A Kinetic Investigation of Phosphoenolpyruvate Carboxylase from *Zea mays*†

James W. Janc,‡,§ Marion H. O'Leary,‖ and W. W. Cleland*,‡

Institute for Enzyme Research and Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53705, and Department of Biochemistry, University of Nebraska, Lincoln, Nebraska 68583

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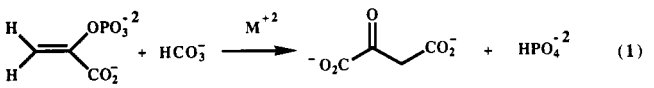
ABSTRACT: The reaction catalyzed by phosphoenolpyruvate carboxylase from *Zea mays* has been studied kinetically. Results of initial velocity patterns and inhibition studies indicate that phosphoenolpyruvate carboxylase has a random sequential mechanism in which there is a high level of synergism in the binding of substrates. The preferred order of addition of reactants is Mg^{2+}, phosphoenolpyruvate, and bicarbonate. The binding of Mg^{2+} is at equilibrium. Values for the various kinetic parameters are K_{iMg} = 2.3 ± 0.4 mM, K_{PEP} = 3.6 ± 0.6 mM, K_{iPEP} = 0.2 ± 0.07 mM, and $K_{bicarbonate}$ = 0.18 ± 0.04 mM. In addition, double inhibition experiments have been performed to examine the nature of the active site interactions with the putative intermediates, carboxy phosphate and the enolate of pyruvate. Highly synergistic inhibition of phosphoenolpyruvate carboxylase was observed in the presence of oxalate and carbamyl phosphate (α = 0.0013). However, an antisynergistic relationship exists between oxalate and phosphonoformate (α = 2.75).

Phosphoenolpyruvate carboxylase [orthophosphate:oxaloacetate carboxy-lyase, phosphorylating (EC 4.1.1.31)] catalyzes the bicarbonate-dependent carboxylation of PEP[1] to form oxalacetate (eq 1). PEP carboxylase is present in plants,



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algae, and bacteria, but the physiological function of the enzyme varies with source (Utter & Kolenbrander, 1972). PEP carboxylase plays a particularly important role in C_4 and crassulacean acid metabolism plants, in which the enzyme catalyzes the initial carbon fixation step of photosynthesis (Hatch, 1987; Kluge & Ting, 1978). Due to the central role of PEP carboxylase in plant physiology, the enzyme has been the focus of much investigation (Utter & Kolenbrander, 1972; O'Leary, 1982, 1992; Andreo et al., 1987).

In spite of the considerable research that has focused on PEP carboxylase, a detailed kinetic investigation of the enzyme has not been reported. Difficulties in eliminating exogenous bicarbonate, particularly at high pH, pose problems in performing kinetic studies. The majority of kinetic studies have simply ignored the kinetics of bicarbonate altogether. O'Leary et al. (1981) have examined the pH dependence of the kinetic parameters V_{max} and $(V/K)_{PEP}$. The V_{max} was found to be independent of pH over the range 7–10. $(V/K)_{PEP}$ is pH independent from pH 9.5 to pH 8.0 but decreases with a slope of 2 below pH 7.5. One of the pK_a values is attributed to the third pK_a of PEP and the second to an enzymatic residue. The pH dependence of $(V/K)_{PEP}$ for PEP carboxylase indicates that it is the trianion of PEP that is bound (O'Leary et al., 1981). Cooperative binding of PEP to PEP carboxylase has been observed under certain circumstances (Uedan & Sugiyama, 1976). Maize PEP carboxylase binds PEP in a sigmoidal fashion at pH 7.0, but at pH 8.0 the binding of PEP is hyperbolic.

Bandurski (1955) demonstrated that Mg^{2+} is necessary for activity of PEP carboxylase. Since then, it has been determined that a variety of metals will support the reaction. PEP carboxylase shows the highest activity with Mg^{2+} or Mn^{2+}, but Co^{2+}, Ni^{2+}, Fe^{2+}, Hg^{2+}, and Zn^{2+} all support the reaction at reduced rates (O'Leary et al., 1981; Nguyen et al., 1988). PEP carboxylase has the highest affinity for PEP when Mn^{2+} is used.

A point of contention is whether free PEP or the metal–PEP complex is the true substrate (O'Leary et al., 1981; Wedding et al., 1988; Outlaw, 1990). Both NMR proton relaxation rate studies and kinetic investigations suggest that the metal serves as a bridge between the enzyme and PEP (Miller et al., 1968; Miziorko et al., 1974). Additionally, these studies demonstrate that Mn^{2+} can bind to the active site of PEP carboxylase in the absence of PEP.

Central to the understanding of any enzyme is knowledge of the kinetic mechanism (i.e., a description of the order of addition of substrates and of product release) (Cleland, 1977). In spite of the considerable amount of study concerning PEP carboxylase, a thorough kinetic investigation of PEP carboxylase has not been reported to date. We report here the results of a kinetic investigation of PEP carboxylase from *Zea mays* in which the kinetic mechanism of the enzyme has been determined. In the three following papers, we take up a number of additional aspects of the mechanism of action of this enzyme.

Materials and Methods

Materials. HEPES (free acid), EPPS (free acid), ADP (dipotassium salt), NADH (disodium salt), glucose 6-phosphate (dipotassium salt), oxalic acid (dipotassium salt), and phosphoglycolate (tricyclohexylammonium salt) were from Sigma Chemical Co. Phosphonoformic acid (trisodium salt), phosphonoacetic acid (free acid), and acetyl phosphate (lithium/potassium salt) were from Aldrich Chemical Co. Sodium bicarbonate and $MgCl_2$ were from Mallinckrodt Chemical Co. Carbamyl phosphate (dilithium salt) was from Boehringer

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‡University of Wisconsin.

§Present address: Department of Chemistry, The Johns Hopkins University, Baltimore, MD 21218.

‖University of Nebraska.

[1] Abbreviations: PEP, phosphoenolpyruvate; EPPS, *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MDH, malate dehydrogenase; PK, pyruvate kinase; LDH, lactate dehydrogenase.

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Mannheim Biochemicals. Phosphonacetamide (dicyclohexylammonium salt) was generously provided by Drs. G. Waldrop and H. K. Schachman. PEP (monocyclohexylammonium salt) was synthesized by Peter Henke as described by Hirshbein et al. (1982). All other chemicals were of reagent grade and were used without further purification. Lactate dehydrogenase (LDH) (porcine muscle), malate dehydrogenase (MDH) (porcine heart), and pyruvate kinase (PK) (rabbit muscle) were from Boehringer Mannheim Biochemicals. PEP carboxylase was purified from *Z. mays* as described by O'Leary et al. (1981) and Diaz (1986) to a specific activity of 20 units/mg. Analysis of the purified enzyme by SDS–polyacrylamide gel electrophoresis revealed that the enzyme was essentially homogeneous (>95% pure).

General Kinetic Methods. Kinetic measurements were made with a Cary 118 spectrophotometer equipped with a thermostated cell compartment. Constant temperature (26 °C) was maintained in the cell compartment using a circulating water bath. Initial velocity measurements were performed in a total volume of 1 mL in cuvettes of 1-cm path length. Reactions were initiated with PEP carboxylase, and initial rates were obtained from the linear portion of the progress curve. The velocities of the PEP carboxylase catalyzed reactions were measured by coupling the oxalacetate produced to MDH and measuring the oxidation of NADH at 340 nm (ϵ_{340} = 6.22 mM^{-1} cm^{-1}). All experiments were performed at pH 7.8. In all cases the activity of PEP carboxylase remained constant for the entire experiment. All solutions used in kinetic assays, except those containing enzymes, were filtered through a disposable 0.2-μm Uniflo syringe filter prior to use.

Initial Velocity Experiments. Bicarbonate stock solutions were made immediately prior to use, and the concentration of bicarbonate was calibrated by end-point analysis with PEP carboxylase. When performing experiments in which the concentration of bicarbonate was varied, it was necessary to take several precautions to minimize the level of exogenous bicarbonate. First, solutions were prepared with water that had been boiled (to degas) and then cooled to room temperature under N_2. Solid KOH was used to adjust the pH. Finally, solutions were stoppered with septa and were sparged with CO_2-free nitrogen for 16 h prior to use. Reactions were carried out in cuvettes that were sealed with septa and had previously been purged with N_2 to dispel CO_2. Hamilton syringes were used to deliver solutions to the cuvette. The concentration of exogenous bicarbonate in the assay was determined by end-point analysis in the absence of added bicarbonate. The true concentration of bicarbonate in each assay was calculated by adding the concentration of exogenous bicarbonate to the concentration of stock bicarbonate.

The concentration of PEP in the stock solutions used in each of the initial velocity patterns was determined by end-point analysis using PK and LDH.

Data Processing. Kinetic data were fitted to the appropriate equation with the FORTRAN programs of Cleland to obtain the desired kinetic parameters (Cleland, 1979). Plots were made using total concentrations of substrates (i.e., not accounting for metal–PEP complexes, which are minor under the conditions used here). Data from the initial velocity pattern

$$v = VA/(K + A) \quad (2)$$

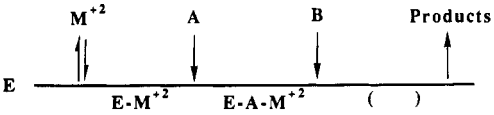
$$v = VAB/(K_{ia}K_b + K_aB + K_bA + AB) \quad (3)$$

$$v = VAB/(K_aB + K_bA + AB) \quad (4)$$

$$v = VAB/(K_{ia}K_b + K_bA + AB) \quad (5)$$

$$v = V/(1 + I/K_I + J/K_J + IJ/\alpha K_I K_J) \quad (6)$$

Scheme I: Kinetic Model Adapted from Morrison and Ebner (1971)



measurements were fitted to eqs 3–5. The data points in the figures represent the experimentally measured values, while the lines represent the fit of the data to the appropriate equation. The data from the double inhibition experiments were fitted to eq 6.

Theory. In an enzymatic reaction in which there are three substrates, there are three initial velocity patterns in which one varies the concentration of one substrate in the presence of different levels of a second substrate, while the concentration of the third substrate is held constant (Viola & Cleland, 1982). Morrison and Ebner (1971) have performed a detailed kinetic analysis of galactosyltransferase for which they proposed a kinetic model in which the metal ion binds at equilibrium followed by the ordered addition of the substrates A and B (Scheme I). The initial rate equation of the model presented in Scheme I was derived using the net rate constant method of Cleland (1975) (eq 7)

$$v = VMAB/(K_{ia}K_bK_{im} + K_aK_{im}B + K_{ia}K_bM + K_aBM + K_bAM + ABM) \quad (7)$$

where V is the maximum velocity, M, A, and B are the concentrations of the metal ion and the two substrates, respectively, K_{im} and K_{ia} are dissociation constants for the reaction of M with free enzyme and the reaction of A with the EM complex, and K_a and K_b are the Michaelis constants for the substrates A and B. The rate equation given in eq 7 may be rearranged according to which pair of substrates is varied to yield eqs 8–10.

varying A and B at a constant level of M:

$$v = VAB/(K_{ia}K_bK_{im}/M + K_aBK_{im}/M + K_{ia}K_b + K_aB + K_bA + AB) \quad (8)$$

varying B and M at a constant level of A:

$$v = VMB/(K_bK_{im}K_{ia}/A + K_{im}BK_a/A + K_bMK_{ia}/A + BMK_a/A + K_bM + BM) \quad (9)$$

varying A and M at a constant level of B:

$$v = VMA/(K_{ia}K_{im}K_b/B + K_aK_{im} + K_{ia}MK_b/B + K_aM + AMK_b/B + AM) \quad (10)$$

The following relationships exist between the apparent dissociation constants obtained from initial velocity experiments and the true kinetic constants (Morrison & Ebner, 1971; Cleland, 1963).

from eq 8, A–B pattern, constant M

$$\text{app } K_b = K_b \quad (11)$$

$$\text{app } K_a = K_a(1 + K_{im}/M) \quad (12)$$

$$\text{app } K_{ia} = K_{ia}(1 + K_{im}/M) \quad (13)$$

from eq 9, B–M pattern, constant A

$$\text{app } K_b = K_b[(1 + K_{ia}/A)/(1 + K_a/A)] \quad (14)$$

$$\text{app } K_m = K_{im}/(1 + A/K_a) \quad (15)$$

$$\text{app } K_{im} = K_{im}/(1 + A/K_{ia}) \quad (16)$$

from eq 10, A–M pattern, constant B

$$\text{app } K_{im} = K_{im} \quad (17)$$

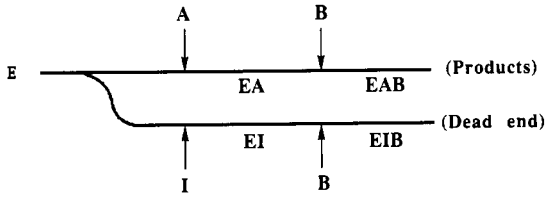
$$\text{app } K_a = K_a[(1 + K_{ia}K_b/K_aB)/(1 + K_b/B)] \quad (18)$$

Table I: Kinetic Constants for PEP Carboxylase[a]

kinetic constant[b]	app value (mM)	true value (mM)	calcd by eq
K_{im}	2.6 ± 0.3[c]	2.6 ± 0.3	17
	1.6 ± 0.2[d]	1.9 ● 0.3	15
	1.8 ± 0.8[d]	8.0 ± 6.0	16
		av 2.3 ± 0.4	
K_a	2.1 ± 0.3[c]	3.6 ± 0.6	18
	5.3 ± 0.6[d]	3.5 ± 0.5	12
		av 3.6 ± 0.6	
K_{ia}	0.3 ± 0.09[d]	0.20 ± 0.07	13
K_b	0.22 ± 0.03[d]	0.22 ± 0.03	11
	0.029 ± 0.008[d]	0.14 ± 0.04	14
		av 0.18 ± 0.04	

[a] Reaction mixtures contained 100 mM EPPS, pH 7.77, 0.2 mM NADH, and 24 units/mL MDH. [b] M = Mg^{2+}, A = PEP, and B = bicarbonate. [c] Value obtained from fit of data to eq 5. [d] Value obtained from fit of data to eq 3.

Scheme II: Illustration of Induced Substrate Inhibition



RESULTS

Initial Velocity Studies. The kinetic mechanism of PEP carboxylase has been examined by determining the three initial velocity patterns. The initial velocity pattern in which bicarbonate was varied at different levels of PEP with a fixed concentration of 5 mM Mg^{2+} is intersecting, indicating the sequential addition of PEP and bicarbonate prior to product release. The data were fitted to eq 3, and the kinetic parameters are summarized in Table I.

The initial velocity pattern obtained by varying the concentration of bicarbonate at different levels of Mg^{2+} at a fixed level of 0.69 mM PEP is intersecting, indicating the sequential addition of Mg^{2+} and bicarbonate. The data were fitted to eq 3, and the kinetic parameters are reported in Table I.

The initial velocity pattern in which PEP was varied at different levels of Mg^{2+} at a constant concentration of 0.25 mM bicarbonate is intersecting, with the intersection point on the vertical axis. This equilibrium-ordered pattern indicates the ordered addition of Mg^{2+} followed by PEP, with the binding of Mg^{2+} at equilibrium. The data were fitted to eq 5, and the kinetic constants are reported in Table I. The apparent Michaelis and dissociation constants determined in this fashion were converted to true values by the use of eqs 11–18 (Table I).

Substrate Inhibition by Bicarbonate Induced by Phosphoglycolate. Dead-end inhibition studies can provide valuable information about the kinetic mechanism of an enzyme. In particular, Fromm (1967) has shown that, in an ordered sequential mechanism, the presence of a dead-end inhibitor that is competitive versus A (the first substrate to bind) should induce substrate inhibition by the second substrate to add (B), provided B can bind to the E·I complex (Scheme II). In a completely ordered mechanism, theory predicts that the substrate inhibition caused by B will be competitive with A at a fixed level of I. The inhibition by B in the presence of I is competitive because an infinite level of A prevents I from combining with E. In order for the inhibition by B to be complete, I must not be able to dissociate from the E·I·B complex. Often there is a strong preference in the order of substrate binding as opposed to a strictly ordered addition of substrates. When this is the case, the presence of I will induce competitive inhibition by B, but the inhibition will be partial. This is because I can dissociate from the E·I·B complex at a finite rate. This phenomenon is highly diagnostic of a random sequential mechanism with a preferred order of substrate addition. In a fully random kinetic mechanism, substrate inhibition by B is not observed because I readily dissociates from the E·I·B complex, resulting in E·B, which is free to combine with A to yield products (Fromm, 1979).

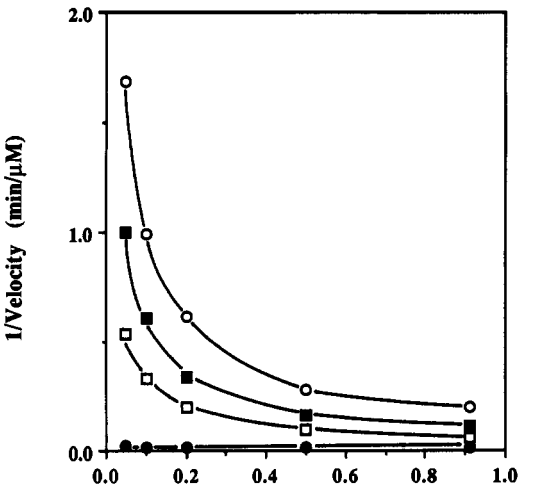


FIGURE 1: Induced substrate inhibition of PEP carboxylase. The concentration of phosphoglycolate was 0.0 mM (●), 5.0 mM (□), 10.0 mM (■), and 20.0 mM (○). Reaction mixtures contained 100 mM EPPS, pH 7.77, 0.2 mM NADH, 1.0 mM PEP, 5 mM $MgCl_2$, and 24 units/mL MDH, and the reaction was started by addition of 0.27 unit of PEP carboxylase.

Induced substrate inhibition by bicarbonate was observed in the PEP carboxylase catalyzed reaction in the presence of phosphoglycolate. A double reciprocal plot in which the concentration of bicarbonate is varied at different levels of phosphoglycolate is shown in Figure 1. The data appear to approach the $1/V$ axis in an asymptotic fashion. This behavior is predicted in the kinetic scenario outlined in Scheme II, where at infinite levels of B all the enzyme is present in the E·I·B complex and as a result the velocity goes to zero.

In an alternative approach to examine the induced substrate inhibition by bicarbonate, an experiment was performed in which PEP was varied at different levels of HCO_3^- while phosphoglycolate and Mg^{2+} concentrations were held constant. Apparent competitive inhibition by bicarbonate was observed (Figure 2). The slope replot appears to be hyperbolic, which implies that the competitive inhibition by bicarbonate in the presence of phosphoglycolate is partial (inset of Figure 2).

Double Inhibition Studies. Important information can be gained about the topology of the active site from double inhibition studies that might not otherwise be learned from single inhibition studies. For example, one can determine if the binding site for two inhibitors overlap (Yonetani & Theorell, 1964). In this report, double inhibition experiments have been employed to characterize the active site of PEP carboxylase.

The general equation used to describe double inhibition experiments is given in eq 6. K_I and K_J are apparent dissociation constants for the inhibitors I and J, respectively. The coefficient α is a measure of the interaction between the two inhibitors in the E·I·J complex. An infinite value of α means that the binding of the two inhibitors is mutually exclusive,

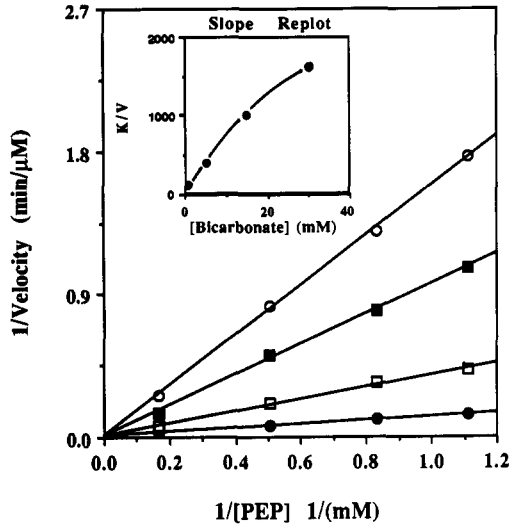
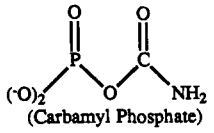
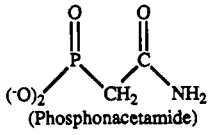
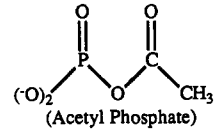
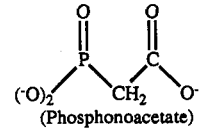
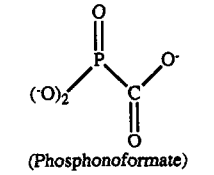
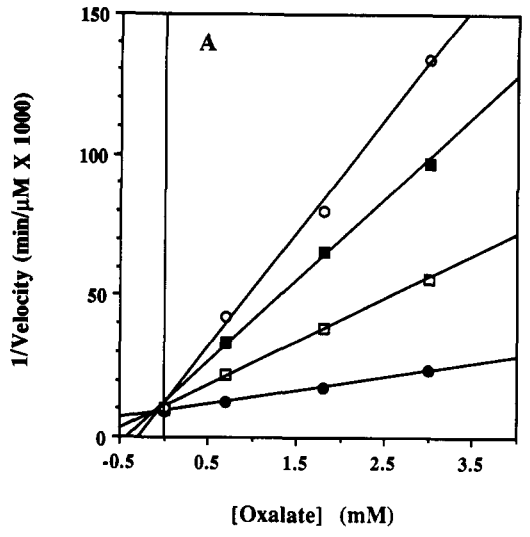


FIGURE 2: Induced substrate inhibition of PEP carboxylase. The concentration of bicarbonate was 1.0 mM (●), 5.0 mM (□), 15.0 mM (■), and 30.0 mM (○). Reaction mixtures were similar to those in Figure 1 with 10 mM phosphoglycolate and 5.0 mM $MgCl_2$.

Table II: Double Inhibition by Oxalate and Various Carboxy Phosphate Analogues

compound	α value	app dissociation constant (mM)
 (Carbamyl Phosphate)	0.0013 ± 0.0002	K_J = 69.5 ± 10.0 αK_J = 0.090 ± 0.003 αK_I = 0.031 ± 0.004
 (Phosphonacetamide)	0.085 ± 0.009	K_J = 25.9 ± 1.3 αK_J = 1.9 ± 0.1 αK_I = 2.0 ± 0.2
 (Acetyl Phosphate)	0.122 ± 0.038	K_J = 42.8 ± 6.4 αK_J = 5.2 ± 1.0 αK_I = 2.9 ± 0.9
 (Phosphonoacetate)	0.46 ± 0.09	K_J = 22.0 ± 1.6 αK_J = 10.2 ± 1.6 αK_I = 10.8 ± 2.2
 (Phosphonoformate)	2.75 ± 1.15	K_J = 11.7 ± 0.4 αK_J = 32 ± 13 αK_I = 65 ± 27

whereas a finite value for α indicates that the inhibitors I and J can occupy the active site simultaneously. Finite α values can be greater than 1, equal to 1, or less than 1. An α value larger than 1 indicates an antisynergistic relationship between the two inhibitors in the E·I·J complex and implies a degree of interference between the two inhibitors. An α value of 1 indicates no interaction between the two inhibitors in the E·I·J complex. An α value of less than 1 indicates a synergistic relationship between the two inhibitors in the E·I·J complex. The parameters αK_I and αK_J are apparent dissociation constants from the E·I·J complex for the inhibitors I and J.



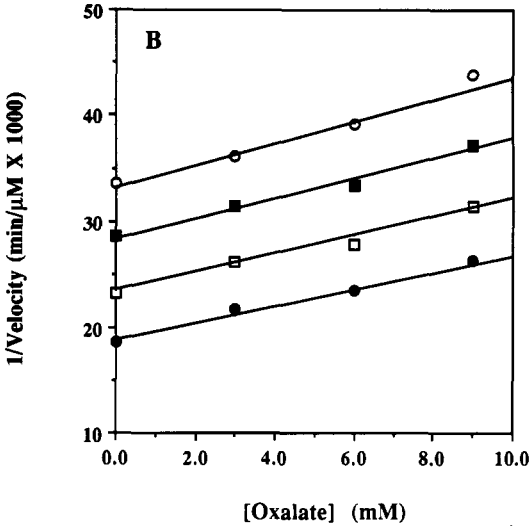


FIGURE 3: (a) Double inhibition of PEP carboxylase by oxalate and carbamyl phosphate. The concentration of carbamyl phosphate was 1.0 mM (●), 4.0 mM (□), 8.0 mM (■), and 12.0 mM (○). (B) Double inhibition of PEP carboxylase by oxalate and phosphonoformate. The concentration of phosphonoformate was 0.0 mM (●), 3.0 mM (□), 6.0 mM (■), and 9.0 mM (○). Reaction mixtures were similar to those in Figure 1 with 10 mM $MgCl_2$, 10 mM glucose 6-phosphate, 5 mM bicarbonate, and 0.336 mM PEP.

Carboxy phosphate and the enolate of pyruvate are thought to be intermediates in the reaction catalyzed by PEP carboxylase (O'Leary et al., 1981). In the present work, double inhibition experiments have been performed with analogues of these putative intermediates. Five separate experiments were performed in which either carbamyl phosphate, phosphonacetamide, acetyl phosphate, phosphonoacetate, or phosphonoformate was supplied as a surrogate for carboxy phosphate. In each of the five experiments, oxalate was supplied as the second inhibitor to mimic the enolate of pyruvate. The results from two representative experiments are shown Figure 3. In each case the data were fitted to eq 6 (Table II). In fitting the data to eq 6, the value for K_I (apparent dissociation constant for oxalate) was supplied as an input parameter. The apparent K_I value for oxalate (23.5 mM) was measured under conditions identical to those used in the double inhibition experiments.

Two patterns are possible in this type of experiment. When α is finite, an intersecting pattern is predicted. When α is infinite, a parallel pattern is observed, and the binding of I and J is mutually exclusive (Yonetani, 1982). Each of the five

experiments performed yielded intersecting patterns, indicating the presence of an E·I·J complex with all five pairs of inhibitors.

Discussion

We have performed a series of kinetic experiments in order to determine the kinetic mechanism of PEP carboxylase from *Z. mays*. First, the initial velocity patterns for PEP carboxylase have been determined. An equilibrium-ordered pattern was obtained when the level of PEP was varied at different levels of Mg^{2+} while the concentration of bicarbonate was held constant. This pattern indicates that the binding of Mg^{2+} is at equilibrium and precedes the binding of PEP. These results appear to be at odds with the findings of Wedding et al. (1988), who suggested that the Mg^{2+}·PEP complex is the active form of the substrate bound by PEP carboxylase. This discrepancy is caused by the high degree of synergism in the binding of PEP to the Enz·Mg^{2+} complex, which makes it appear that the Mg^{2+}·PEP complex is bound by the enzyme. It is also clear that Mg^{2+} and Mn^{2+} are able to bind to PEP carboxylase in the absence of PEP (Miller et al., 1968; Miziorko et al., 1974).

Intersecting patterns were obtained when bicarbonate and PEP were varied at a constant level of Mg^{2+} and when bicarbonate and Mg^{2+} were varied at a fixed level of PEP. These data are consistent with the sequential addition of PEP and bicarbonate after Mg^{2+} has bound. The relative order of addition of bicarbonate and PEP cannot be determined from these data alone. A variety of methods are available for determining the order of addition of substrates in an enzyme-catalyzed reaction, including dead-end inhibition studies, product inhibition studies, and isotope effect studies (Cleland, 1986). In this report we have determined the order of substrate addition by examining the induced substrate inhibition by bicarbonate in the presence of phosphoglycolate. The inhibition by bicarbonate was found to be competitive with respect to PEP; however, the slope replot appeared slightly hyperbolic, indicating the partial nature of the induced substrate inhibition. The dissociation of phosphoglycolate was decreased by at least a factor of 170, however, in the presence of bicarbonate. This result is consistent with a random sequential mechanism in which the strongly preferred order of substrate addition is PEP followed by bicarbonate. Linear competitive-induced substrate inhibition would be seen in a completely ordered sequential mechanism.

O'Leary and Paneth (1986) have examined the bicarbonate dependence of the $^{18}(V/K)$ isotope effect for the bridging oxygen of PEP. At 2 mM bicarbonate $^{18}(V/K)$ was 1.006. At high levels of bicarbonate (200 mM) the value decreased to 0.994 (O'Leary & Paneth, 1986). In an ordered sequential mechanism, infinite levels of the second substrate to add will eliminate the apparent V/K isotope effect for the first substrate (Cook & Cleland, 1981). The results obtained by O'Leary and Paneth were interpreted to be consistent with an ordered mechanism in which PEP binds prior to bicarbonate and there is an inverse isotope effect on the binding of PEP.

Double inhibition experiments have been employed to characterize the active site interactions with the putative intermediates carboxy phosphate and the enolate of pyruvate. Oxalate has been tested as an analogue of the enolate of pyruvate. Carbamyl phosphate, phosphonacetamide, acetyl phosphate, phosphonoacetate, and phosphonoformate have each been examined as analogues of carboxy phosphate. Strong synergistic inhibition of PEP carboxylase was observed with oxalate and either carbamyl phosphate (α = 0.0013) or phosphonacetamide (α = 0.085). In both cases the carboxy phosphate surrogates are neutral at the carbonyl end and are

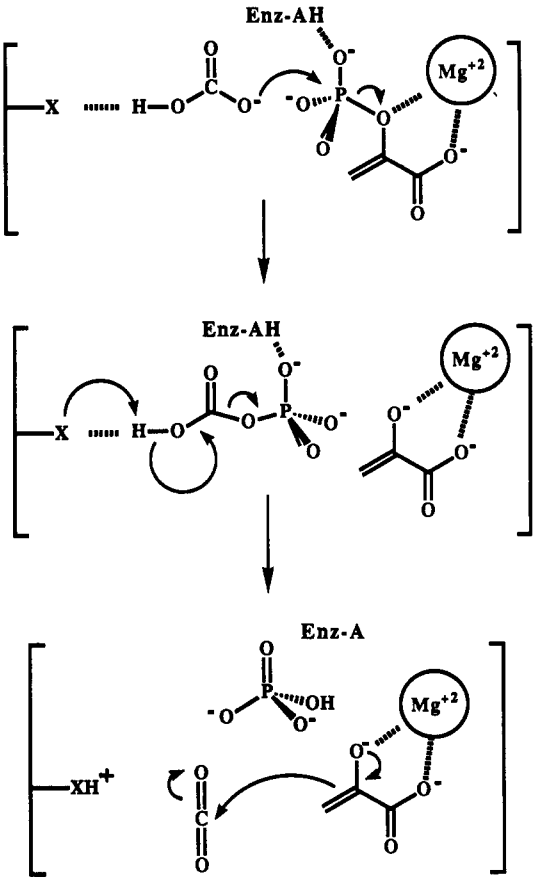


FIGURE 4: Possible mechanism for PEP carboxylase.

potential H-bond donors. Acetyl phosphate shows much weaker synergistic inhibition (α = 0.122). Acetyl phosphate does not possess the ability to H-bond to its methyl group, which may account for the weaker synergistic inhibition observed. The final two experiments involved inhibitors that possess a carboxyl group at the carbonyl end of the carboxy phosphate surrogate. An α value of 0.46 was obtained when phosphonoacetate and oxalate were examined. This value implies a weaker interaction (less synergism) than was measured with the three previous pairs of inhibitors. Finally, the last pair of inhibitors examined, phosphonoformate and oxalate, were antisynergistic (α = 2.75).

These data suggest that a negative charge at the carbonyl end of the carboxy phosphate surrogate is unfavorable. Thus carboxy phosphate is presumably strongly bound only when its carboxyl group is protonated, as it would be immediately following phosphorylation of bicarbonate by PEP. In addition, the presence of an H-bond donor at the carbonyl end of the inhibitor appears to produce a favorable interaction, as evidenced by the strong synergistic inhibition seen with carbamyl phosphate or phosphonacetamide. Taken together, these results are consistent with the involvement of an active site base that may be H-bonded to the amide hydrogen atoms of these two inhibitors (X in Figure 4).

Carbamyl phosphate has been examined in alternate substrate studies of PEP carboxylase by Gonzalez et al. (1987), who report that PEP carboxylase catalyzes the bicarbonate-dependent hydrolysis of carbamyl phosphate at approximately 1% of the carboxylation rate. Our study did not directly examine the hydrolysis of carbamyl phosphate but does support the finding that carbamyl phosphate can bind to the active site of PEP carboxylase.

A role for the metal ion in the reaction catalyzed by PEP carboxylase is proposed in Figure 4, in which the metal ion

is coordinated to the bridging oxygen of PEP. In this coordination scheme the metal would function as a Lewis acid, stabilizing the enolate of PEP and thus catalyzing phosphoryl transfer. Support for the coordination of the metal to the bridging oxygen has largely come from inhibition studies of PEP carboxylase with analogues of PEP in which the bridging oxygen atom of PEP has been replaced by a methylene group or a sulfur. Phosphono-PEP and 3-phosphonopropionate have been found to be poor inhibitors of PEP carboxylase (O'Leary, 1983). The K_I for phosphothioglycolate, an analogue of phosphoglycolate in which the bridging oxygen has been replaced with a sulfur atom, is 100-fold greater than that of phosphoglycolate (Sikkema & O'Leary, 1988).

The formation of CO_2 and phosphate as intermediates is implied by studies with fluoro-PEP reported in the third paper of this series (Janc et al., 1992). The mechanism in Figure 4 requires the intervention of at least one acid–base catalyst (X in Figure 4). This group functions as a proton acceptor in the decomposition of carboxy phosphate to CO_2 and PO_4^{3-}. Subsequently, this same group might donate a proton to PO_4^{3-}, forming HPO_4^{2-}. Alternatively, as shown in Figure 4, different catalytic groups might be involved in proton removal and proton donation. This X group may be the one seen in the pH dependence of the steady-state kinetics by O'Leary et al. (1981) and might be the histidine identified in site-directed mutagenesis studies of the enzyme from *Escherichia coli* by Terada and Izui (1991).

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