinase from higher plants is not activated by $Fru2,6P_2$ [2–4]. Instead, $Fru2,6P_2$ activates an enzyme called PFP, which catalyses the reversible phosphorylation of Fru6P using PP_i as a phosphoryl donor [2,3,5,6]. This enzyme is widely distributed in plants but its role and the significance of the activation by $Fru2,6P_2$ remain controversial.

PFP is activated by lower concentrations of $Fru2,6P_2$ when assayed in the forward (glycolytic) rather than the reverse (gluconeogenic) direction [5,6]. These differences might be due to Fru6P increasing the affinity for $Fru2,6P_2$, while P_i decreases the affinity [5,6] and it was suggested that the physiological role of PFP is in the glycolytic direction [5]. Many later studies have assumed that increasing $Fru2,6P_2$ would stimulate glycolysis by activating PFP, in an analogous manner to the activation of phosphofructokinase by $Fru2,6P_2$ [3,4,7,8]. However, based on a comparison of $Fru2,6P_2$ levels and PFP activities in different tissues, it has also been suggested that PFP may operate as a gluconeogenic enzyme [9,10] or that it may provide the PP_i needed for sucrose mobilisation via sucrose synthase [11–15].

Recently, analogs of $Fru2,6P_2$ have become available, in which the phosphoryl group on the second carbon is replaced by a phosphonoyl group [16] or a methyl phosphonyl group [17]. The methylphosphonoyl analog (fig.1A) is isosteric to $Fru2,6P_2$ but non-isopolar. The phosphonoyl

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Abbreviations: FBPase, fructose-1,6-bisphosphatase; Fru1, $6P_2$, fructose 1,6-bisphosphate; $Fru2,6P_2$, fructose 2,6-bisphosphate; Fru6P, fructose 6-phosphate; PFP, pyrophosphate: fructose-6-phosphate phosphotransferase; P_i, inorganic phosphate; PP_i, inorganic pyrophosphate

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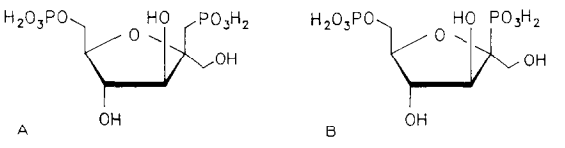

Fig.1. Structures of the analogs to Fru2,6P_2. (A) Isosteric analog; (B) isopolar analog.

analog (fig.1B) is isopolar to Fru2,6P_2, but non-isosteric. In this article we compare their effectiveness as inhibitors of spinach leaf cytosolic FBPase, and as activators of potato tuber PFP. The results show that a single type of site is responsible for activating catalysis by PFP, irrespective of whether this enzyme is catalysing the forward (PP_i-consuming, glycolytic) or reverse (PP_i-generating, gluconeogenic) reaction. This has implications for the role of PFP and Fru2,6P_2 in plant metabolism.

2. MATERIALS AND METHODS

Analogs of Fru2,6P_2 were synthesised as in [16,17]. Spinach leaf cytosolic FBPase was partially purified as in [1] and potato tuber PFP was partially purified as in [5]. All analogs and other biochemicals (except NaDH) were incubated for 10 min at pH 2 to ensure they did not contain traces of Fru2,6P_2. Enzymes were assayed in 50 mM imidazole-HCl (pH 7.3), 4 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA. For the assay of FBPase activity and PFP activity in the reverse direction, 0.4 mM NADP, 14 U/ml phosphoglucose isomerase and 3 U/ml glucose-6-phosphate dehydrogenase were included. For the assay of PFP in the forward direction, 0.12 mM NADH, 2 U/ml glycerol-3-phosphate dehydrogenase, 7 U/ml triose phosphate isomerase and 2 U/ml aldolase were included. All assays of PFP included 8 mM P_i. For concentrations of Fru6P, Fru1,6P_2, Fru2,6P_2 and analogs see the figure legends. To calculate K_a values [18], double reciprocal plots were used to obtain values for the y-axis intercept ($= V_{max}^{app}$) and the slope ($= K_s^{app}/V_{max}^{app}$) at different activator concentrations. The corresponding value obtained in the absence of an activator was subtracted, yielding the terms Δintercept and Δslope. Plots of $(\Delta\text{intercept})^{-1}$ and $(\Delta\text{slope})^{-1}$ against $(\text{activator concentration})^{-1}$ yield straight lines which intersect in the negative region on the x-axis. K_a is estimated as $K_a = (-\beta/\alpha)$ (x-axis intercept), where $\beta = (V_{max}^{app}$ with saturating activator$) \times (V_{max}$ without activator$)^{-1}$ and $\alpha = (K_s^{app}$ with saturating activator$) \times (K_s^{app}$ without activator$)^{-1}$. In cases where the activator does not alter the V_{max}, only Δslope can be estimated.

3. RESULTS

3.1. *Comparison of the cytosolic FBPase and PFP*

Both analogs inhibited spinach leaf cytosolic FBPase (fig.2A), but the isosteric analog was 3-times more effective than the isopolar analog. Both analogs also activated PFP when it was catalysing the forward (fig.2B) and the reverse (fig.2C) reaction. However, the isopolar analog was now 13-fold more effective than the isosteric analog. Differences in the sensitivity of rat liver phosphofructokinase and FBPase to the Fru2,6P_2 analogs α- and β-D-arabinose-1,5-bisphosphate have also been found [19]. Apparently, there are differences between the Fru2,6P_2 binding sites, depending on the enzyme. This could reflect the different action of Fru2,6P_2, which increases the affinity of PFP for Fru6P and Fru1,6P_2 (see section 1) while it binds close to the active site of the FBPase and competes with binding of Fru6P and Fru1,6P_2 [20]. This selectivity might be of practical

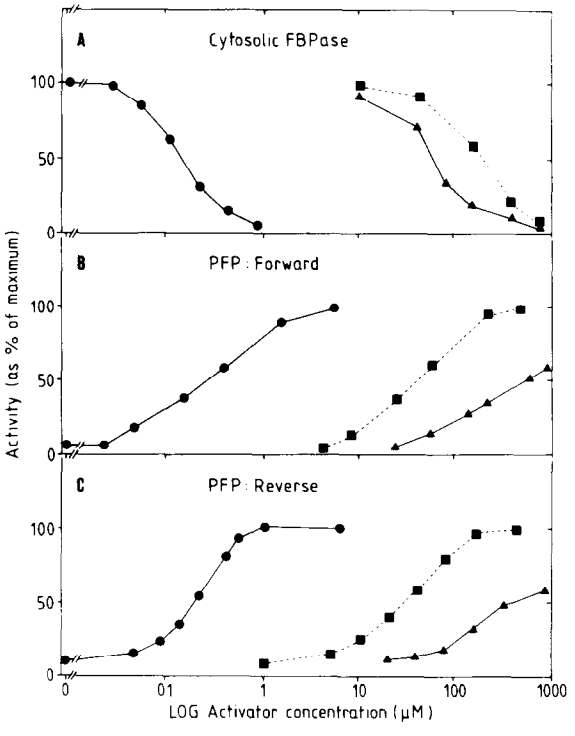

Fig.2. Sensitivity of spinach leaf cytosolic FBPase and potato tuber PFP. (A) FBPase, assayed with 40 μM FBP; (B) PFP (forward direction) assayed with 0.83 mM Fru6P and 0.5 mM PP_i; (C) PFP (reverse direction) assayed with 20 μM Fru1,6P_2 and 8 mM P_i. The activity is given as a percentage of the V_{max} activity, with increasing (note log scale) concentrations of Fru2,6P_2 (●—●), an isosteric but non-isopolar phosphonate analog (▲—▲) and an isopolar but non-isosteric phosphonate analog (■---■).

value. For example, 100 μM of the isosteric analog almost completely inhibits the cytosolic FBPase, but hardly activates the reverse reaction of PFP, providing a useful method for distinguishing these reactions in extracts.

3.2. *Forward and reverse reaction of PFP*

P_i is needed as a substrate for the reverse reaction, but it also decreases the affinity for Fru2,6P_2 (see section 1). We therefore included an identical P_i concentration in the assay for the forward reaction. Provided this is done, both reactions are activated by similar concentrations of Fru2,6P_2 (cf. fig.2B and C). The analogs act at 150-fold (isopolar analog) and 2000-fold (isosteric analog) higher concentrations than Fru2,6P_2.

Fig.3 demonstrates the kinetic properties of PFP in more detail. Each of the activators increased V_{max} by about 10-fold in the forward direction (solid line) but not in the reverse direction (dotted line). The absence of any effect on the V_{max} of the reverse reaction could just reflect the fact that Fru1,6P_2 can substitute for Fru2,6P_2 and activate PFP, if the concentration is high enough [2]. The K_s^{app} (Fru6P) and the K_s^{app} (Fru1,6P_2) decreased in parallel over the concentration range where an activator was effective. The K_s^{app} (Fru1,6P_2) decreased about 25-fold from 0.26 mM to about 0.01 mM, while the K_s^{app} (Fru6P) decreased by about 10-fold from 4.5 mM to 0.46 mM. Thus, rising concentrations of activator lead to a simultaneous stimulation of catalysis in both directions.

We also compared the binding constants for ac-

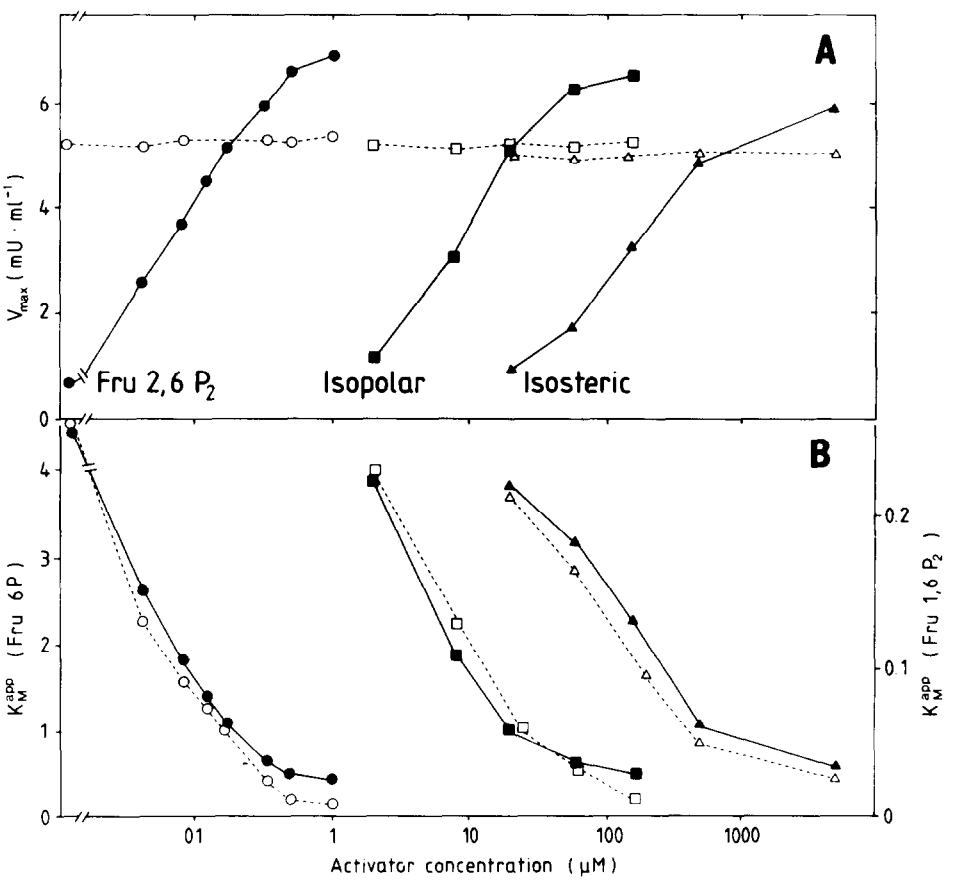

Fig.3. Response of substrate affinity and maximal PFP activity. (A) V_{max} activity; (B) affinity for Fru6P and Fru1,6P_2. Fru2,6P_2 (●,○), the isopolar (■, □) or the isosteric (▲, △) analog were added at different concentrations (note log scale). At each concentration, the substrate saturation curve was measured in the forward (●, ■, ▲) or in the reverse (○, □, △) direction.

tivation of the forward and reverse reactions. It is important to note that Fru6P and Fru1,6P_2 alter the affinity for Fru2,6P_2, just as Fru2,6P_2 alters the affinity for Fru6P and Fru1,6P_2. A meaningful comparison can therefore only be made by measuring K_a, the binding constant obtained by extrapolation to zero Fru6P or zero Fru1,6P_2. These were calculated using a method [18] which is related to the derivation of a K_i value for mixed-type and partial inhibitor (see section 2 for details). Derivative plots are shown for Fru2,6P_2 activation of the forward (fig.4A) and reverse (fig.4B) reactions. They yield K_a values of 0.91 and 0.99 μM, respectively. Similar plots (not shown) for the forward and reverse reactions gave K_a values of 100 and 136 μM for the isopolar analog, and 2480 and 2040 μM for the isosteric analog. Thus, the forward and reverse reactions show the same selectivity and the same binding constants for three different activators. We conclude that a single type of binding site is responsible for the activation of catalysis in both directions.

3.3. *Physiological significance*

These results show that the differing sensitivity of PFP to Fru2,6P_2 in the forward and the reverse assay is an artefact, due to binding of Fru2,6P_2 being more strongly favoured in the conditions used to assay the forward reaction. Provided the P_i concentration is standardised and kinetic analysis is used to eliminate the influence of Fru1,6P_2 and Fru6P, it can be shown that the forward and reverse reactions are activated in parallel by comparable concentrations of Fru2,6P_2, acting at a single type of binding site.

This suggests that the role of Fru2,6P_2 may need to be re-evaluated. In vivo, PFP will be operating in the presence of all four of its potential substrates/products, and an increase of the Fru2,6P_2 concentration will activate the forward and the reverse reaction in parallel. The direction of the resulting flux will depend upon the prevailing mass action ratio, while the rate will also depend upon kinetic factors. Two considerations suggest that PFP could operate in either direction in vivo. Firstly, the reaction catalysed by PFP is probably close to equilibrium in plants [14,21]. Secondly, the K_m values for Fru6P, Fru1,6P_2, P_i and PP_i [5,6] compare favourably with the available estimates of the cytosolic metabolite concentrations in plants [21]. Thus, the kinetic properties of PFP do not provide any reason to expect this enzyme acts preferentially as a glycolytic enzyme, nor do they allow us to predict that increasing concentrations of Fru2,6P_2 will favour the glycolytic reaction. Rather, alterations of Fru2,6P_2 may act to engage and disengage a basically reversible reaction, and the direction and rate of the resulting flux will depend upon the requirements of the cell.

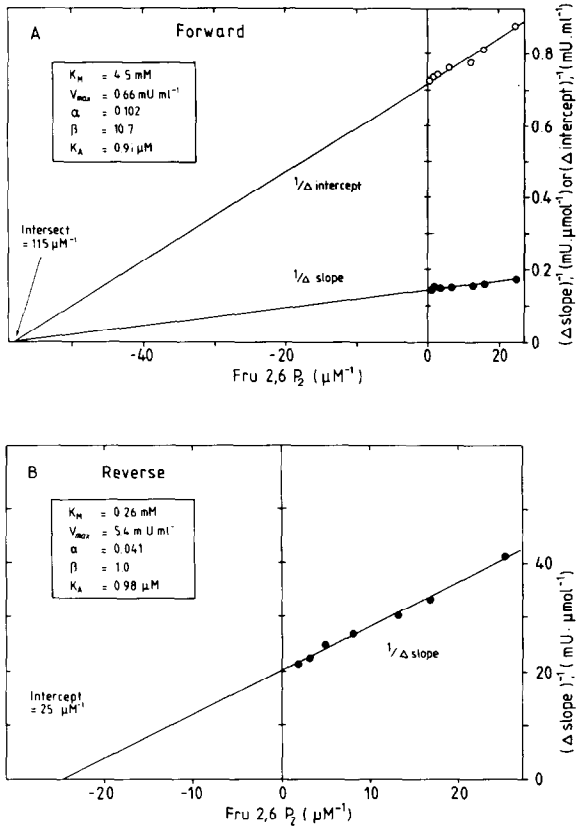

Fig.4. Derivative plots to estimate the K_a for Fru2,6P_2 of the potato tuber PFP. (A) Assayed while catalysing the forward (glycolytic) reaction; (B) assayed while catalysing the reverse (gluconeogenic) reaction. The analysis is based on [20]. The K_a values are estimated from the intercept on the x-axis (see section 2).

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