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Metabolite Activation of Crassulacean Acid Metabolism and C_4 Phospho*enol*pyruvate Carboxylase[1]

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ABSTRACT

The effects of glycine, alanine, serine, and various phosphorylated metabolites on the activity of phospho*enol*pyruvate (PEP) carboxylase from *Zea mays* and *Crassula argentea* were studied. The maize enzyme was found to be activated by amino acids at a site that is separate from the glucose 6-phosphate binding site. The combination of glycine and glucose 6-phosphate synergistically reduced the apparent K_m of the enzyme for PEP and increased the apparent V_{max}. Of the amino acids tested, glycine showed the lowest apparent K_a and caused the greatest activation. D-Isomers of alanine and serine were more effective activators than the L-isomers. Unlike the maize enzyme, the *Crassula* enzyme was not activated by amino acids. Activation of either the *Crassula* or maize enzyme by glucose 6-phosphate occurred without dephosphorylation of the activator molecule. Furthermore, the *Crassula* enzyme was activated by two compounds containing phosphonate groups whose carbon-phosphorus bonds were not cleaved by the enzyme. A study of analogs of glucose 6-phosphate with *Crassula* PEP carboxylase revealed that the identity of the ring heteroatom was a significant structural feature affecting activation. Activation was not highly sensitive to the orientation of the hydroxyl group at the second or fourth carbon positions or to the presence of a hydroxyl group at the second position. However, the position of the phosphate group was found to be a significant factor.

PEP[2] carboxylase (EC 4.1.1.31) catalyzes an essentially irreversible reaction between PEP and HCO_3^- in the presence of magnesium, forming OAA and Pi. The enzyme was first isolated from spinach leaves (24), but has since been shown to exist in virtually all plants (1). This enzyme plays a crucial role in the photosynthetic carbon metabolism of the CAM plant *Crassula argentea* and the C_4 plant *Zea mays* (16). The activity of PEP carboxylase is regulated by metabolic effectors and by the phosphorylation status of the enzyme (10). PEP carboxylase is inhibited by OAA, L-malate, and, in the case of C_4 plants, aspartate (16). The enzyme is activated by various phosphorylated metabolites such as Glc-6P (27), triose phosphate (5), AMP (20), and carbamyl phosphate (9).

Glc-6P and other phosphorylated metabolites regulate the activity of the *Crassula* or maize PEP carboxylase by reducing its K_m for PEP (5, 9, 16, 20). These compounds have generally been assumed to activate by a conventional allosteric mechanism. However, a recent report suggests an alternative mechanism, namely, that dephosphorylation of these activators at the catalytic site may elevate the level of carboxyphosphate, an unstable intermediate whose formation is rate limiting in the PEP carboxylase reaction (26). Many of the basic kinetic responses of the maize and *Crassula* enzymes to Glc-6P are similar (27). It is not clear whether the two enzymes respond similarly to various amino acids that have been reported to activate the maize enzyme (5, 7, 15). Although structurally quite different from phosphorylated activators of the enzyme, amino acids also reduce the K_m of the maize enzyme for PEP (5).

Our goals were to investigate the activation of PEP carboxylase by amino acids, to reexamine the activation mechanism of phosphorylated compounds, and to elucidate the structural features of Glc-6P that make it a strong activator. Our studies, in addition to revealing spatially distinct activation sites for Glc-6P and amino acids, have also demonstrated a strong synergistic interaction of these activators.

MATERIALS AND METHODS

Materials

Hydroxymethylphosphonate and phenylphosphonate were obtained from Johnson Matthey/Alfa. TSK DEAE 650M column packing was obtained from Supelco. Bio-Spin 6 chromatography columns were obtained from Bio-Rad Laboratories. All other reagents, unless otherwise specified, were obtained from Sigma Chemical Co.

Enzyme Purification

The day form of *Crassula argentea* PEP carboxylase was purified as previously described by Meyer et al. (14) with the following modifications. The extraction buffer contained 100 mM instead of 50 mM Hepes, pH 8.0, 2% (w/v) instead of 1% (w/v) PVP, and 1 mM benzamidine. All subsequent buffers, except for the final dialysis buffer, also contained 1 mM benzamidine. The PEG fractionation procedure included a 5 to 15% instead of 5 to 12% PEG-6000 cut prior to loading the extract onto a DEAE column. The enzyme had a specific

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[2] Abbreviations: PEP, phospho*enol*pyruvate; OAA, oxaloacetic acid; Glc-6P, D-glucose 6-phosphate; thio-Glc-6P, 5-thio-D-glucose 6-phosphate; Man-6P, D-mannose 6-phosphate; Gal-6P, D-galactose 6-phosphate; dGlc-6P, 2-deoxy-D-glucose 6-phosphate; myo-In-1P, D-myo-inositol 1-phosphate; Glc-1P, α-D-glucose 1-phosphate; Man-1P, α-D-mannose 1-phosphate; GlcN-6P, D-glucosamine 6-phosphate.

activity of 18 IU/mg at 25°C and a pH of 7.0. The protein concentration was 1.8 mg/mL as determined by the Bio-Rad protein assay, a dye binding technique based on the method of Bradford (3).

For the phosphatase studies (see Table II), crude maize PEP carboxylase (3.0 IU/mg) from Sigma Chemical Co. was desalted on a Bio-Spin 6 chromatography column and used without further purification. Although the specific activity suggests that the enzyme was no more than 14% pure, interference from NADH oxidase activity was not significant and no nonspecific phosphatase activity was present. For all other studies involving the maize enzyme, PEP carboxylase was purified from *Zea mays* L. (var Golden Cross Bantam) seedlings grown in a greenhouse for 4 weeks in commercial potting soil. Leaf tissue was harvested at midday and PEP carboxylase was purified as described by McNaughton et al. (13) with the following modifications. Enzyme was precipitated from the crude homogenate with 60% rather than 50% $(NH_4)_2SO_4$. After the hydroxylapatite column, fractions were concentrated by precipitation with 60% $(NH_4)_2SO_4$, desalted on Sephadex G-25M, and applied to a DEAE column (1.6 × 19 cm), rather than a Mono Q column. The enzyme was eluted from the DEAE column with a 0 to 200 mM gradient of KCl. Active fractions were pooled, precipitated with 60% $(NH_4)_2SO_4$, and desalted on Sephadex G-25M. Purified enzyme was mixed with an equal volume of glycerol and stored at −70°C. The resulting enzyme had a specific activity of 21 IU/mg (pH 7.8, 25°C) and showed a single band of 109 kD following SDS-PAGE. This procedure (13), which includes 10 μg/mL of chymostatin in all buffers, has been shown to prevent proteolysis of the enzyme.

Coupled Enzyme Assays

The routine assay mixture for both enzymes contained 13 IU of malate dehydrogenase (EC 1.1.1.37), 19 IU of lactate dehydrogenase (EC 1.1.1.27), and 0.1 mM NADH. The concentrations of the coupling enzymes used in these assays were sufficient, as calculated by the method of Segel (21), to achieve 99% of the final steady-state rate within 1 s. Buffer, $MgCl_2$, HCO_3^-, PEP, PEP carboxylase, and activator concentrations are indicated in the text where appropriate. All assays were run at 25°C in a Gilford Response spectrophotometer. Reactions were initiated by the addition of PEP carboxylase. Rates were measured by following the disappearance of NADH as a function of time. All reported rates are from linear portions of absorbance versus time curves (usually between 0 and 2 min).

Phosphate Assays

The assay mixture for the maize enzyme contained 25 mM Mops, pH 7.0, 3 mM $MgCl_2$, 10 mM HCO_3^-, 3 mM PEP, and 0.1 IU of PEP carboxylase as described by Walker et al. (25). The mixture for assays of the *Crassula* enzyme contained 50 mM Pipes, pH 7.0, 5 mM $MgCl_2$, 4 mM HCO_3^-, 0.2 mM PEP, and 0.06 IU of PEP carboxylase. These assay conditions provided subsaturating concentrations of PEP for both types of the enzyme. Reactions were initiated by the addition of PEP carboxylase. Aliquots (200 μL) were withdrawn at timed intervals and their Pi content determined chemically by the method of Kaplan and Pedersen (11) as modified by Walker et al. (25). Absorbances were measured at 700 nm as suggested by Baginski et al. (2). Rates were calculated from linear portions of absorbance versus time curves (usually between 0 and 2 min).

Data Analyses

Rate (IU/mg protein) data were fitted to the modified Michaelis-Menten equation below:

$$v = \frac{V_{max} \cdot S^n}{K_m{}^n + S^n}$$

where v, V_{max}, S, and n represent observed velocity, maximal velocity, total substrate concentration, and the Hill coefficient, respectively. Activation data were fitted to the cooperative saturation function below:

$$\frac{v - v_0}{v_0} = \frac{Act_{max} \cdot A^n}{K_a{}^n + A^n}$$

where v, v_o, Act_{max}, n, and A represent observed velocity, initial velocity, maximal activation, Hill coefficient, and total activator concentration, respectively.

RESULTS AND DISCUSSION

Activation of Maize PEP Carboxylase by Amino Acids

Gly, Ser, and Ala activated PEP carboxylase from maize (Table I; Fig. 1), but had no effect on the enzyme from *Crassula* (data not shown). This lack of activation of the CAM enzyme by amino acids extends the findings of Nishikido and Takanashi (15), who observed Gly activation of PEP carboxylase from monocotyledonous C_4 plants, but were unable to observe activation of the enzyme from C_3 and dicotyledonous C_4 plants. This fundamental difference in amino acid sensitivity prompted us to examine more closely activation of the maize enzyme by amino acids.

The first set of experiments was designed to see whether

Table I. *Activator Competition Studies with Maize PEP Carboxylase*

The standard coupled assay mixture was modified to include 50 mM Hepes, pH 7.8, 10 mM $MgCl_2$, 10 mM HCO_3^-, 0.2 mM PEP, and 0.02 IU of maize PEP carboxylase. Glc-6P (5 mM), Gly (10 mM), DL-Ala (10 mM), Man-6P (10 mM), and phenylphosphonate (10 mM) were added as indicated.

Compound(s)	Observed Activation
	%
Glc-6P	601
Man-6P	508
Phenylphosphonate	337
Gly	190
DL-Ala	169
Glc-6P + Man-6P	584
Glc-6P + phenylphosphonate	447
Glc-6P + Gly	745
Glc-6P + DL-Ala	721
Gly + DL-Ala	196

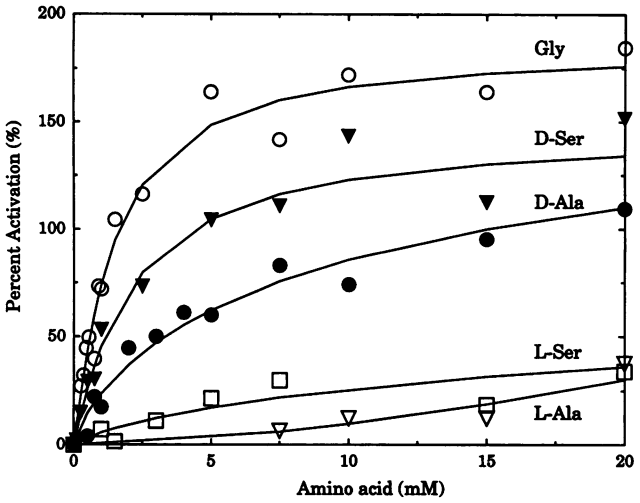

Figure 1. Activation of maize PEP carboxylase by Gly and D- and L-isomers of Ser and Ala. The coupled assay mixture was as described in the legend of Table I, except that these assays contained 0.5 mM PEP and 0.007 IU of PEP carboxylase.

amino acids such as DL-Ala or Gly compete with Glc-6P at a common regulatory site on the enzyme. If amino acids activate by binding to the same site as Glc-6P, rates obtained in the presence of saturating levels of both types of activators should not exceed rates obtained with the same effectors tested singly. When tested individually, Glc-6P, DL-Ala, and Gly strongly activated maize PEP carboxylase (Table I). Our data are consistent with previous reports of activation by these compounds (5, 7, 22). Assays containing near saturating levels of both Glc-6P and DL-Ala or Glc-6P and Gly showed synergism in that greater activation was obtained with the combination of activators than was obtained with either activator alone. In fact, the effects of these activators are nearly additive. These results suggest the presence of an amino acid activation site that is spatially distinct from the Glc-6P binding site. In contrast, the combination of DL-Ala and Gly produced no more activation than would have been expected from either activator alone. This suggests that DL-Ala and Gly compete for a single binding site. Similarly, the combination of Glc-6P and other phosphorylated activators produced no more activation than that caused by Glc-6P alone. Podesta et al. (17) have reported that Gly can protect maize PEP carboxylase from the inhibitory effects of glyphosine, whereas Glc-6P cannot. Their findings support the conclusion that Glc-6P and Gly bind at separate sites on the enzyme.

To further probe the synergistic effects of Glc-6P and Gly on the activity of the C_4 enzyme, we tested the effects of these compounds on the apparent K_m for total PEP in the presence and absence of saturating levels of the activators (Fig. 2). In the absence of activator, the K_m was 0.93 ± 0.15 mM, whereas in the presence of saturating levels of Glc-6P or Gly, it dropped to 0.089 ± 0.007 or 0.33 ± 0.02 mM, respectively. In the presence of both Glc-6P and Gly, the apparent K_m dropped to 0.036 ± 0.005 mM. As these data indicate, Glc-6P lowered the apparent K_m (total PEP) 10-fold, Gly lowered the K_m 3-fold, and a mixture of Glc-6P and Gly lowered the K_m 26-fold. The V_{max} increased 56% in the presence of Glc-6P and showed a 21% increase in the presence of Gly, whereas in the presence of both Glc-6P and Gly, the V_{max} increased 76%. Thus, the combination of Gly and Glc-6P synergistically activated the enzyme by reducing the K_m and raising the V_{max} beyond the levels seen with either activator alone.

Changes in the level of Glc-6P in the cytosol of C_4 mesophyll cells indirectly reflect the output of the Calvin cycle in the bundle sheath cells and play an important role in coordinating the rate of carboxylation of PEP with the turnover of the Calvin cycle (5). Separate activation sites for amino acids and sugar phosphates may allow the maize PEP carboxylase to respond simultaneously to regulatory signals from the Calvin cycle and photorespiration. In this view, the accumulation of Gly would signal a need for greater flux through the C_4 pathway to minimize photorespiration in the bundle sheath cells. Outwardly, C_4 plants show no apparent photorespiration due to lower rates of phosphoglycolate production by Rubisco and efficient trapping of the resulting CO_2. Although the level of photorespiration of a C_4 plant is much lower than that of a C_3 plant, it is not insignificant (4). In plants such as maize, for example, photorespiration can generate Gly and Ser at levels that are roughly 20% of a C_3 plant (4, 23).

Activation of PEP carboxylase by Gly suggests the possibility that photorespiratory intermediates generated in the bundle sheath cells may move into mesophyll cells. Although there are no experimental data to support this speculation, it has previously been suggested that Gly transport may occur between mesophyll and bundle sheath cells of *Moricandia arvensis*, a C_3–C_4 intermediate species (18). A study of intact maize mesophyll protoplasts has shown that mesophyll cells do respond to exogenous Gly (6). The addition of 20 mM Gly

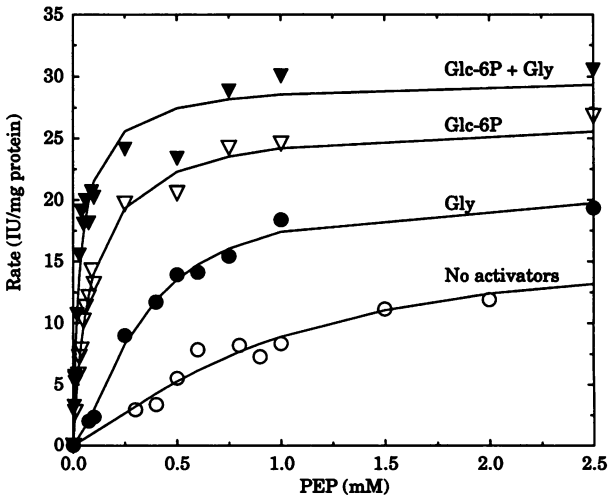

Figure 2. Substrate saturation curves for maize PEP carboxylase with different combinations of Gly and Glc-6P. The coupled assay mixture was as described in the legend of Table I, except that these assays contained 0.01 IU of PEP carboxylase. Glc-6P (15 mM) and Gly (10 mM) were added as indicated.

doubles the rate of CO_2 fixation in mesophyll cells, whereas it inhibits CO_2 fixation in bundle sheath cells (6). However, further data on the actual concentration of Gly in C_4 mesophyll and bundle sheath cells are needed to support this speculative physiological role for Gly activation. The insensitivity of CAM PEP carboxylase to amino acids may reflect the temporal separation of the PEP carboxylase and Rubisco activities within the cell. Because photorespiration products such as Gly and Ser would be generated during the day and CAM PEP carboxylase is active at night, the enzyme would not be expected to respond to these metabolites.

In subsequent experiments, we found that the amino acid activation site was not specific for L-amino acids. Surprisingly, at saturating levels of the amino acids, D-Ala activated the enzyme 2-fold more than L-Ala, and D-Ser activated the enzyme 3-fold more than L-Ser (Fig. 1). Gly was a better activator than either the D- or L- forms of Ser and Ala. Gly bound with an apparent K_a of 1.44 ± 0.26 mM and an Act_{max} of 185 ± 12% (Fig. 1). In contrast, the other amino acids bound with higher apparent K_a values and gave lower values of Act_{max}. The L-isomers were particularly weak activators (Fig. 1). Thus, the site appears to interact preferentially with Gly. The lower activation by L-isomers compared with D-isomers may simply reflect specificity of the binding site for the hydrogen atom R group of Gly. Although L-amino acids with bulkier side chains would suffer steric hindrance when binding to a site designed to accommodate hydrogen, D-amino acids would dock with a hydrogen atom rather than the R group in that position, and thereby mimic Gly to some extent.

It has been suggested that Gly activates maize PEP carboxylase, at least in part, by altering the OAA_{enol}/OAA_{keto} equilibrium in favor of OAA_{keto}, as opposed to acting as a direct effector of PEP carboxylase (25). In the presence of magnesium, OAA_{keto} can form binary metal-OAA_{keto} complexes and follow two possible paths: it can either nonenzymically decarboxylate to form pyruvate or tautomerize to form metal-OAA_{enol} (25). Only the OAA_{keto} is recognized by malate dehydrogenase; therefore, any OAA_{enol} that is produced remains undetected in coupled assays (25). We compared the extent of Gly activation in coupled and phosphate assays to test the possibility that coupled assays overestimate activation of maize PEP carboxylase by Gly.

As shown in Table II, coupled and phosphate assays gave comparable rates, both in the presence and absence of Gly. If Gly activated solely by altering the OAA_{enol}/OAA_{keto} equilibrium, the ratio of the rates observed in coupled and phosphate assays should have been considerably less than one in the absence of Gly and equal to one in the presence of Gly. Although our data allow for the possibility that Gly has some small effect on the OAA_{enol}/OAA_{keto} equilibrium, the substantial activation of maize PEP carboxylase by Gly that is observed equally in both coupled and phosphate assays is more consistent with a direct mechanism involving binding of Gly to the enzyme.

Table II. *Relative Rates of OAA and Pi Production by Crassula and Maize PEP Carboxylases*

The coupled assay mixture for each enzyme was as described in "Materials and Methods" for the phosphate assays, except that they contained 19 IU of lactate dehydrogenase, 13 IU of malate dehydrogenase, 0.1 mM NADH, and, in the case of *Crassula*, 0.03 IU of PEP carboxylase.

Enzyme Source	Activator[a]	OAA/Pi[b]
Crassula	None	1.09 ± 0.15
	Glc-6P	1.16 ± 0.06
	Phenylphosphonate	1.04 ± 0.21
	Hydroxymethylphosphonate	1.08 ± 0.09
Maize	None	0.95 ± 0.19
	Glc-6P	0.91 ± 0.20
	Gly	1.12 ± 0.06

[a] All activators were at 10 mM. [b] Ratio of rates from coupled and phosphate assays.

Table III. *Activation of Crassula PEP Carboxylase by Phosphate- and Phosphonate-Containing Compounds*

The coupled assay mixture was modified to include 50 mM Pipes, pH 7.0, 5 mM $MgCl_2$, 4 mM HCO_3^-, 0.05 mM PEP, and 0.03 IU of PEP carboxylase.

Compound	K_a	n	Act_{max}
	mM		%
Hydroxymethylphosphonate	0.38 ± 0.06	1.5 ± 0.4	87 ± 6
Glc-6P	0.12 ± 0.01	1.14 ± 0.07	151 ± 3
Phenylphosphonate	0.06 ± 0.01	1.2 ± 0.2	171 ± 6
Phenylphosphate	0.023 ± 0.005	1.1 ± 0.3	63 ± 5

Phosphatase Studies

PEP carboxylase is activated by many phosphate-containing compounds, some of which may be dephosphorylated by the enzyme (9, 26). Walker et al. (26) suggested that such phosphatase activity may be mechanistically related to the activation process. We reexamined the phosphatase activity of the enzyme after discovering that two phosphonate-containing compounds, which are potentially more stable to hydrolysis because of their C-P bonds, significantly activated *Crassula* PEP carboxylase. Table III shows, for example, that phenylphosphonate is a better activator, in terms of apparent binding strength and maximum activation, than Glc-6P. In

Table IV. *Kinetic Parameters for Glc-6P and its Analogs with Crassula PEP Carboxylase*

The coupled assay mixture was modified as described in the legend of Table III.

Compound	K_a	n	Act_{max}
	mM		%
thio-Glc-6P	0.011 ± 0.003	1.17 ± 0.25	114 ± 11
Man-6P	0.10 ± 0.01	1.09 ± 0.16	135 ± 6
Gal-6P	0.11 ± 0.01	1.62 ± 0.23	101 ± 3
Glc-6P	0.12 ± 0.01	1.14 ± 0.07	151 ± 3
dGlc-6P	0.24 ± 0.04	0.93 ± 0.11	104 ± 4
myo-In-1P	0.45 ± 0.16	1.45 ± 0.36	83 ± 18
Glc-1P	0.66 ± 0.08	1.12 ± 0.11	136 ± 7
GlcN-6P	0.93 ± 0.12	1.37 ± 0.20	104 ± 6
Man-1P	1.29 ± 0.31	1.35 ± 0.34	74 ± 8

terms of maximum activation, phenylphosphonate is also a better activator than phenylphosphate, which has previously been shown to activate maize PEP carboxylase (19). Hydroxymethylphosphonate was a weaker activator, and its saturation curve showed slight sigmoidicity ($n = 1.5 \pm 0.4$), but this cooperativity may not be statistically significant.

Both the maize and *Crassula* enzymes were found to lack phosphatase activity toward Glc-6P. When these enzymes were assayed for phosphatase activity in the absence of PEP and in the presence of 10 mM Glc-6P, no Pi was detected over a 3-min period under conditions where a rate as small as 1.5 nmol min^{-1} could have been detected. This finding is in agreement with Gonzalez and Andreo (8), who were also unable to detect phosphatase activity toward Glc-6P with the maize enzyme. In addition to this, the *Crassula* enzyme also lacked phosphatase activity toward hydroxymethylphosphonate and phenylphosphonate. Rates measured in terms of OAA or phosphate production were very similar to each other and showed similar stimulation by all the activators tested (Table II). Therefore, activation of the enzyme by phosphorylated compounds entails equal increases in OAA and phosphate production, indicating that PEP is the only compound dephosphorylated in these assays.

Glc-6P Analog Studies

As the apparent K_a values of the Glc-6P analogs in Table IV indicate, the most significant structural feature affecting activation was the identity of the ring heteroatom. When the ring oxygen in Glc-6P was replaced by a sulfur as in thio-Glc-6P, the apparent K_a dropped by a factor of 10. Thio-Glc-6P did, however, show lower maximal activation than Glc-6P. These effects may possibly be related to the fact that C–S bonds are longer than C–O bonds and cause the ring to pucker slightly differently. Although the nature of the heteroatom does seem to be a significant factor, the ability of myo-In-1P, where the heteroatom is replaced by a carbon atom, to activate the enzyme indicates that a ring heteroatom is not an absolute requirement for binding and activation of *Crassula* PEP carboxylase.

Epimers of Glc-6P with respect to the second and fourth carbon atoms, Man-6P and Gal-6P, bound to the enzyme with nearly the same strength as Glc-6P, but led to lower maximal activations. Gal 6-P showed greater cooperativity ($n = 1.62 \pm 0.23$) than other test compounds, but the reason for this difference is not known. The apparent binding of dGlc-6P was 2-fold weaker than Glc-6P. GlcN-6P, an analog of Glc-6P where the second position hydroxyl is replaced by an amino group, had one-eighth the binding strength of Glc-6P, but still activated the enzyme by about 100%. Leblova et al. (12) have recently reported that maize PEP carboxylase is activated by 2-deoxy-2-fluoro glucosephosphate. From these observations, one can conclude that the presence of a hydroxyl group at carbon-2 of Glc-6P is not crucial for activation but does contribute favorably to binding strength.

The position of the phosphate on the ring is also an important factor affecting activation. For example, Glc-1P activated only 15% less than Glc-6P, but its binding strength was 5.5-fold lower. This effect of position is even more evident with mannose, where Man-1P activated 60% less than Man-6P and bound with 10-fold lower affinity. Man-1P is considerably less effective than Glc-1P. The reason for this difference is unclear because Man-6P and Glc-6P have nearly identical maximal activation and apparent K_a values.

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