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Communication

Kinetic Studies of the Form of Substrate Bound by Phosphoenolpyruvate Carboxylase[1]

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ABSTRACT

Phosphoenolpyruvate carboxylase isolated from maize (*Zea mays* L.) leaves was assayed with varying concentrations of free phosphoenolpyruvate at several fixed-varying concentrations of free magnesium higher than required to saturate the enzyme reaction. These assays produced velocity data which were found to form a family of individual lines when plotted against free phosphoenolpyruvate or against total phosphoenolpyruvate, but not when plotted against the concentration of the complex of phosphoenolpyruvate with magnesium. In this latter case, the points from all the fixed-varying concentrations fell on the same line, which can be fitted to a modified Michaelis-Menten equation with a multiple correlation coefficient $R^2 = 0.995$. Similar results were obtained when the enzyme from the C_4 plant maize was assayed with manganese rather than magnesium and when phosphoenolpyruvate carboxylase from leaves of the C_3 plant wheat (*Triticum vulgare* Vill.) was assayed with magnesium. However, at pH 7.0 the enzyme from the Crassulacean acid metabolism plant *Crassula argentea* did not produce a satisfactory single line when plotted against the complex of metal ion and substrate, but did so when the assay pH was raised to 8.0. It is concluded that in general the preferred form of substrate for phosphoenolpyruvate carboxylase is the complex of phosphoenolpyruvate with the metal ion.

Enzymes whose primary substrate is an anion, but whose activity requires a cation which is not tightly bound and thus must be supplied from the reaction medium, are subject to some uncertainty as to the nature of the true substrate, whether the anionic substrate is bound from solutions as the free, uncomplexed form, or whether only the metal ion-anion complex can be bound. Close examination, however, often reveals that the enzyme discriminates between the free and the metal ion complex of the substrate. Enzymes which use nucleoside phosphates seem to bind only the dication complex of the nucleotide (8). In the case of an enzyme whose role in plants is closely related to that of PEPC[3] the NAD^+ malic enzyme, the substrate malate, NAD^+, and ionic effectors all are bound in the free form, although a divalent cation is required for activity (4).

Since its discovery by Bandurski and Greiner (2), PEPC has been the subject of considerable research (1, 15, 16, 19), including various studies of the nature of the true substrate of the enzyme, reaching various conclusions. In early studies, Mildvan's group concluded that the magnesium ion served as a bridge between PEP and the enzyme (9, 10), although the question of whether free metal ion and PEP could bind separately in that conformation or whether only the preformed complex can bind was left open. Later, Mukerji (11) concluded on the basis of kinetic studies that corn leaf PEPC activity was dependent on only the MgPEP complex. More recently, O'Leary (14) has concluded, based on the fact that the dissociation constant for MgPEP is larger than the K_m of PEPC for magnesium (15) and that dissociation constants for the complexes of three metal ions with PEP did not parallel the variation of V_m/K_m with these same metals—although the variation of K_m was parallel with the K_d values of PEP with the three metal ions used—that, "it is unlikely that the active substrate bound by the enzyme is the binary metal-phosphoenolpyruvate complex." This was later cited (16) as evidence that the substrate for maize PEPC is the trianion of PEP.

Others (1) have found by chemical modification of the active site of the maize enzyme indications that, while Mg^{2+} is not essential for the binding of PEP to the enzyme, its presence increases the affinity of the enzyme for PEP. We also have recently observed that the binding of PEP to PEPC measured by fluorescence shows that, while some PEP appears to bind in the absence of metal ion, the addition of magnesium causes a very substantial increase in PEP binding (20).

We, like some others, have been studying PEPC from several plant sources under the assumption that only the trianionic form of PEP can bind to PEPC and expressing substrate concentrations in terms of free ions (1, 3, 7, 12, 14, 21, 22). In the course of a recent study of the regulation of PEPC by binding of PEP at both the active site and an activation site—apparently that used by glucose 6-phosphate—we found that some observations were much more readily explained if it were assumed that PEP binds as the complex with magnesium rather than as the free ion.

For these reasons we have undertaken a study of the binding of PEP to several different forms of PEPC in the hope of resolving the question of which form of the substrate is preferred by this enzyme.

MATERIALS AND METHODS

Enzymes. PEPC (EC 4.1.1.31) samples isolated from plants with three different types of photosynthetic mechanism were used in these studies. One of these enzymes was isolated from *Crassula argentea* Thunb. in our laboratory essentially by the procedure described previously (7). The purification procedure included a separation on a Fractogel TSK DEAE column (EM

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[3] Abbreviations: PEPC, phosphoenolpyruvate carboxylase; Aces, *N*-2-acetamido-2-aminoethanesulfonic acid; PEP, phosphoenolpyruvate; OAA, oxaloacetate.

Science) followed by a fractionation on a hydroxyapatite column (HTP, Bio-Rad) and an additional second separation on a DEAE column. This enzyme had a specific activity of 25 units/mg and appeared to be homogeneous on the basis of SDS gel electrophoresis. Enzyme from leaves of the C_4 plant *Zea mays* L. was supplied by Calbiochem-Behring with a specific activity of 5 units/mg. The third form of PEPC came from leaves of the C_3 plant *Triticum vulgare* Vill. This was supplied by Boehringer Mannheim Biochemicals, with a specific activity of 5 units/mg. Although the latter two enzyme forms were not homogeneous, there appeared to be little or no competing activity present with activity *versus* enzyme concentration linear over a wide range.

Enzyme Assay. Activity was determined with the modified assay of Meyer *et al.* (7) using both malate dehydrogenase and lactate dehydrogenase as coupling enzymes. The question of the role of the metal ion in substrate binding seemed likely to be solved more readily with this assay, which records the pyruvate produced by metal ion decarboxylation of OAA as well as OAA which is captured by malate dehydrogenase. The assay, which accounts for the entire activity of PEPC, should permit more accurate evaluation of the nature of substrate binding to the enzyme and its subsequent decarboxylation since the ability of different metal ions to decarboxylate OAA as well as their effectiveness under different conditions varies, causing inaccuracies in the rates obtained using only malate dehydrogenase as a coupling enzyme. The standard assay contained 50 mM Aces (pH 7.0), 0.2 mM NADH, 5 mM HCO_3, 3 units each of malate dehydrogenase and lactate dehydrogenase, and varying concentrations of PEP and metal ion as indicated in the presentation of individual experiments. At pH 8.0, 50 mM Hepes served as the buffer instead of Aces. Assays were carried out in accordance with protocols written by a computer program which provided information on total and free forms of the assay constituents (4). This program used the following dissociation constants: MgAces = 572 mM; MgNADH = 22.1 mM; $MgHCO_3$ = 99.4 mM; MgPEP = 9.58; MnAces = 398 mM; MnNADH = 12.7 mM; $MnHCO_3$ = 4.5 mM; MnPEP = 2.0 mM. These constants were gathered from several sources (5, 6, 17) and corrected for pH and ionic strength (13). Assays of 1.0 mL were run in cell compartments, controlled at 25°C, of either a Cary model 219 or a Beckman DU-50 spectrophotometer. The reaction was started by the addition of PEPC, and the rate was calculated from the initial 30 s of the reaction.

The velocity data obtained were fitted against the selected form of substrate by a nonlinear least squares fitting procedure (4) to a modified Michaelis-Menten equation:

$$v = V_m \times S^{nH}/(K_m^{nH} + S^{nH}) \quad (1)$$

where v, V_m, K_m, and S have the usual meaning and nH is the Hill number.

RESULTS AND DISCUSSION

Maize PEPC. Since both the report of Mukerji (11) that PEPC uses the magnesium complex of PEP and that of O'Leary (16) that PEPC binds the trianion of PEP referred to the C_4 enzyme from corn leaves, we chose to first use this enzyme in our studies. The assays of Figure 1 were run with varying PEP concentrations with three fixed-varying concentrations of magnesium, consisting of 1.0, 10.0, and 20.0 mM free Mg^{2+}. (Note that the points for the two highest PEP concentrations are missing in the 20.0 mM Mg^{2+} line; this is because of solubility problems at the high concentrations required.) The rationale for this sort of experiment (4) is that varying PEP concentrations at increasing concentrations of magnesium, all saturating for the reaction, will produce a family of lines when plotted against the concentrations of various forms of the substrate *except* when the form of substrate used is that which binds and is processed by the enzyme, in which case all three lines will fall in the same line. As may be seen in Figure 1, these data plotted against concentration of ionic PEP or total (ionic + complexed) PEP produce distinct lines for each concentration of magnesium, lowest with 1.0 mM Mg^{2+} and highest with 20.0 mM Mg^{2+}. Only when velocity is plotted against the concentration of MgPEP, as in Figure 1C, do the points from all three lines fall on a single fitted line. The statistical evaluation of the fit and the kinetic parameters obtained are given in Table I.

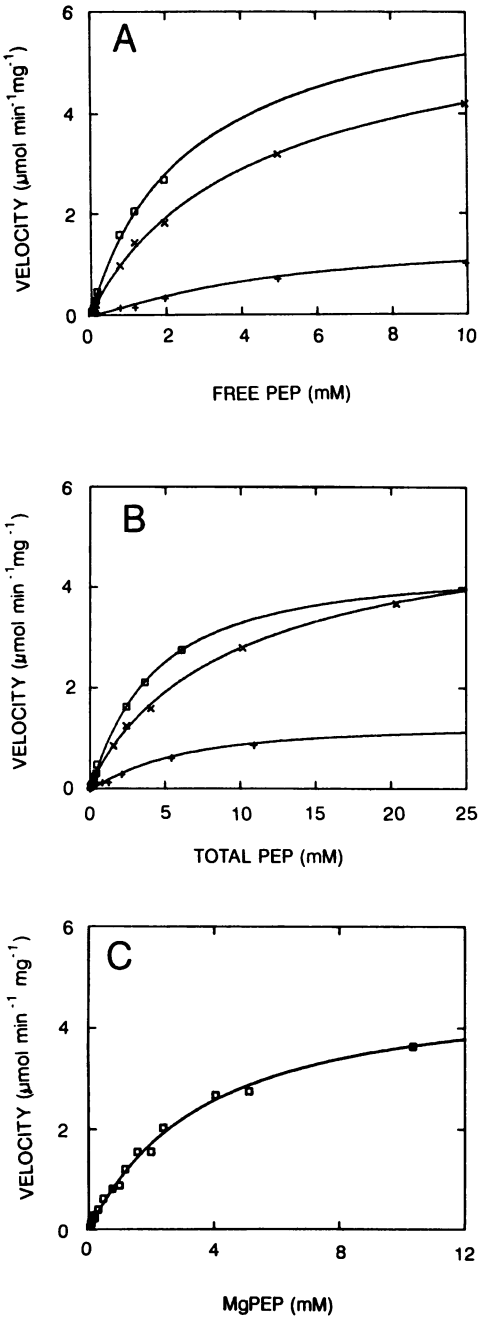

FIG. 1. Activity of corn leaf PEPC as a function of the concentration of different forms of PEP. A, Activity *versus* free PEP. Lowest line (+), assays with 1.0 mM Mg^{2+}; intermediate line (×), assays with 10.0 mM Mg^{2+}; and upper lines (□), assays with 20 mM Mg^{2+}. B, Activity *versus* total PEP. Sequence of lines is the same as in A. C, Activity *versus* MgPEP. Points from all three lines of A and B fitted to a single line by Equation 1.

***Crassula* and Wheat PEPC.** Since we have noted some rather

Table I. *Kinetic Parameters of PEP Carboxylases Based on MgPEP as Substrate*

Values derived from a nonlinear least squares fit of enzyme rates as a function of MgPEP concentration to equation 1. Each line consisted of 24 to 30 individual points obtained from two or three sets of PEP concentration series at different fixed saturating levels of free metal ion. Except as indicated, all rates were determined at pH 7.0 with the standard assay described in "Materials and Methods."

Enzyme Source	Metal Ion	V_m	K_m	V_m/K_m	Hill No.	R^{2a}	F^b
		$\mu mol\ min^{-1}\ mg^{-1}$	mM	$min^{-1}\ mg^{-1}$			
Maize Leaf	Mg	4.78 ± 0.01	3.55 ± 0.02	1.35 ± 0.02	1.03 ± 0.02	0.995	1683
Maize Leaf	Mn	2.55 ± 0.02	0.15 ± 0.03	16.48 ± 0.03	1.51 ± 0.04	0.987	631
Wheat Leaf	Mg	4.31 ± 0.04	0.92 ± 0.13	4.69 ± 0.14	0.81 ± 0.10	0.916	88
Crassula leaf, pH 8	Mg	21.03 ± 0.01	0.35 ± 0.04	60.32 ± 0.05	1.44 ± 0.06	0.988	425

[a] Multiple correlation coefficient (16) of fit to equation 1. [b] F ratio for fitted line (16).

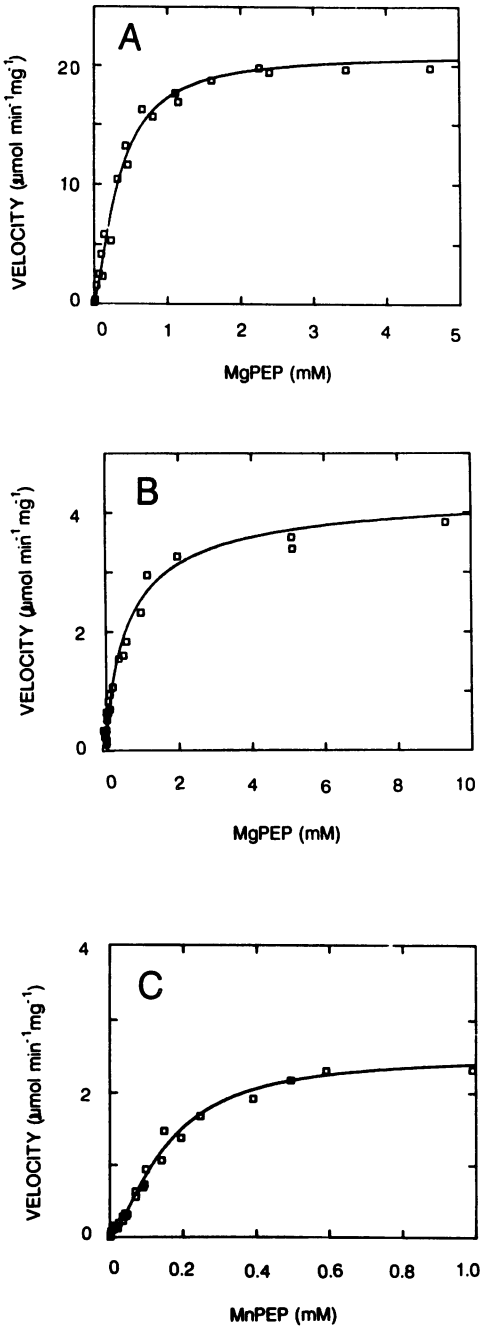

FIG. 2. Substrate preferences of PEPC under different conditions. A, Enzyme from *Crassula* leaves, activity from assays run at 10 and 20 mM of Mg^{2+} but at pH 8.0, fitted to Equation 1. B, Enzyme from wheat leaves run at pH 7.0 at three different levels of Mg^{2+} as in Figure 1 fitted to Equation 1. C, Enzyme from corn leaf run at pH 7.0 with 1.0 mM Mn^{2+}, 2.0 mM Mn^{2+} and 5.0 mM Mn^{2+} fitted to Equation 1.

striking differences in the characteristics of the C_4 PEPC from corn and the CAM enzyme from *Crassula*, including reverse responses to temperature (21), it seemed desirable to compare the two with respect to nature of the preferred substrate. One possibly relevant difference is that we (21, 22) and others (12) have found the CAM enzyme at pH 7.0 to have a K_m for free PEP much lower than the C_4 enzyme. When the CAM enzyme was studied in the same way as the corn enzyme in Figure 1 at pH 7.0, the results (not shown) were similar to those shown in Figure 1 except that the fit of all lines to total PEP was better than the fit to MgPEP. If the pH of the assay is raised from 7.0 to 8.0, the results shown in Figure 2A are obtained, where the fit to MgPEP (Table I) is better than to total PEP ($R^2 = 0.899$, $F = 59.3$). This difference of the CAM enzyme is explored in a paper to be published elsewhere.

PEP carboxylase from wheat, a C_3 plant, appears to be like the enzyme from a C_4 plant in the form of substrate bound, preferring the MgPEP complex, although it is intermediate between the C_4 and CAM enzymes with respect to K_m, Table I. Our value for K_m for MgPEP is much higher than the free PEP values reported for some C_3 plants (3), although this may reflect differences in assay composition and procedure.

Maize PEP Carboxylase with Manganese. Thus, while three different forms of PEPC all appear to preferentially use the complex of PEP with magnesium as their substrate, although with some differences depending on pH, the question of whether the preference is the same with other metal ions remains. The use of Mn^{2+} answers this question with respect to the C_4 enzyme as shown in Figure 2C where it may be seen that a single line fits well to data obtained with three different saturating concentrations of manganese, in this case 1, 2, and 5 mM. The lower concentration of metal ion is permitted in this case because, as Table I shows, the K_m for MnPEP is much lower than for MgPEP.

Kinetic Parameters of PEPC. The kinetic parameters derived from the fitting of the lines of Figure 1C and Figure 2, A, B, and C are summarized in Table I. Here it is apparent that when run with manganese the C_4 enzyme has a K_m for MnPEP more than 20-fold lower than for MgPEP. The maximal velocity, however, is reduced to about half of that with magnesium. The wheat enzyme, with about the same activity, has a K_m about one third that of the C_4 enzyme run at the same pH and with the same metal ion. The CAM enzyme, run at pH 8.0, has a smaller K_m than that from wheat, and when run at pH 7.0 the K_m is even lower (0.14 mM). The maximal velocity at pH 7.0 is the same as at pH 8.0.

Conclusions. This kinetic study of the substrate preference of PEPC strongly supports the results of Mukerji (11) indicating that the maize enzyme binds MgPEP rather than trianionic PEP or both forms of the substrate. We have extended this observation to include similar behavior of the maize enzyme with manganese and of the wheat enzyme with magnesium. The CAM enzyme appears to bind both forms of the substrate at pH 7, but at pH 8 it also binds only the MgPEP. We view this difference as a manifestation of the very high affinity of the CAM enzyme for PEP and magnesium, and the difference in substrate binding at different pHs will be followed in a succeeding paper.

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