Re-examination of the roles of PEP and Mg^{2+} in the reaction catalysed by the phosphorylated and non-phosphorylated forms of phosphoenolpyruvate carboxylase from leaves of *Zea mays*

Effects of the activators glucose 6-phosphate and glycine

Alejandro TOVAR-MÉNDEZ, Rogelio RODRÍGUEZ-SOTRES, Dulce M. LÓPEZ-VALENTÍN and Rosario A. MUÑOZ-CLARES[1]

Departamento de Bioquímica, Facultad de Química, Universidad Nacional Autónoma de México, México D.F., 04510, México

To study the effects of phosphoenolpyruvate (PEP) and Mg^{2+} on the activity of the non-phosphorylated and phosphorylated forms of phosphoenolpyruvate carboxylase (PEPC) from *Zea mays* leaves, steady-state measurements have been carried out with the free forms of PEP (*f*PEP) and Mg^{2+} (*f*Mg^{2+}), both in a near-physiological concentration range. At pH 7.3, in the absence of activators, the initial velocity data obtained with both forms of the enzyme are consistent with the exclusive binding of MgPEP to the active site and of *f*PEP to an activating allosteric site. At pH 8.3, and in the presence of saturating concentrations of glucose 6-phosphate (Glc6P) or Gly, the free species also combined with the active site in the free enzyme, but with dissociation constants at least 35-fold that estimated for MgPEP. The latter dissociation constant was lowered to the same extent by saturating Glc6P and Gly, to approx. one-tenth and one-sixteenth in the non-phosphorylated and phosphorylated enzymes respectively. When Glc6P is present, *f*PEP binds to the active site in the free enzyme better than *f*Mg^{2+}, whereas the metal ion binds better in the presence of Gly. Saturation of the enzyme with Glc6P abolished the activation by *f*PEP, consistent with a common binding site, whereas saturation with Gly increased the affinity of the allosteric site for *f*PEP. Under all the conditions tested, our results suggest that *f*PEP is not able to combine with the allosteric site in the free enzyme, i.e. it cannot combine until after MgPEP, *f*PEP or *f*Mg^{2+} are bound at the active site. The physiological role of Mg^{2+} in the regulation of the enzyme is only that of a substrate, mainly as part of the MgPEP complex. The kinetic properties of maize leaf PEPC reported here are consistent with the enzyme being well below saturation under the physiological concentrations of *f*Mg^{2+} and PEP, particularly during the dark period; it is therefore suggested that the basal PEPC activity *in vivo* is very low, but highly responsive to even small changes in the intracellular concentration of its substrate and effectors.

INTRODUCTION

Phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) from maize leaves catalyses the essentially irreversible formation of oxaloacetate and P_i from phosphoenolpyruvate (PEP) and bicarbonate. This reaction is the first step in the assimilation pathway of atmospheric CO_2 in C_4 plants such as maize. The importance of this step in the photosynthetic metabolism of C_4 plants is underscored by the abundance of the PEPC protein in mesophyll cells of leaves of these plants, accounting for approx. 10–15% of the total soluble protein [1,2].

At physiological pH, PEPC from leaves of C_4 plants is subjected to complex allosteric regulation involving homotropic co-operativity and heterotrophic effects by a number of physiological effectors. The enzyme is also subject to covalent modification consisting of the phosphorylation–dephosphorylation of an N-terminal Ser residue [3]. Regarding the positive effectors, it is considered that the enzyme is activated by two kinds of metabolite: (1) hexose and triose phosphates [4–9], which bind to the so-called glucose 6-phosphate (Glc6P) allosteric site [10], and (2) neutral amino acids, mainly Gly, Ala and Ser [7,9,11–14], which bind to the Gly allosteric site [13]. In addition, several authors have suggested that PEP itself might behave as an allosteric activator of the non-phosphorylated form of the enzyme, on the basis of the observed changes in fluorescence on binding of ligands to PEPC [15] and of steady-state kinetic studies ([16]; C. Mújica-Jiménez, A. Castellanos-Martínez and R. A. Muñoz-Clares, unpublished work). The fact that some PEP analogues are good activators of the non-phosphorylated form of the enzyme [16–18] gives additional support to that proposal.

Mg^{2+} ions are essential for the activity of PEPC [19]. The chemical mechanism of the PEPC-catalysed reaction involves the formation of the enolate form of pyruvate, which is stabilized by Mg^{2+} [20]. In addition, Mg^{2+} can form a binary complex with PEP with a moderate stability constant [21–23], raising the question of whether the complex or the free species combines with the catalytic site of PEPC. The answer to this question, although of great importance to the understanding of the *in vivo* regulation of the enzyme, is still a matter of debate. From kinetic studies of the non-phosphorylated enzyme performed with total PEP and Mg^{2+} concentrations, it has been concluded that the trianionic form of PEP binds to the active site of maize leaf PEPC [24] and that Mg^{2+} binds before PEP [25]. No activation by free PEP (*f*PEP) was detected in these studies. In contrast, from steady-state studies, also of the non-phosphorylated enzyme, in which the MgPEP complex was considered the variable substrate [16,26–28], it was concluded that the binary MgPEP complex is the substrate of the reaction and that either free Mg^{2+} (*f*Mg^{2+}) [26] or *f*PEP [16] behaves as an activator. Thus the role that Mg^{2+}, PEP or MgPEP has in the kinetics of the maize leaf PEPC-catalysed reaction is not clear at present. In addition,

Abbreviations used: *f*Mg^{2+}, free Mg^{2+}; *f*PEP, free PEP; Glc6P, glucose-6-phosphate; PEP, phosphoenolpyruvate; PEPC, phosphoenolpyruvate carboxylase.

[1] To whom correspondence should be addressed.

neither of these studies could be considered conclusive, given that the enzyme used in them was prepared in the absence of protease inhibitors, and it is known that the removal of an N-terminal peptide [20] in the first minutes after extraction alters the kinetic properties of the enzyme [29,30].

Because in order to achieve a complete understanding of the operation of PEPC *in vivo* is of pivotal importance to establish its kinetic mechanism, we studied the non-truncated enzyme by performing steady-state measurements with controlled concentrations of *f*PEP and fMg^{2+} to determine: (1) the relative affinities of the active site for MgPEP and the free species, (2) if appropriate, the order of binding of the free species, and (3) activation by *f*PEP. In the present study we also examined the effects of saturating concentrations of Glc6P and Gly on the above-mentioned kinetic properties of the enzyme, to gain further insight into the mechanism of the allosteric regulation and to determine the allosteric site involved in the *f*PEP activation. In addition we considered it of interest to investigate not only the non-phosphorylated form of the enzyme, which is present in leaves during the dark period of the diurnal cycle [31,32], but also the phosphorylated, day-time form of the enzyme [31,32]. Our results provide experimental evidence indicating that the Mg^{2+} complex of PEP is the true substrate of the reaction catalysed by PEPC from maize leaves in the absence of activators and is the preferred substrate in their presence, and that *f*PEP is an activator that binds to the Glc6P allosteric site. The possible physiological implications of these findings are discussed. A preliminary account of part of this work has been published [33].

MATERIALS AND METHODS

Chemicals and biochemicals

PEP (monocyclohexylammonium salt), NADH (disodium salt), Glc6P, Gly, pig heart malic dehydrogenase and Hepes were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). EDTA (disodium salt) were from Merck KGaA (Darmstadt, Germany). All other chemicals of analytical grade were from standard suppliers.

Enzyme purification and assay

The non-phosphorylated night form of PEPC was purified from *Zea mays* L. leaves kept in darkness for 10 h before extraction as described elsewhere [10]. The sensitivity of the enzyme to malate, measured as IC_{50}, under the conditions described in [30], was typical of the non-truncated, non-phosphorylated form [30], and did not change on incubation with alkaline phosphatase under the conditions described in [31]. The specific activity of the enzyme preparation used, determined in a standard assay in the presence of 5 mM total PEP and 10 mM total Mg^{2+} at pH 7.3 and 30 °C, was 33 units/mg of protein. This enzyme preparation was phosphorylated *in vitro* by the method described in [34]. The degree of phosphorylation was assessed by (1) the change in IC_{50} for malate, which increased from 0.18 mM for the non-phosphorylated form to a maximum of 1.54 mM after prolonged incubation with the kinase, and (2) the activity at the subsaturating concentrations at which the IC_{50} was determined, which approximately doubled in the phosphorylated form. The addition of fresh ATP and kinase did not cause further increases in the maximum IC_{50} value reached. After exhaustive incubation with alkaline phosphatase, the IC_{50} for malate and the activity at the subsaturating substrate concentrations reverted to those of the non-phosphorylated enzyme. The specific activity of the enzyme determined at saturating substrate did not change upon phosphorylation.

Kinetic studies

Steady-state initial velocity studies were performed at 30 °C in a final volume of 0.5 ml of 100 mM Hepes/KOH buffer, pH 7.3 or 8.3, containing 1 mM EDTA, 10 mM $NaHCO_3$, 0.2 mM NADH, 4 units of malate dehydrogenase, varied concentrations of *f*PEP and fMg^{2+}, both in the range from 0.25–4 mM, and the concentrations of Glc6P or Gly stated for each experiment. The amounts of total magnesium (as $MgCl_2$) and total PEP used to give the desired concentrations of the free species were calculated as described in [16]. The ligand–Mg dissociation constants used were: 5.55 mM for PEP [21], 15.4 mM for Glc6P [23] and 50.12 mM for Gly [35]. The other dissociation constants used were as in [16]. Typically, assays were initiated by the addition of 15 μg of PEPC. A thermostatically controlled Beckman DU-7500 spectrophotometer, equipped with a kinetics software package, was used for these measurements. Initial velocities are expressed in units (μmol of product formed per minute). Each point shown in the figures is the average of duplicate or triplicate determinations.

Data analysis

PEPC kinetic data were analysed by non-linear regression calculations with a commercial computing program formulated with the algorithm of Marquardt [36]. Initial velocity data at several concentrations of the fixed substrate were first individually fitted to either the Michaelis–Menten equation [eqn. (1)] for hyperbolic kinetics, or to the Hill equation [eqn. (2)] for sigmoidal kinetics:

$$v = V[S]/(K_s + [S]) \quad (1)$$

$$v = V[S]^h/(S_{0.5}{}^h + [S]^h) \quad (2)$$

where v is the experimentally determined initial velocity; V is the maximal velocity; [S] is the concentration of the variable substrate; K_s and $S_{0.5}$ are the concentration of substrate at half-maximal velocity, and h is the Hill coefficient.

On the basis of the corresponding double-reciprocal plots, the mechanism was identified, and each data set was globally fitted to the corresponding initial velocity equation derived from that particular mechanism, always assuming rapid equilibrium conditions. The rapid equilibrium assumption needs to be validated by additional experiments, although, as shown below, the kinetic data in this paper are consistent with rate equations derived for rapid equilibrium models. Eqns. (3) and (4) correspond to mechanisms where there is exclusive binding of the MgPEP complex to the active site and activation by *f*PEP or competitive inhibition by fMg^{2+} respectively:

$$v = V([S][M]/K_o)^h/[K_{sm}{}^h/(1 + [S]/K_a) + ([S][M]/K_o)^h] \quad (3)$$

$$v = V([S][M]/K_o)^h/(K_{sm}{}^h(1 + [M]/K_i) + ([S][M]/K_o)^h) \quad (4)$$

where [S] is the concentration of *f*PEP, [M] the concentration of fMg^{2+}, K_o the dissociation constant of the substrate–metal complex, K_{sm} the concentration of the substrate–metal complex at half-maximal velocity, K_a the activation constant for *f*PEP, i.e. the dissociation constant of *f*PEP from the allosteric site, and K_i the inhibition constant for fMg^{2+}.

Eqns. (5) to (8) were used when the substrate–metal complex and the free species bound to the active site, either in an ordered fashion with the substrate combining first and with [eqn. (5)] or without [eqn. (6)] activation by *f*PEP, or in a random fashion and with [eqn. (7)] or without [eqn. (8)] activation by *f*PEP:

$$v = V[S][M]/(\alpha K_s K_m(1+[S]/K_s)/(1+[S]/K_a)+[S][M]) \quad (5)$$

$$v = V[S][M]/(\alpha K_s K_m(1+[S]/K_s)+[S][M]) \quad (6)$$

$$v = V[S][M]/(\alpha K_s K_m(1+[S]/K_s+[M]/K_m+[S][M]/K_a \times K_m+[S]^2/K_s K_a)/(1+[S]/K_a)+[S][M]) \quad (7)$$

$$v = V[S][M]/(\alpha K_s K_m(1+[S]/K_s+[M]/K_m)+[S][M]) \quad (8)$$

where K_s and K_m are the concentrations of fPEP and fMg^{2+} at half-maximal velocity respectively and α is the interaction factor that describes the influence that the binding of one of the ligands, free substrate or free metal ion, has on the binding of the other ligand.

Eqn. (9) is a modification of eqn. (6) containing additional terms to account for inhibition by Mg^{2+} and was used to analyse the initial velocity patterns of the non-phosphorylated enzyme in the presence of Glc6P at pH 8.3:

$$v = V[S][M]/(\alpha K_s K_m(1+[S]/K_s+[M]/K_i+[S][M]/K_s \times K_i)/(1+[M]/\delta K_i)+[S][M]) \quad (9)$$

where K_i is the inhibition constant for the metal, i.e. the dissociation constant from the allosteric site, and δ the interaction factor describing the influence that the binding of the non-productive metal ion has on the binding of the productive metal ion or on the binding of the substrate–metal complex, and vice versa.

In the experiments in which the concentration of the activator was varied at constant concentration of substrates, eqn. (10) was used:

$$(v_a - v_0)/v_0 = \mathrm{Act}_{max}[A]^h/(A_{0.5}{}^h+[A]^h), \quad (10)$$

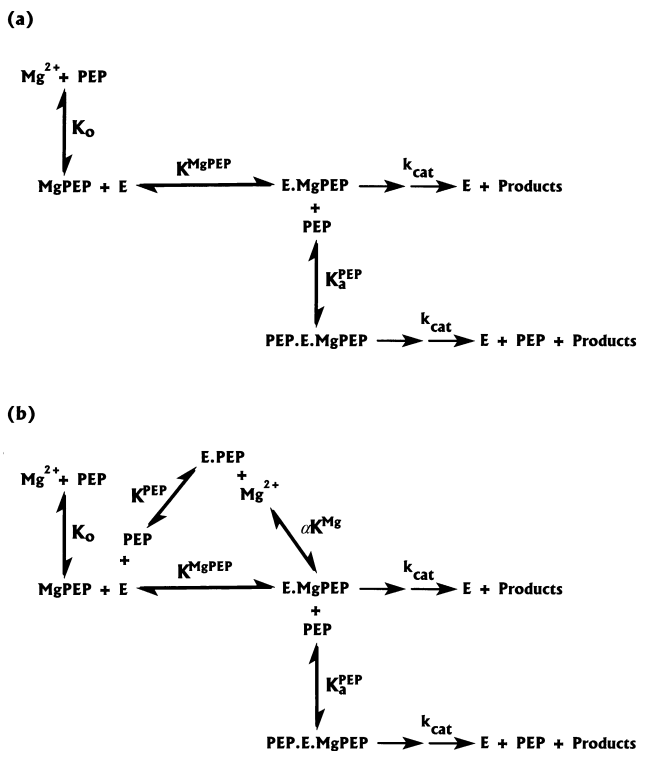
where v_0 and v_a are the initial velocities in the absence and presence of activator respectively, Act_{max} is the maximum activation, [A] is the activator concentration, and $A_{0.5}$ is an apparent activation constant that equals the concentration of activator giving half-maximal activation at a given substrate concentration.

The points in the figures are the experimentally determined values; the curves are calculated from the global fit of these data to the appropriate equation. The best fits were determined by the relative fit error, error of the constants and absence of significant correlation between the residuals and other relevant variables such as observed velocities, substrate concentration and data number.

RESULTS

Kinetics in the absence of activators

Initial velocity studies were performed by varying fPEP concentration while keeping fMg^{2+} concentration at different fixed levels, and vice versa. The concentration range used, from 0.25 to 4 mM in both cases, was chosen to include and not greatly exceed the physiological concentrations of PEP [9] and fMg^{2+} [37]. At pH 7.3, the saturation curves of both forms of PEPC for either fPEP or fMg^{2+} were sigmoidal and yielded maximal velocity values that were independent of the concentration of the fixed substrate when individually fitted to the Hill equation [eqn. (2)] (results not shown), suggesting that the MgPEP complex is the true substrate of the enzyme. However, if MgPEP were the only kinetically significant species, we should obtain similar apparent Hill coefficients (h) when the concentrations of the fixed species, either fPEP or fMg^{2+}, were the same, but we consistently observed higher h values when the variable substrate was fPEP than when it was fMg^{2+} (results not shown). Lower Hill coefficients in the metal saturation curves than in the PEP saturation curves were also observed previously by others [38].



Scheme 1 PEP and Mg^{2+} binding to maize leaf PEPC in the absence of activators

Proposed mechanism for the non-phosphorylated and phosphorylated forms of the enzyme at pH 7.3 (**a**), and for the non-phosphorylated form of the enzyme at pH 8.3 (**b**).

These differences in the behaviour of the free species could be accounted for by the mechanism shown in Scheme 1(a), in which there is exclusive binding of the complex MgPEP to the catalytic site and of fPEP to an allosteric site in an obligate order, i.e. fPEP cannot add to the allosteric site until after the substrate MgPEP has added to the active site. Binding of fPEP to the allosteric site results in activation, because the apparent K^{MgPEP} is decreased by the factor $(1+[f\mathrm{PEP}]/K_a{}^{PEP})$ but has no effect on the catalytic constant. As an approximation to allow mathematical treatment of the experimental data, the initial velocity data were analysed by using the velocity equation that accounts for the main features of this model, modified to allow for the co-operativity of substrate binding [eqn. (3)]. A good fit was obtained for both enzyme forms, non-phosphorylated and phosphorylated, which shows that the simplified analysis is valid at least for determining the maximal velocity and half-saturating concentrations of MgPEP and fPEP. Figures 1(a) and 1(b) show the data and the fits of the non-phosphorylated enzyme, and the estimated kinetic constants are given in Table 1.

At pH 8.3, the saturation curves for fMg^{2+} of the non-phosphorylated form of PEPC were hyperbolic and the individual fit of each line to the Michaelis–Menten equation gave apparent V values independent of the concentration of fPEP (results not shown). Interestingly, saturation curves for fPEP were sigmoidal. This non-symmetrical sigmoidicity is suggestive of the same kinetic mechanism as that found at pH 7.3 (Scheme 1a), with the only difference that the binding of MgPEP to the active site is now non-co-operative. Binding of fPEP to an allosteric site will introduce $[f\mathrm{PEP}]^2$ terms in the numerator and denominator of the velocity equation, accounting for the sigmoidicity of the

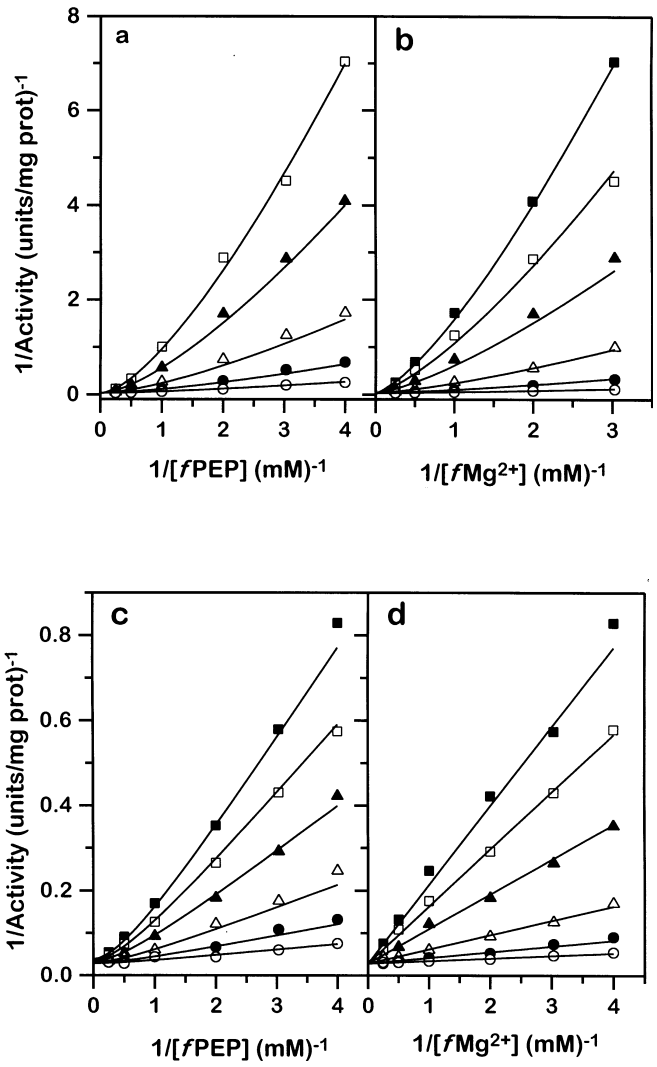


Figure 1 Double-reciprocal plots of initial velocity of the non-phosphorylated form of maize leaf PEPC at saturating bicarbonate concentration

Initial velocities were measured at pH 7.3 (**a**, **b**) or pH 8.3 (**c**, **d**) with *f*PEP (**a**, **c**) or *f*Mg^{2+} (**b**, **d**) as the variable substrate at the following fixed concentrations of the other: 0.25 (■), 0.33 (□), 0.5 (▲), 1.0 (△), 2.0 (●) and 4.0 (○) mM. Assays were performed under the standard conditions described in the Materials and methods section. The points are the experimental values and the curves are the best fit of the experimental data to eqn. (3) (**a**, **b**) or eqn. (5) (**c**, **b**).

*f*PEP saturation curves. In fact, the data obtained at pH 8.3 fit very well to eqn. (3), giving $V = 36.1 \pm 0.9$ units/mg of protein, $K^{MgPEP} = 0.30 \pm 0.02$ mM, $K_a^{PEP} = 3.7 \pm 0.9$ mM and $h = 1.06 \pm 0.07$. It can be seen that the h was close to 1 at this pH, and that the affinities of the active and allosteric sites for their respective ligands were increased by the same factor (approx. 3.5-fold) compared with those obtained at pH 7.3.

Attempts were made to fit the initial velocity data obtained at pH 7.3 and 8.3 to velocity equations derived for alternative mechanisms, such as those in which there is binding of the free species to the active site in a random or ordered fashion. All of these equations gave poor fits of the initial velocity data obtained at pH 7.3, with undefined kinetic constants. However, at pH 8.3 we obtained a good fit of the data to eqn. (5), which described an ordered mechanism in which PEP adds to the active site in rapid equilibrium before Mg^{2+} and there is activation by *f*PEP (Scheme 1b). In the latter fit, the error affecting the dissociation constant of PEP from the active-site–PEP complex, K^{PEP}, or of Mg^{2+} from the active-site–PEP–Mg complex, αK^{Mg}, were high (Table 1), which is understandable if we consider that the highest *f*PEP concentration used in this study is one-fifth of the estimated dissociation constant of the active-site–PEP complex. The absence of a term for the dissociation constant of Mg^{2+} from the active-site–Mg binary complex does not necessarily indicate that this complex does not form, but rather than the Mg^{2+} concentrations used in our study might not have been high enough to observe it. Similarly, at pH 7.3 the dissociation constant of the possible complexes of the free species from the active site of the enzyme might have been so high that the levels of these complexes were not kinetically significant in the concentration range used by us.

Kinetics in the presence of saturating concentrations of Glc6P

The results shown above support that *f*PEP binds to an activating allosteric site as a non-essential activator. Binding of *f*PEP to the Glc6P-allosteric site was proposed previously as a result of studies on changes in the fluorescence on binding of ligands to PEPC [15], or of kinetic studies with PEP analogues as PEPC activators [16–18]. To test whether Glc6P competes for the *f*PEP-binding site, we performed steady-state measurements of PEPC in the presence of a saturating concentration of Glc6P. Because the concentration at which Glc6P saturates the allosteric site depends on the degree of saturation of the active site [34], we first investigated the saturation kinetics of both forms of PEPC by Glc6P at the lowest and highest substrate concentrations used in our experiments. On the basis of the results obtained, given in

Table 1 Kinetic constants of maize leaf PEPC at fixed, saturating bicarbonate concentration

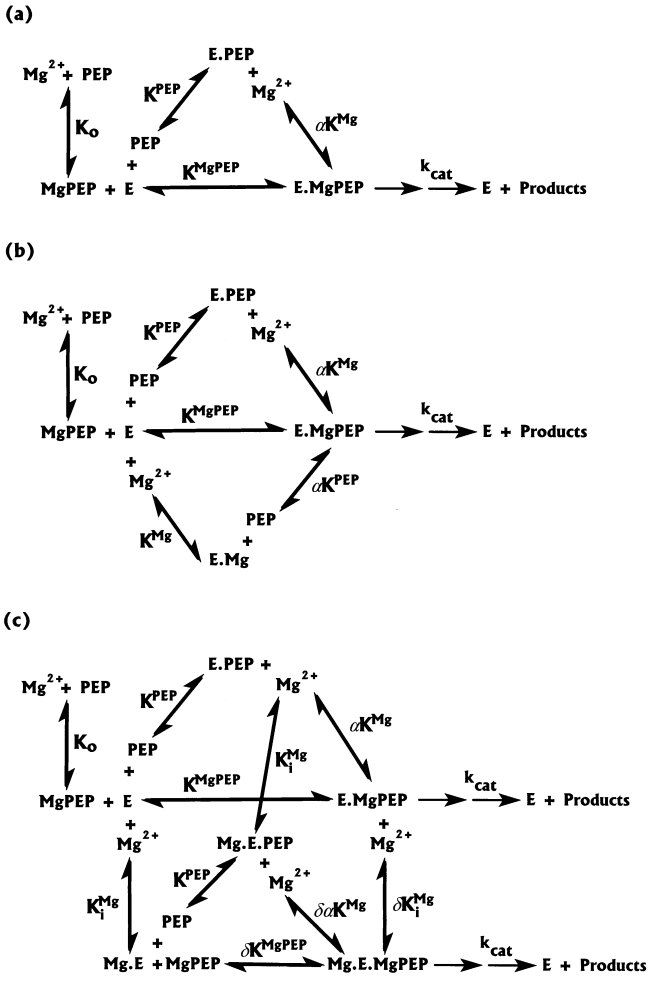
Means ± S.E.M. for the kinetic constants were calculated from a fit of the initial velocity data obtained at pH 7.3 to eqn. (3), or of those obtained at pH 8.3 to eqn. (5). $K^{MgPEP} = K^{PEP}\alpha K^{Mg}/K_0 = \alpha K^{PEP}K^{Mg}/K_0$; K_0 (dissociation constant of the MgPEP complex) = 5.55 mM [21].

			Non-phospho-PEPC		Phospho-PEPC
Constant	Description	pH ...	7.3	8.3	7.3
V (units/mg of protein)			32.0 ± 0.5	36.2 ± 0.9	31.8 ± 0.4
K^{MgPEP} (mM)	Dissociation constant of MgPEP from E.MgPEP		0.83 ± 0.03	0.36 ± 0.05	0.55 ± 0.02
K^{PEP} (mM)	Dissociation constant of PEP from E.PEP		–	19.7 ± 5.8	–
αK^{Mg} (mM)	Dissociation constant of Mg^{2+} from E.MgPEP		–	0.10 ± 0.03	–
K_a^{PEP} (mM)	Dissociation constant of PEP from the allosteric site		4.78 ± 0.45	1.32 ± 0.51	7.00 ± 0.82
h	Hill coefficient		1.35 ± 0.03	1.03 ± 0.05	1.26 ± 0.02

Table 2 Kinetic constants for maize leaf PEPC activator Glc6P

Means ± S.E.M. for the kinetic constants were calculated from a fit of the initial velocity data to eqn. (10). *f*PEP and *f*Mg^{2+} were used at equal concentrations. Bicarbonate concentration was fixed at 10 mM.

	Assay conditions			Kinetic constants		
Enzyme form	pH	[Free species] (mM)	[MgPEP] (mM)	$A_{0.5}$ (mM)	Act_{max}	*h*
Non-phospho-PEPC	7.3	0.25	0.011	3.7 ± 0.2	39.0 ± 1.2	1.81 ± 0.13
		4	2.88	0.77 ± 0.09	0.37 ± 0.01	1.24 ± 0.29
	8.3	0.25	0.011	1.6 ± 0.1	11.3 ± 0.2	1.70 ± 0.11
		4	2.88	0.57 ± 0.05	0.39 ± 0.01	1.05 ± 0.10
Phospho-PEPC	7.3	0.25	0.011	4.1 ± 0.3	28.9 ± 0.9	1.97 ± 0.20
		4	2.88	1.0 ± 0.1	0.46 ± 0.02	1.08 ± 0.17



Scheme 2 PEP and Mg^{2+} binding to maize leaf PEPC in the presence of Glc6P

Proposed mechanism for the non-phosphorylated (**a**) and phosphorylated (**b**) forms of the enzyme at pH 7.3, and for the non-phosphorylated form at pH 8.3 (**c**).

Table 2, we decided to use 20 mM Glc6P at pH 7.3 and 10 mM Glc6P at pH 8.3. Concentrations of Glc6P above 20 mM could not be used at pH 7.3 because they produced significant inhibition at the lowest substrate concentration. The same Glc6P con-

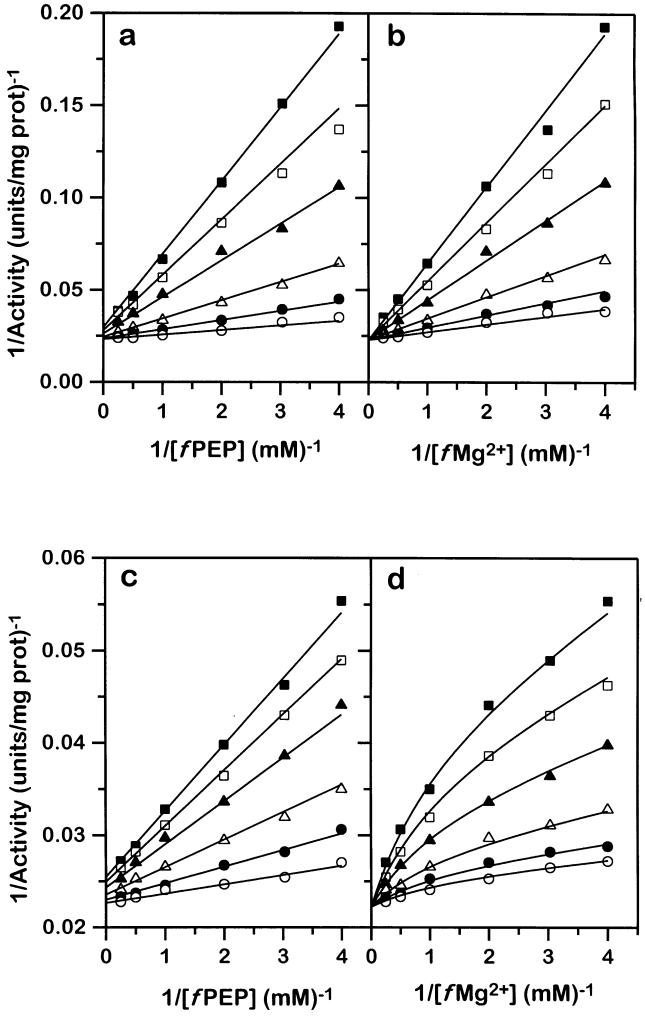


Figure 2 Double-reciprocal plots of initial velocity of the non-phosphorylated form of maize leaf PEPC at saturating bicarbonate concentration in the presence of Glc6P

Initial velocities were measured at pH 7.3 (**a**, **b**) or pH 8.3 (**c**, **d**) with *f*PEP (**a**, **c**) or *f*Mg^{2+} (**b**, **d**) as the variable substrate at the following fixed concentrations of the other: 0.25 (■), 0.33 (□), 0.5 (▲), 1.0 (△), 2.0 (●) and 4.0 (○) mM. Assays were performed under the standard conditions described in the Materials and methods section in the presence of 20 (**a**, **b**) or 10 (**c**, **d**) mM Glc6P. The points are the experimental values and the curves are the result of the overall fit of the experimental data to eqn. (6) (**a**, **b**) or eqn. (9) (**c**, **d**).

Table 3 Kinetic constants for maize leaf PEPC at fixed, saturating concentrations of bicarbonate and Glc6P

The concentration of Glc6P in the assay medium was 20 mM at pH 7.3 and 10 mM at pH 8.3. Means ± S.E.M. for the kinetic constants were calculated from a fit of the initial velocity data obtained at pH 7.3 to eqn. (6) or eqn. (7) for the non-phosphorylated or phosphorylated enzymes respectively, and of those obtained at pH 8.3 to eqn. (9). See Table 1 for a description of K^{MgPEP}.

			Non-phospho-PEPC		Phospho-PEPC
Constant	Description	pH ...	7.3	8.3	7.3
V (units/mg of protein)			44.0 ± 0.4	45.1 ± 0.3	44.9 ± 0.4
K^{MgPEP} (μM)	Dissociation constant of MgPEP from E.MgPEP		79 ± 2	7 ± 1	33 ± 1
K^{PEP} (mM)	Dissociation constant of PEP from E.PEP		5.7 ± 0.7	2.2 ± 0.2	1.7 ± 0.1
K^{Mg} (mM)	Dissociation constant of Mg^{2+} from E.Mg		–	–	4.9 ± 0.8
αK^{Mg} (μM)	Dissociation constant of Mg^{2+} from E.MgPEP		76 ± 9	18 ± 4	107 ± 2
αK^{PEP} (μM)	Dissociation constant of PEP from E.MgPEP		–	–	37 ± 3
K_i^{Mg} (mM)	Dissociation constant of Mg^{2+} from the non-catalytic site of the free enzyme (Mg.E)		–	0.15 ± 0.05	–
δK^{MgPEP} (μM)	Dissociation constant of MgPEP from the complex Mg.E.MgPEP		–	38 ± 7	–
$\delta\alpha K^{Mg}$ (μM)	Dissociation constant of Mg^{2+} from the catalytic site of the complex Mg.E.MgPEP		–	96 ± 20	–
δK_i^{Mg} (mM)	Dissociation constant of Mg^{2+} from the non-catalytic site of the complex Mg.E.Mg or Mg.E.MgPEP		–	0.80 ± 0.29	–

Table 4 Kinetic constants for maize leaf PEPC activator Gly

Means ± S.E.M. for the kinetic constants were calculated from a fit of the initial velocity data to eqn. (10). fPEP and $f Mg^{2+}$ were used at equal concentrations. Bicarbonate concentration was fixed at 10 mM. Abbreviation: n.d., activation not detected.

	Assay conditions			Kinetic constants		
Enzyme form	pH	[Free species] (mM)	[MgPEP] (mM)	$A_{0.5}$ (mM)	Act_{max}	h
Non-phospho-PEPC	7.3	0.25	0.011	22.9 ± 1.4	40.8 ± 0.9	1.25 ± 0.07
		4	2.88	0.41 ± 0.04	0.28 ± 0.01	1.02 ± 0.12
	8.3	0.25	0.011	5 ± 1.3	5.5 ± 0.7	0.93 ± 0.37
		4	2.88	n.d.	n.d.	n.d.
Phospho-PEPC	7.3	0.25	0.011	19.3 ± 2.2	28.8 ± 1.1	1.17 ± 0.14
		4	2.88	0.61 ± 0.05	0.28 ± 0.01	1.05 ± 0.08

centration was used for the non-phosphorylated as for the phosphorylated forms of the enzyme, because we found only a small increase in $A_{0.5}$ after phosphorylation. The latter finding contradicts the previous report of decreases in the apparent activation constant of Glc6P brought about by phosphorylation [34].

The saturation kinetics of both fPEP and $f Mg^{2+}$ at pH 7.3 in the presence of 20 mM Glc6P were hyperbolic, in line with previous findings of a decrease in the co-operative homotropic effects by this activator [1,14,39]. These observations rule out activation by fPEP, otherwise sigmoidal kinetics would have been obtained when fPEP was the varied substrate. Double-reciprocal plots were constructed for both sets of data. For the non-phosphorylated enzyme, the family of lines obtained when $f Mg^{2+}$ was varied and fPEP was held constant intersected on the $1/v$-axis, whereas the family of lines obtained when fPEP was varied and $f Mg^{2+}$ was held constant intersected to the left of the $1/v$-axis. These results are consistent with a rapid-equilibrium ordered mechanism in which fPEP binds before $f Mg^{2+}$ (Scheme 2a). Accordingly we obtained a good global fit of the data to eqn. (6), as shown in Figures 2(a) and 2(b). With the phosphorylated enzyme, both families of lines intersected at the left of the $1/v$-axis (results not shown), suggesting a rapid-equilibrium random binding of the free species (Scheme 2b); the best fit was obtained by using eqn. (7). The estimated kinetic parameters are given in Table 3.

At pH 8.3, double-reciprocal plots of $1/v$ against $1/[f$PEP$]$ at fixed levels of $f Mg^{2+}$ were linear (Figure 2c) but plots of $1/v$ against $1/[f Mg^{2+}]$ were non-linear at all fPEP concentrations (Figure 2d). The best fit of these data, shown in Figures 2(c) and 2(d), was obtained with eqn. (9), which was derived for the mechanism shown in Scheme 2(c). The estimated kinetic parameters are included in Table 3. This mechanism is similar to that described for pH 7.3, but here two Mg^{2+} ions bind to the enzyme, one as a substrate and the other as an inhibitor interfering with the binding of the catalytic Mg^{2+} and of MgPEP, but not with the binding of fPEP. A modified equation, including terms accounting for an effect of the inhibitor Mg^{2+} in the binding of fPEP, gave poorer fits to the experimental data. Therefore it seems that the sites at which the two metal ions bind partly overlap. In this respect it is interesting that two binding sites for metal ions [38] or concave-downward double-reciprocal plots of $1/v$ against $1/[f Mg^{2+}]$ [26] have been reported before for maize leaf PEPC, although the latter report concluded that the non-linearity of the double-reciprocal plots was due to activation by high concentrations of the metal ion.

Interestingly, no activation by fPEP was observed in the presence of saturating Glc6P, regardless of the form of the enzyme or of the pH of the assay, indicating either that binding of fPEP and Glc6P is mutually exclusive or that both activators share the same mechanism of activation.

Kinetics in the presence of saturating concentrations of Gly

The effects of Gly and Glc6P on the kinetics of maize leaf PEPC are additive [13,14], even at saturating concentrations of both

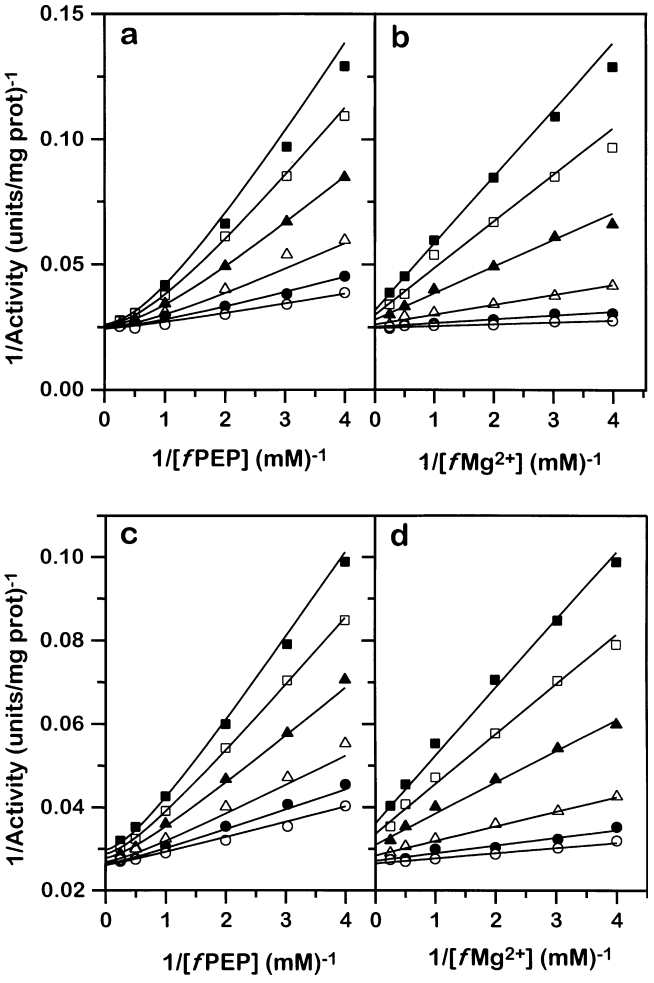


Figure 3 Double-reciprocal plots of initial velocity of the non-phosphorylated form of maize leaf PEPC at saturating bicarbonate concentration in the presence of Gly

Initial velocities were measured at pH 7.3 (**a**, **b**) or pH 8.3 (**c**, **d**) with *f*PEP (**a**, **c**) or $f Mg^{2+}$ (**b**, **d**) as the variable substrate at the following fixed concentration of the other: 0.25 (■), 0.33 (□), 0.5 (▲), 1.0 (△), 2.0 (●) and 4.0 (○) mM. Assays were performed under the standard conditions described in the Materials and methods section in the presence of 100 (**a**, **b**) or 50 (**c**, **d**) mM Gly. The points are the experimental values and the lines are the best fit of the experimental data to eqn. (8).

activators (A. Tovar-Méndez and R. A. Muñoz-Clares, unpublished work). Therefore we expected to observe activation by *f*PEP at saturating concentrations of Gly if *f*PEP bound to the Glc6P allosteric site. In contrast, if *f*PEP bound to the same site as Gly, or to a third allosteric site eliciting the same allosteric transition as Gly, Gly should abolish the activation by *f*PEP.

To test whether the activation by *f*PEP could be abolished by saturation of the allosteric Gly site, the response of PEPC to varying the concentration of *f*PEP and *f*Mg was studied in the presence of saturating concentrations of the activator. As with Glc6P, the affinity of the enzyme for Gly greatly increased as the substrate concentration did (Table 4). Therefore, to ensure saturation by the activator in the whole range of substrate concentrations used in our experiments, the concentrations of Gly used in this study were chosen on the basis of the apparent activation constants determined at the lowest substrate concentrations. Because Gly did not produce enzyme inhibition even at very high concentrations, we used Gly concentrations that were at least 3-fold the apparent activation constants estimated at the lowest substrate concentration.

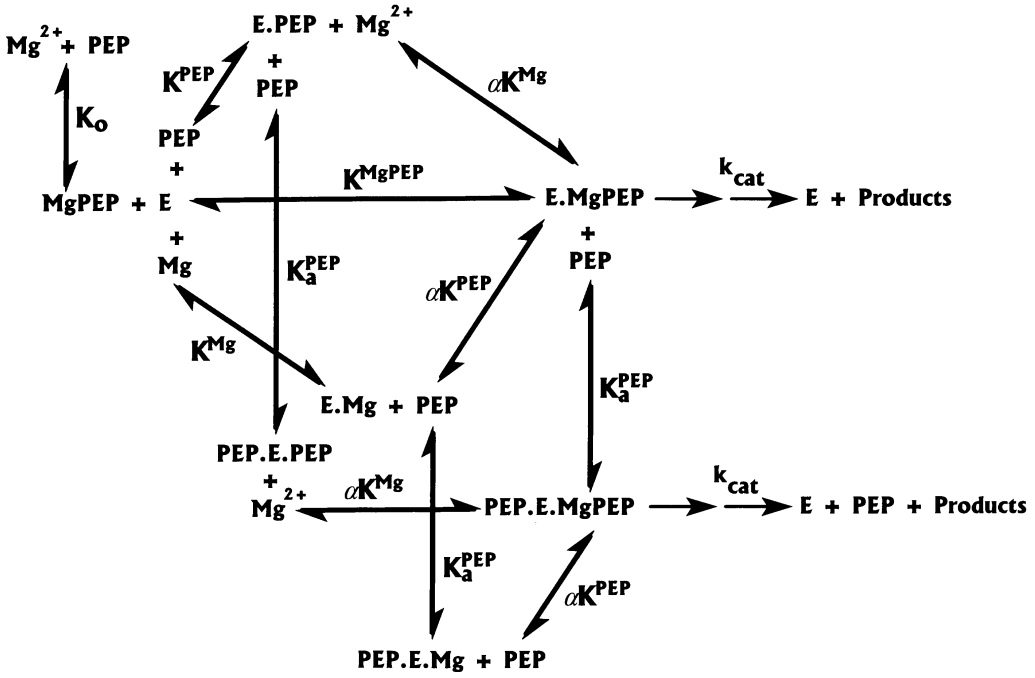
The initial velocity patterns of Figure 3, obtained with the non-phosphorylated enzyme at pH 7.3 in the presence of 100 mM Gly (Figures 3a and 3b) or at pH 8.3 in the presence of 50 mM Gly (Figures 3c and 3d), are consistent with random binding of the MgPEP complex and the free species and with activation by *f*PEP (Scheme 3). The experimental data were best fitted by eqn. (8), derived from the mechanism outlined in Scheme 3, assuming that all ligands interacted with the enzyme in a rapid-equilibrium fashion. Saturation with Gly eliminated the positive co-operativity in the binding of the substrate MgPEP or of the free species to the catalytic site. The sigmoidal nature of the saturation kinetics observed only when *f*PEP was the variable substrate [Figures 3(a) and 3(c) show the corresponding double-reciprocal plots] arises from the heterotropic effects produced by the binding of *f*PEP to the allosteric site, as discussed above. Similar results were obtained with the phosphorylated form of the enzyme (results not shown). The kinetic parameters in Table 5 show that the effect of Gly on the affinity of the active site for MgPEP is similar to that of Glc6P, lowering the K^{MgPEP} to approx. one-tenth and one-sixteenth in the non-phosphorylated and phosphorylated forms respectively. Glycine also increased the affinity of the active site for the free species, mostly for $f Mg^{2+}$, which resulted in a preferred pathway of binding of $f Mg^{2+}$ before *f*PEP. In contrast, binding of Glc6P favoured the binding of *f*PEP to the free enzyme, as shown above. It is then very clear that occupation of the Glc6P allosteric site has different consequences on the kinetics of the enzyme from occupation of the Gly-allosteric site, although both are activating sites. This finding indicates that the allosteric properties of maize leaf PEPC cannot be explained by a two-state model, as suggested previously by binding studies [10,40].

Interestingly, Gly notably increases the affinity of the allosteric site for *f*PEP. Thus, at pH 7.3, the estimated activation constants for *f*PEP in the non-phosphorylated and phosphorylated forms were approx. one-sixth and one-quarter in the presence of Gly of those in its absence, and approx. one-quarter at pH 8.3 in the non-phosphorylated enzyme. This result supports that Gly increases the binding of ligands to both the active and the Glc6P-allosteric sites, as suggested before [14]. The finding that *f*PEP behaves as an activator even in the presence of saturating Gly concentrations shows clearly that Gly and *f*PEP do not share either the same binding site or the same mechanism of activation.

DISCUSSION

MgPEP is the true substrate of maize leaf PEPC at the physiological concentrations of PEP, Mg^{2+} and H^+ in the absence of activators, and the preferred substrate in their presence

Divalent metal ions, mainly Mg^{2+}, are essential activators of all known PEPCs. But whether the metal activates them by complexing with the substrate PEP or by complexing with the enzyme is still a matter of debate. Several studies on PEPC from maize leaves concluded that the MgPEP complex is the true substrate of the enzyme [16,26–28]. However, a more recent study claimed that PEPC binds the free species in an ordered fashion, with Mg^{2+} binding before PEP [25]. This conclusion was drawn from results obtained in initial velocity studies performed on the non-phosphorylated, truncated enzyme at non-physiological pH 7.8, with varied total PEP concentrations at several fixed total Mg^{2+} concentrations. They argued that the previous claims of preferential binding of the MgPEP complex to the enzyme were based on a misinterpretation of the experimental



Scheme 3 PEP and Mg^{2+} binding to the non-phosphorylated and phosphorylated forms of maize leaf PEPC in the presence of Gly at pH 7.3 and 8.3

Table 5 Kinetic constants of maize leaf PEPC at fixed, saturating concentrations of bicarbonate and Gly

The concentration of Gly in the assay medium was 100 mM at pH 7.3 and 50 mM at pH 8.3. Means ± S.E.M. for the kinetic constants were calculated from a fit of the initial velocity data to eqn. (8). See Table 1 for a description of K^{MgPEP}.

			Non-phospho-PEPC		Phospho-PEPC
Constant	Description	pH ...	7.3	8.3	7.3
V (units/mg of protein)			41.3 ± 0.5	38.7 ± 0.3	40.2 ± 0.4
K^{MgPEP} (μM)	Dissociation constant of MgPEP from E.MgPEP		68 ± 7	33 ± 2	34 ± 2
K^{PEP} (mM)	Dissociation constant of PEP from E.PEP		19.6 ± 5.4	4.7 ± 0.6	9.8 ± 2.9
K^{Mg} (mM)	Dissociation constant of Mg^{2+} from E.Mg		4.9 ± 0.9	1.8 ± 0.2	1.2 ± 0.3
αK^{PEP} (μM)	Dissociation constant of PEP from E.MgPEP		78 ± 21	99 ± 13	160 ± 47
αK^{Mg} (μM)	Dissociation constant of Mg^{2+} from E.MgPEP		19 ± 4	37 ± 4	19 ± 2
K_a^{PEP} (mM)	Dissociation constant of PEP from the allosteric site		0.6 ± 0.1	1.2 ± 0.4	1.7 ± 0.6

data that arose when the high degree of synergism in the binding of PEP to the enzyme–metal complex was not taken into account. We did find such a synergism, which could be assessed by the estimated interaction factors in the cases in which there is random addition of the free species. These interaction factors were 0.02 and 0.016 for the phosphorylated enzyme at pH 7.3 in the presence of Glc6P or Gly respectively, and 0.004 and 0.020 for the non-phosphorylated enzyme in the presence of Gly at pH 7.3 and 8.3 respectively. Therefore the binding of one of the free species to the active site enhances the binding of the second at least 50-fold. However, it is clear from the results of steady-state studies reported here that in the absence of effectors and at physiological pH values and concentrations of free species, neither of the free species, PEP or Mg^{2+}, binds to the active site of maize leaf PEPC; only the complex MgPEP does, regardless of the state of phosphorylation of the enzyme. When the affinity of the active site for its ligands was increased by raising the pH of the assay medium to 8.3, or by including Glc6P or Gly, we observed the binding of the free species to the active site. However, even under these conditions the MgPEP complex was still the substrate preferentially bound for the free enzyme. Thus the dissociation constants of the MgPEP complex from the free non-phosphorylated and phosphorylated forms of the enzyme are around 1/35 to 1/70 those of fPEP or $f Mg^{2+}$ (Tables 1, 3 and 5), Given the stability constant value of the MgPEP complex [21–23] and the concentration of $f Mg^{2+}$ present in the cytoplasm of vegetal cells (0.4 mM) [37], the cytosolic concentration of fPEP is only around 10-fold that of MgPEP, so formation of the active-site–MgPEP complex would be favoured over formation of the active-site–fPEP complex. However, it is clear that if the concentration of both free species and their respective dissociation constants from the active site are considered, equal amounts of the enzyme–MgPEP complex are formed through the pathway involving MgPEP and through the pathway involving the free species, because there is a high synergism in the binding of the second species once the other has been bound.

At pH 8.3 in the absence of Glc6P and Gly, the results were consistent with a rapid-equilibrium ordered addition of *f*PEP before Mg^{2+}, which is the same mechanism as that found in the presence of Glc6P, as would be expected if *f*PEP and Glc6P bound to the same allosteric site. This mechanism is opposite to the ordered binding of fMg^{2+} before *f*PEP found by Janc et al. [25]. The reason for the discrepancies between our results and those of these authors are not clear at present, although they might be related to their use of total concentrations of PEP and Mg^{2+} in their kinetic studies or, most probably, to differences in this respect between the non-truncated and truncated enzymes. The N-terminal region of maize leaf PEPC might therefore influence not only the phosphorylation status of the enzyme [41,42] and therefore its sensitivity to malate [29] but also the relative affinities of the active site for MgPEP, PEP and Mg^{2+}.

*f*PEP activates maize leaf PEPC by binding to the Glc6P allosteric site

Our initial-velocity results are consistent with the activation of *f*PEP of maize leaf PEPC regardless of the phosphorylation status of the enzyme, the pH of the assay medium and the presence of activators other than those that bind to the Glc6P site. An alternative mechanism, in which MgPEP is the only substrate and fMg^{2+} inhibits the reaction by competition with MgPEP, could also account for our results in the absence of activators. In this mechanism, the metal ion might bind to an allosteric site or to the active site, but in the latter case binding of fMg^{2+} would not lead to a productive enzyme–Mg complex, i.e. fMg^{2+} would not be the first substrate to add, but an inhibitor that would form a true dead-end enzyme–Mg complex unable to bind PEP. Because at fixed MgPEP the concentration of fMg^{2+} decreases by a fixed factor when the concentration of *f*PEP increases, and vice versa, it is not experimentally feasible to separate the effects of fMg^{2+} from those of *f*PEP. On the basis of χ^2 values and error of the constants, poorer fits of the data were obtained with eqn. (4), derived from the mechanism in which there is inhibition by fMg^{2+}, than with eqn. (3), which describes activation by *f*PEP. Moreover, the results obtained in the presence of saturating Gly could not be fitted by equations in which the terms corresponding to activation by *f*PEP were eliminated and terms corresponding to inhibition by fMg^{2+} were included. Additional experimental evidence supporting activation by *f*PEP was obtained in independent experiments in which desensitization of the enzyme to activation by Glc6P by means of chemical modification with PLP [10] also abolished the activation by *f*PEP (results not shown).

In none of the conditions in which activation by *f*PEP was detected, the data could be fitted to rate equations corresponding to mechanisms in which *f*PEP adds to the allosteric site in the free enzyme, suggesting that this complex does not form at the concentrations of *f*PEP used in our study. Our results do not allow us to conclude whether *f*PEP is able or not to add to the allosteric site in the enzyme–Mg complex, which forms in the presence of saturating Gly, because similar fits were obtained in both cases, although it was clear that *f*PEP binds to the enzyme–PEP and enzyme–PEPMg complexes. We therefore concluded that under physiological conditions *f*PEP cannot bind to the allosteric site until a ligand of the active site, whether MgPEP, PEP or Mg^{2+}, is bound. In such a mechanism, binding of *f*PEP to the allosteric site results in activation because the apparent dissociation constant of the complex MgPEP is lowered by the factor $(1+[fPEP]/K_a^{PEP})$ resulting from the formation of the complex PEP–enzyme–MgPEP. In addition to this activating effect, due exclusively to the kinetic mechanism, it might be expected that *f*PEP could induce a conformational change in subunit to which it is bound, as most allosteric activators do, leading to (1) an increase in the affinity of the enzyme for the second substrate bicarbonate, which has been shown to bind after MgPEP [25], (2) an increase in the k_{cat}, i.e. an increase in the rate-limiting step of the reaction, or (3) increased affinities of the other subunits for MgPEP or allosteric activators. Although, before ruling out any effect of the binding of *f*PEP to the allosteric site on the binding of bicarbonate, experiments at subsaturating concentrations of this substrate must be performed, the good fit of our data to the equation derived from the mechanisms outlined in Schemes 1 and 3 do not support the suggestion that *f*PEP either induces an allosteric transition or affects the allosteric transition triggered by the substrate MgPEP. Therefore the activating effects of *f*PEP seem to arise exclusively from the kinetics of the reaction.

Physiological implications of the kinetic mechanism of maize leaf PEPC

The fMg^{2+} concentration in the cytoplasm of leaf cells has been reported to be approx. 0.4 mM [37] and that of total PEP approx. 0.1 and 4 mM during the dark and light periods respectively [9]. As discussed above, these concentrations are certainly consistent with exclusive binding of MgPEP to the active site of the non-phosphorylated and phosphorylated forms of maize leaf PEPC in the absence of effectors. The physiological role of Mg^{2+} in regulating of the enzyme activity is only that of a substrate, mainly as part of the MgPEP complex, whereas that of PEP is as a substrate and activator of the enzyme.

Given the low concentrations of MgPEP *in vivo*, and the kinetic properties of the enzyme described here, the degree of saturation of the enzyme will be very low, particularly during the dark period. Therefore the enzyme will be almost inactive when the C_4 cycle is not in operation, avoiding an unnecessary use of PEP. In addition the enzyme would be highly responsive to the increases in the levels of substrate and allosteric activators, which affect mainly the affinity for the substrate, brought about by illumination.

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