Mechanistic Studies of Phosphoenolpyruvate Carboxylase from *Zea mays* with (*Z*)- and (*E*)-3-Fluorophosphoenolpyruvate as Substrates†

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ABSTRACT: The catalytic mechanism of phosphoenolpyruvate (PEP) carboxylase from *Zea mays* has been studied using (*Z*)- and (*E*)-3-fluorophosphoenolpyruvate (F-PEP) as substrates. Both (*Z*)- and (*E*)-F-PEP partition between carboxylation to produce 3-fluorooxalacetate and hydrolysis to produce 3-fluoropyruvate. Carboxylation accounts for 3% of the reaction observed with (*Z*)-F-PEP, resulting in the formation of (*R*)-3-fluorooxalacetate, and for 86% of the reaction of (*E*)-F-PEP forming (*S*)-3-fluorooxalacetate. Carboxylation of F-PEP occurs on the 2-*re* face, which corresponds to the 2-*si* face of PEP. The partitioning of F-PEP between carboxylation and hydrolysis is insensitive to pH but varies with metal ion. Use of ^{18}O-labeled bicarbonate produces phosphate that is multiply labeled with ^{18}O; in addition, ^{18}O is also incorporated into residual (*Z*)- and (*E*)-F-PEP. The $^{13}(V/K)$ isotope effect on the carboxylation of F-PEP catalyzed by PEP carboxylase at pH 8.0, 25 °C, is 1.049 ± 0.003 for (*Z*)-F-PEP and 1.009 ± 0.006 for (*E*)-F-PEP. These results are consistent with a mechanism in which carboxylation of PEP occurs via attack of the enolate of pyruvate on CO_2 rather than carboxy phosphate. In this mechanism phosphorylation of bicarbonate to give carboxy phosphate and decarboxylation of the latter are reversible steps. An irreversible step, however, precedes partitioning between carboxylation to give oxalacetate and release of CO_2, which results in hydrolysis of PEP.

One of the most effective tools for studying enzyme mechanisms is the use of alternate substrates, among which fluorinated analogues have been widely used (Abeles & Alston, 1990). The fluorinated analogue of PEP, 3-fluorophosphoenolpyruvate (F-PEP),[1] has been successfully employed in studying the mechanisms of a variety of PEP-utilizing enzymes. F-PEP has been shown to be a substrate for pyruvate kinase (Stubbe & Kenyon, 1972; Duffy & Nowak, 1984), enolase (Stubbe & Kenyon, 1972; Duffy & Nowak, 1984), phosphoenolpyruvate carboxykinase (Duffy & Nowak, 1984), and pyruvate phosphate dikinase (Duffy & Nowak, 1984). In addition, F-PEP has been used to determine the stereochemistry of the reaction catalyzed by phosphoenolpyruvate carboxykinase (Hwang & Nowak, 1986). Recently, two independent studies of the substrate activity of F-PEP with maize PEP carboxylase have been reported (Diaz et al., 1988; Gonzalez & Andreo, 1988). Both investigations demonstrated that the primary reaction of F-PEP with PEP carboxylase was hydrolysis, resulting in the formation of 3-fluoropyruvate. Diaz et al. (1988) reported that 14% of F-PEP was carboxylated to form 3-fluorooxalacetate (F-OAA). Gonzalez and Andreo (1988) reported that the carboxylation of F-PEP accounted for only 10% of the consumption of F-PEP and that the remaining 90% was hydrolyzed to 3-fluoropyruvate.

We have reexamined the substrate activity of F-PEP with PEP carboxylase from *Zea mays* using ^{19}F and ^{31}P NMR and isotope effects to characterize the reaction. We have determined the partitioning of (*Z*)- and (*E*)-F-PEP individually and have characterized the dependence on pH and divalent cation. We have examined the incorporation of ^{18}O from bicarbonate to the product phosphate and to residual F-PEP. Additionally, the $^{13}(V/K)$ isotope effect for the carbon arising from bicarbonate has been measured with either PEP or F-PEP as the substrate.

Materials and Methods

Materials. CHES (free acid) was from Research Organics. HEPES (free acid), EPPS (free acid), NADH (disodium salt), glucose 6-phosphate (dipotassium salt), fluoropyruvic acid (free acid), and EDTA (disodium salt) were from Sigma Chemical Co. 1-Propanol was from Mallinckrodt Chemical Co. $Na_2{}^{13}CO_3$ and $NaH^{13}CO_3$ were from Cambridge Isotope Laboratories. $H_2{}^{18}O$ (95% enriched) was from EG and G Mound Applied Technologies. PEP (monocyclohexylammonium salt) was synthesized by Peter Henke as described by Hirschbein et al. (1982). F-PEP (monocyclohexylammonium salt) was synthesized by Dr. E. Diaz by the method of Stubbe and Kenyon (1972) as modified by Duffy and Nowak (1984), and on the basis of ^{19}F NMR the isomeric composition was 87% (*Z*) and 13% (*E*). 3-Fluorooxalacetate

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[1] Abbreviations: PEP, phosphoenolpyruvate; F-PEP, 3-fluorophosphoenolpyruvate; F-OAA, 3-fluorooxalacetate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; EPPS, *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid; MDH, malate dehydrogenase; G6PDH, glucose-6-phosphate dehydrogenase; LDH, lactate dehydrogenase; PK, pyruvate kinase.

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was synthesized by Debra Bradshaw by the method of Blank et al. (1955) and Kun et al. (1958). (2*R*,3*R*)- and (2*R*,3*S*)-3-fluoromalate were synthesized enzymatically with MDH (Sigma) from 3-fluorooxalacetate as described by Goldstein et al. (1978). Fluorolactate was synthesized by reduction of fluoropyruvate with NADH catalyzed by LDH. All other chemicals were of reagent grade and were used without further purification.

Carbonic anhydrase (bovine erythrocytes), glucose-6-phosphate dehydrogenase (G6PDH) (*Leuconostoc mesenteroides*), aldehyde dehydrogenase (bakers' yeast), alcohol dehydrogenase (bakers' yeast), and malic enzyme (chicken liver) were from Sigma Chemical Co. L-Lactate dehydrogenase (LDH) (porcine muscle), L-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 Methods. Kinetic measurements were made with a Cary 118 spectrophotometer equipped with a thermostated cell compartment in which a constant temperature was maintained in the cell compartment using a circulating water bath. ^{19}F nuclear magnetic resonance spectra were recorded at 376.50 MHz with a Bruker AM-400 Fourier transform spectrometer. Fluorine chemical shifts were externally referenced to $CFCl_3$ ($\delta = 0.0$). ^{31}P NMR spectra were recorded at 202.42 MHz with a Bruker AM-500 Fourier transform spectrometer. Phosphorus chemical shifts were externally referenced to 200 mM D_3PO_4 ($\delta = 0.0$). A Finnigan Delta E isotope ratio mass spectrometer was used for measurement of the isotopic composition of the carbon in CO_2.

Photoisomerization of F-PEP. Samples of F-PEP [87% (*Z*), 13% (*E*)] were dissolved in 100 mM EPPS at pH 8.0 and were photoisomerized in quartz EPR tubes by exposure to UV light from a 100-W Hanovia lamp. The ratio of isomers was monitored by ^{19}F NMR. Samples were illuminated for no longer than 10 min to avoid excessive heating. After illumination samples were incubated in the dark for 5 min, and then the illumination was repeated as necessary. Five 10-min exposures produced a 70/30 mixture of (*Z*)- and (*E*)-F-PEP. Further exposure did not alter the ratio of (*Z*) to (*E*). No decomposition of F-PEP was observed under the conditions used for the photoisomerization as judged by ^{19}F NMR.

Quantitation of the Products in the PEP Carboxylase Catalyzed Reaction with (Z)- and (E)-F-PEP as Substrates. Four reactions were performed, the first two containing F-PEP [87.7% (*Z*), 12.3% (*E*)] with one of these containing PEP carboxylase and one not. The other two reactions were performed with photoisomerized F-PEP [70.4% (*Z*), 29.6% (*E*)]. Again, one reaction contained PEP carboxylase, and the other did not. Reactions were initiated with 0.52 unit of PEP carboxylase and incubated for 5 h at 23 °C. After 5 h, 400 μL of each reaction was withdrawn and diluted with 171 μL of 100 mM EDTA. The solutions were filtered, using an Amicon Centricon-30 (30 000 MW cutoff) ultrafiltration apparatus to remove the protein, and the filtrate was examined by ^{19}F NMR to analyze the isomeric composition of the products of the PEP carboxylase catalyzed reaction.

Effects of MDH Concentration on the Partitioning of F-PEP by PEP Carboxylase. The partitioning of F-PEP catalyzed by PEP carboxylase between F-OAA and 3-fluoropyruvate was examined in the presence of increasing concentrations of MDH. Reaction mixtures contained 100 mM HEPES (pH 8.0), 5 mM $MgCl_2$, 100 mM glucose 6-phosphate, 25 mM $NaHCO_3$, 1 mM NADH, 1 mM dithiothreitol, 173 units of carbonic anhydrase, 61 units of LDH, 140 units of G6PDH, 25 mM F-PEP [87% (*Z*), 13% (*E*)], and a variable amount of MDH in 750 μL. Four reactions were carried out in which the amount of MDH supplied was varied from 150 to 600 units. Reactions were initiated with 0.51 unit of PEP carboxylase and were incubated for 4 h at 25 °C. A control was performed in which PEP carboxylase was omitted. After 4 h, reactions were stopped by the addition of 171 μL of 100 mM EDTA to 400 μL of the reaction mixture. Samples were filtered with a Centricon-30 apparatus, and the products were quantitated using ^{19}F NMR.

Metal Ion Dependency of the Partitioning of F-PEP by PEP Carboxylase. The partitioning of F-PEP catalyzed by PEP carboxylase with either Mg^{2+}, Co^{2+}, or Ni^{2+} at 5 mM or Mn^{2+} at 1 mM was examined. Reactions were initiated with 1.4 units of PEP carboxylase and incubated at 23 °C for 16 h. Reactions were stopped by addition of 214 μL of 100 mM EDTA to 500 μL of the reaction mixture. Protein was removed by ultrafiltration using a Centricon-30 apparatus, and the products were quantitated using ^{19}F NMR.

pH Dependence of the Partitioning of F-PEP by PEP Carboxylase. The PEP carboxylase catalyzed partitioning of F-PEP between hydrolysis and carboxylation was examined at pH 7.5, 8.0, and 9.0. Reaction mixtures contained 100 mM HEPES (pH 7.5 and 8.0) or 100 mM CHES (pH 9.0), 5 mM $MgCl_2$, 100 mM glucose 6-phosphate, 25 mM $NaHCO_3$, 1 mM NADH, 1 mM dithiothreitol, 173 units of carbonic anhydrase, 61 units of LDH, 260 units of MDH, 140 units of G6PDH, and 25 mM F-PEP [87% (*Z*), 13% (*E*)] in 750 μL. PEP carboxylase (1.0 unit) was used to initiate the reactions, which were incubated at 23 °C. After 5 h, reaction mixtures were diluted with 100 mM EDTA so that the final concentration of EDTA was 30 mM. Reactions were filtered with a Centricon-30 apparatus and assayed by ^{19}F NMR to quantitate the products.

Synthesis of ^{18}O-Labeled Bicarbonate. ^{18}O-Labeled bicarbonate was synthesized by incubating 64 mg of $Na_2{}^{13}CO_3$ and 25 mg of $NaH^{13}CO_3$ in 1 mL of $H_2{}^{18}O$ and 200 μL of D_2O for 120 h. The reaction was then assayed by ^{13}C NMR, and the composition of the resulting bicarbonate was estimated to be 39% 3-^{18}O, 37% 2-^{18}O, 17% 1-^{18}O, and 7% 0-^{18}O on the basis of peak heights. These values correspond to 70% overall incorporation of ^{18}O in bicarbonate.

^{18}O Transfer Experiment. Reaction mixtures contained 3.375 mL of buffer [125 mM EPPS (pH 8.0) containing 10 mM $MgCl_2$] that had been sparged with CO_2-free N_2, as well as 125 μL of 1.4 M ^{18}O-labeled bicarbonate and 250 μL of 274 mM F-PEP [87% (*Z*), 13% (*E*)]. Separate reactions were performed with and without 5 mg (13 000 units) of carbonic anhydrase in addition to the normal assay components. Reactions were initiated with 250 μL of PEP carboxylase (427 units/mL) and were incubated for 2 min, after which time three drops of 18 N H_2SO_4 was added to stop the reactions. The pH of each solution was then rapidly adjusted to 6.0 with saturated NaOH, and the protein was removed by ultrafiltration with a Centricon-30 filter. Each filtrate was lyophilized, and the resulting solid was dissolved in 5 mL of H_2O and adjusted to pH 11.0. The reaction mixtures were loaded onto separate Chelex-100 columns (0.6 × 5.0 cm; Na^+ form) and eluted with 5 mL of H_2O. The eluant from each column was lyophilized and then dissolved in 0.35 mL of D_2O, 0.25 mL of H_2O, and 100 μL of 60 mM EDTA. The ^{31}P NMR

spectrum of each reaction mixture was recorded at 202 MHz.

Isotope Effect Studies of PEP Carboxylase with PEP as a Substrate. The ^{13}C isotope effect for the reaction catalyzed by PEP carboxylase for the carbon arising from bicarbonate was measured by the method of successive substrate fractions described by Winkler et al. (1983) and Arnelle and O'Leary (1992). The procedure involves carrying out the PEP carboxylase catalyzed reaction in a syringe and sampling the reaction at intervals to analyze the isotopic composition of the remaining bicarbonate. At the same time another sample is taken to determine the fraction of reaction. Experiments were performed in disposable plastic syringes (30 mL) equipped with a Luer Lok which was attached a Pharmaseal three-way valve. The reaction mixture for the isotope effect determinations contained 100 mM HEPES (pH 8.0), 30 mM bicarbonate, 15 mM PEP, 5 mM $MgCl_2$, 1 mM NADH, 1 mM dithiothreitol, 50 mM glucose 6-phosphate, 5000 units of MDH, 5200 units of carbonic anhydrase, and 6500 units of G6PDH in 25 mL. It was critical for the initial concentration of bicarbonate to be known accurately in order to allow accurate fractional conversions to be determined. Prior to the addition of PEP carboxylase, the concentration of bicarbonate in the reaction mixture was determined by end-point analysis with PEP carboxylase. Reactions were initiated by drawing 1 unit of PEP carboxylase into the syringe, at which time the syringe was inverted several times to mix the solution. A small stirring bar was present in the syringe to aid in mixing. At this point any air bubbles were expelled from the syringe. Samples (2 mL) were taken every 20–30 min after the addition of PEP carboxylase. The samples were directly injected from the syringe into a sealed reaction flask, equipped with a vacuum adapter and a side arm with a glass stopcock and a septum. Reaction flasks contained 1 mL of 18 N sulfuric acid and had previously been purged of CO_2 with CO_2-free N_2. CO_2 was isolated by a high-vacuum distillation using two dry ice/2-propanol traps and a liquid N_2 trap. CO_2 was purified by bulb to bulb distillation, and the isotopic content of the resulting CO_2 was determined by isotope ratio mass spectrometry.

At the same time that a sample was taken for mass spectral analysis, a sample of the reaction mixture was also taken for malate analysis to determine the fraction of reaction corresponding to each CO_2 sample. A 600-μL aliquot of the reaction mixture was mixed with 200 μL of 2 N H_2SO_4, vortexed, and filtered with a Centricon-30 ultrafiltration apparatus to remove the protein. The concentration of malate was determined by end-point analysis with malic enzyme.

An isotope effect experiment was performed in which the glucose 6-phosphate/G6PDH nucleotide recycling system used in the experiment described above was replaced with a 1-propanol/alcohol dehydrogenase/aldehyde dehydrogenase nucleotide recycling system. The reaction mixture contained 50 mM 1-propanol, 2800 units of alcohol dehydrogenase, and 250 units of aldehyde dehydrogenase in place of glucose 6-phosphate and G6PDH. With the exception of the nucleotide recycling system, the experiment was identical to that described above.

A control experiment was performed in which PEP carboxylase was omitted from the reaction mixture. Samples were taken for mass spectral analysis to determine whether the isotopic composition of the source CO_2 was constant in the absence of PEP carboxylase. In addition, samples of the reaction mixture were analyzed for malate throughout the course of the experiment.

Isotope Effect Studies with F-PEP as a Substrate. Determinations of the $^{13}(V/K)$ isotope effect for the carbon arising from bicarbonate with F-PEP were performed using essentially the same protocol described above with PEP. Reaction mixtures contained 100 mM HEPES (pH 8.0), 5 mM $MgCl_5$, 100 mM glucose 6-phosphate, 23 mM $NaHCO_3$, 1 mM NADH, 1 mM dithiothreitol, 26 mM F-PEP, 2400 units of MDH, 3100 units of carbonic anhydrase, 1100 units of LDH, and 2500 units of G6PDH in 12 mL. Before use in these experiments the F-PEP was dissolved in 100 mM HEPES, pH 8.0, and frozen with liquid nitrogen, and the sample was lyophilized. Water was added, and the sample was again frozen and lyophilized. This procedure removed a volatile contaminant, apparently introduced during recrystallization, which interfered with the subsequent mass spectrometry.

Reactions were initiated with 15 units of PEP carboxylase. The initial concentration of bicarbonate was assayed by end-point analysis with PEP carboxylase. Samples (1.0 mL) of the reaction were taken every 30–40 min to determine the isotopic composition of the residual bicarbonate. To determine the fraction of reaction, 0.2-mL samples were diluted with 0.2 mL of 60 mM EDTA and filtered with a Centricon-30 filter. The fraction of reaction was determined by assaying the concentration of residual F-PEP with the PK/LDH couple. The assay determined the amount of F-PEP consumed, but in order to determine the f-value, it was necessary to determine how much of the F-PEP consumed was carboxylated. The ratio of hydrolysis to carboxylation was determined by comparing the concentration of 3-fluorolactate to the sum of the concentrations of the two isomers of 3-fluoromalate by ^{19}F NMR. From the partitioning of F-PEP and the amount of F-PEP consumed, it was possible to calculate the amount of bicarbonate consumed to determine the fraction of reaction. Three separate experiments were performed to determine $^{13}(V/K)$ in which the ratio of (*Z*)- to (*E*)-F-PEP was 87/13. Two experiments were performed with F-PEP that had been photoisomerized to produce a (*Z*) to (*E*) ratio of 78/22.

Relative V/K Values for (Z)- and (E)-F-PEP. The relative V/K values for (*Z*)- and (*E*)-F-PEP when used as substrates for PEP carboxylase were determined by a competitive method using the data obtained from the isotope effect experiments. The relative concentrations of (*Z*)- and (*E*)-F-PEP were determined by ^{19}F NMR both before the reactions were initiated and at a time corresponding to the last sample taken for isotopic analysis by withdrawal of aliquots of the reaction mixture at these times as described above. The fraction of reaction for each isomer was determined from integrations of all peaks in the ^{19}F NMR spectrum.

Isotope Effect Nomenclature. The nomenclature used throughout this report is that of Northrop (1977) in which the leading superscript denotes the isotope responsible for the effect on the given kinetic or thermodynamic parameter. For example, the ^{13}C isotope effect on V/K, written as $^{13}(V/K)$, represents V/K for the ^{12}C-containing species divided by V/K for the ^{13}C-containing species.

Data Processing. The $^{13}(V/K)$ isotope effects with PEP or with F-PEP as the substrate were measured using the method of internal competition, in which changes in the isotopic composition of the residual bicarbonate were measured over the course of the experiment (O'Leary, 1980). The kinetic isotope effect is calculated from

$$^{13}(V/K) = \frac{\ln (1 - f)}{\ln [(1 - f)(R_s/R_0)]} \quad (1)$$

where R_s is the $^{13}C/^{12}C$ ratio of the carbon of interest in the substrate after fraction of reaction f. R_0 is the isotopic ratio

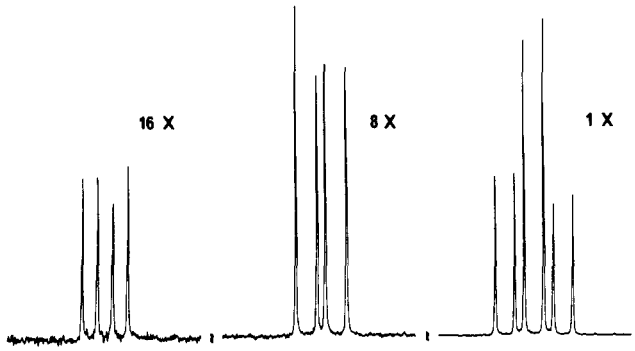


FIGURE 1: ^{19}F NMR spectra of the three reaction products of PEP carboxylase catalyzed carboxylation and hydrolysis of (*Z*)- and (*E*)-F-PEP coupled to the reactions catalyzed by excess MDH, LDH, and NADH. ^{19}F NMR data: for 3-fluorolactate (right), −228.80 ppm (dt, $^2J_{HF}$ = 47.53 Hz, $^3J_{HF}$ = 31.75 Hz); for (2*R*,3*R*)-3-fluoromalate (left), −188.14 ppm (dd, $^2J_{HF}$ = 49.70 Hz, $^3J_{HF}$ = 24.85 Hz); for (2*R*,3*S*)-3-fluoromalate (center), −196.85 ppm (dd, $^2J_{HF}$ = 48.57 Hz, $^3J_{HF}$ = 34.64 Hz).

before any reaction has occurred. Equation 1 can be rewritten as

$$\ln R_s = \ln R_0 + \left[\frac{1 - {}^{13}(V/K)}{{}^{13}(V/K)}\right][\ln (1 - f)] \quad (2)$$

Equation 2 has the form of a straight line if ln R_s is plotted versus ln $(1 - f)$. The isotopic composition of the substrate is measured at various fractions of reaction, and the data are fitted to eq 2 by least squares linear regression, with weighting proportional to the inverse standard error squared of each point obtained from the mass spectral analysis. The isotope effect is obtained from the slope by eq 3, with errors obtained by appropriate propagation.

$$^{13}(V/K) = 1/(1 + \text{slope}) \quad (3)$$

Relative V/K values were determined using eq 4, where f_Z and f_E are the fractional conversions of the (*Z*) and (*E*) isomers of F-PEP, respectively, to products, regardless of the identity of the products.

$$(V/K)_Z/(V/K)_E = \ln (1 - f_Z)/[\ln (1 - f_E)] \quad (4)$$

Results

Identification of the Products in the Reaction of (Z)- and (E)-F-PEP with PEP Carboxylase. Two samples of F-PEP with different ratios of (*Z*) and (*E*) isomers were incubated with PEP carboxylase, and the reaction was monitored by ^{19}F NMR. The carboxylation of a mixture of (*Z*)- and (*E*)-F-PEP resulted in the formation of (*R*)- and (*S*)-3-fluorooxalacetates which were reduced by NADH and MDH. The hydrolysis of F-PEP catalyzed by PEP carboxylase resulted in the production of 3-fluoropyruvate which in the presence of LDH and excess NADH was converted to 3-fluorolactate. The ultimate products were identified as 3-fluorolactate, (2*R*,3*R*)-3-fluoromalate, and (2*R*,3*S*)-3-fluoromalate by comparison to authentic samples (Figure 1). The assignments are consistent with those of Keck et al. (1980) and Hwang and Nowak (1986).

The results in Table I show that when the proportion of the (*E*) isomer in the (*Z*)/(*E*) mixture of F-PEP is increased, the proportion of the (2*R*,3*S*) isomer in the 3-fluoromalate product mixture is increased to the same extent. This indicates not only that the (*E*) isomer is a substrate for carboxylation catalyzed by PEP carboxylase but also that the (2*R*,3*S*)-fluoromalate isomer results from the carboxylation of the (*E*) isomer. From the ratio of (*Z*) to (*E*) at time 0 and the ratio of products after all the F-PEP had been consumed, it was calculated that 97% of (*Z*)-F-PEP was hydrolyzed to fluoropyruvate and only 3% was carboxylated to produce (2*R*,3*R*)-3-fluoromalate after reduction by NADH. In contrast, 86% of the (*E*) isomer was carboxylated, resulting in the formation of (2*R*,3*S*)-3-fluoromalate after reduction.[2] Only 14% of the (*E*)-F-PEP was hydrolyzed to fluoropyruvate.[3]

MDH Concentration Dependence. The above experiment was repeated at varying MDH concentrations in order to ensure that the two isomers of F-OAA were reduced by MDH faster than decarboxylation or racemization could occur. The ratio of fluoromalate to fluorolactate was constant, indicating that the reduction of F-OAA was rapid compared to decarboxylation. In addition, the ratio of (2*R*,3*R*)-3-fluoromalate to (2*R*,3*S*)-3-fluoromalate was constant, indicating that reduction of F-OAA was rapid relative to racemization. Goldstein et al. (1978) and Hwang and Nowak (1986) reported that racemic 3-fluorooxalacetate was converted by MDH to a mixture of (2*R*,3*R*)-3-fluoromalate and (2*R*,3*S*)-3-fluoromalate without significant stereoselectivity.[4]

Table I: Partition Analysis of (*Z*)- and (*E*)-F-PEP[a]

ratio of (*Z*)- and (*E*)-F-PEP in substrate	% contribution to product		
	FLac[b]	(2*R*,3*R*)-FMal[c]	(2*R*,3*S*)-FMal[d]
87.7/12.3	86.3	2.9	10.8
70.4/29.6	72.4	2.1	25.5

[a] Reaction mixtures contained 100 mM HEPES (pH 8.0), 26 mM F-PEP (isomer content shown), 5 mM $MgCl_2$, 100 mM glucose-6-P, 25 mM $NaHCO_3$, 1 mM NADH, 1 mM dithiothreitol, 173 units of carbonic anhydrase, 61 units of LDH, 300 units of MDH, and 140 units of G6PDH in 750 μL. Reactions were initiated with 0.52 unit of PEP carboxylase and incubated at 23 °C for 5 h. Reactions were assayed by ^{19}F NMR. When PEP carboxylase was omitted, no products were observed. [b] 3-Fluorolactate. [c] (2*R*,3*R*)-3-Fluoromalate. [d] (2*R*,3*S*)-3-Fluoromalate.

Table II: Metal Ion Dependence of the Partitioning of F-PEP[a]

metal ion	% contribution			
	FLac[b]	(2*R*,3*R*)-FMal[c]	(2*R*,3*S*)-FMal[d]	(2*R*,3*R*)-FMal/(2*R*,3*S*)-FMal
Mg^{2+}	85.7	3.2	11.1	0.29
Mn^{2+}	89.5	1.0	9.5	0.10
Co^{2+}	92.1	0.8	7.1	0.11
Ni^{2+}	92.5	0.9	6.6	0.14

[a] Reaction mixtures contained 100 mM HEPES (pH 7.5), 100 mM glucose-6-P, 25 mM $NaHCO_3$, 1 mM NADH, 1 mM dithiothreitol, 173 units of carbonic anhydrase, 61 units of LDH, 260 units of MDH, 140 units of G6PDH, and 26 mM F-PEP [87% (*Z*), 13% (*E*)] in 750 μL. Mg^{2+}, Ni^{2+}, and Co^{2+} concentrations were 5 mM; the Mn^{2+} concentration was 1 mM. Reactions were initiated with 1.4 units of PEP carboxylase and incubated at 23 °C for 16 h. Reactions were assayed by ^{19}F NMR. [b] 3-Fluorolactate. [c] (2*R*,3*R*)-3-Fluoromalate. [d] (2*R*,3*S*)-3-Fluoromalate.

[2] While (*Z*)-F-PEP is only carboxylated 3% of the time, (*Z*)-Cl-PEP is reported to undergo 25% carboxylation and 75% hydrolysis (Liu et al., 1990). The reason for the difference may lie in the enhanced willingness of carbon bonded to chlorine to assume carbanionic character and the decreased ability of carbon bonded to fluorine to do this (Adolph & Kamlet, 1966). This does not explain the different degree of carboxylation observed with (*Z*)- and (*E*)-F-PEP, but until the degree of carboxylation of (*E*)-Cl-PEP is determined, further comparison is not possible.

[3] These calculations assume that the carboxylation is stereospecific, as indicated by the fact that changes in the ratio between the fluoromalate isomers after reduction of the product parallel the change in the ratio of F-PEP isomers used as substrate.

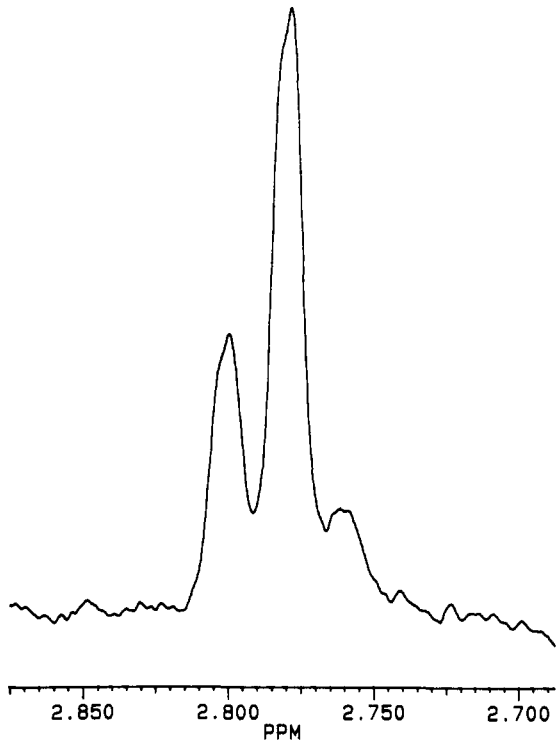


FIGURE 2: ^{31}P NMR spectrum of the phosphate product of the ^{18}O transfer experiment. The relative integrals of the peaks are 0.43, 1.00, and 0.17.

pH Dependence. The partitioning of F-PEP between carboxylation and hydrolysis was examined at pH 7.5, 8.0, and 9.0. Both the ratio of fluoromalate to fluorolactate and the ratio of (2*R*,3*R*)-3-fluoromalate to the (2*R*,3*S*) isomer were identical at each pH examined, indicating that the partitioning of F-PEP by PEP carboxylase is insensitive to pH in this pH range.

Metal Ion Dependence. The metal ion dependence of the partitioning of F-PEP was examined with Mg^{2+}, Mn^{2+}, Co^{2+}, or Ni^{2+}. The partitioning of F-PEP between carboxylation and hydrolysis was found to vary with metal ion (Table II).

^{18}O Transfer Experiment. ^{18}O-Labeled bicarbonate (70% ^{18}O) was supplied as a substrate together with F-PEP in the reaction catalyzed by PEP carboxylase. Two reactions were performed, one with and one without carbonic anhydrase. Reactions were run to 12% completion, and products and substrate were examined by ^{31}P NMR. The ^{31}P NMR spectrum of the reaction containing carbonic anhydrase showed one resonance at 2.8 ppm corresponding to unlabeled inorganic phosphate in addition to the unreacted (*Z*)- and (*E*)-F-PEP. When 100 μL of 200 mM Na_2HPO_4 was added to the reaction, the resonance corresponding to unlabeled phosphate increased in intensity (data not shown), indicating that this peak was indeed phosphate. The ^{31}P NMR spectrum of the phosphate produced in the reaction that did not contain carbonic anhydrase is shown in Figure 2. Three resonances were

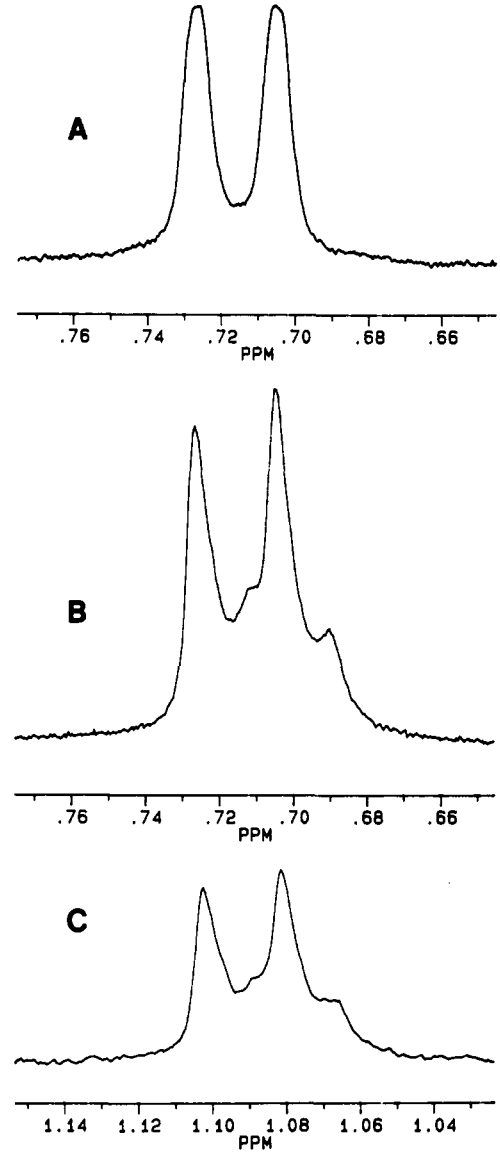


FIGURE 3: (A) Proton-decoupled ^{31}P NMR spectrum of (*Z*)-F-PEP in the reaction that contained $HC^{18}O_3^-$ and carbonic anhydrase. (B, C) Proton-decoupled ^{31}P NMR spectrum of (*Z*)-F-PEP (B) or (*E*)-F-PEP (C) in the reaction that did not contain carbonic anhydrase. The relative areas of the major and minor components in (B) are 1.00 and 0.53.

observed, corresponding to phosphate labeled with zero (downfield resonance), one, or two (upfield resonance) atoms of ^{18}O. The separation between the three resonances was 0.021 ppm, consistent with the isotopic shift reported by Cohn and Hu (1978). The proton-decoupled spectrum of the region corresponding to (*Z*)-F-PEP of the reaction that contained carbonic anhydrase is shown in Figure 3A. The doublet observed results from the fluorine coupling ($^4J_{FP}$ = 4.2 Hz). The same region of the proton-decoupled ^{31}P NMR spectrum of the reaction that did not contain carbonic anhydrase is shown in Figure 3B. The shoulders on the upfield side of the peaks in Figure 3B correspond to (*Z*)-F-PEP containing one atom of ^{18}O and are approximately 0.02 ppm upfield of the resonance corresponding to unlabled (*Z*)-F-PEP. A shoulder was observed on each peak of the phosphate resonance corresponding to the (*E*) isomer as well (Figure 3C). The proportion of residual F-PEP containing ^{18}O was about 35% in each case, which by comparison with the expected 75% exchange at isotopic equilibrium and the 70% initial ^{18}O labeling in bicarbonate represents a fractional approach to isotopic

[4] It has been reported that when excess F-OAA is present with a limiting amount of MDH, only the (2*R*,3*R*) isomer of 3-fluoromalate is produced in the presence of NADH (Skilleter et al., 1972). Goldstein et al. (1978) assumed that these results were in error because the (2*R*,3*S*) isomer was lost during purification. We have duplicated the results of Skilleter et al. (1972) and conclude that the (3*R*) isomer of F-OAA is the preferred substrate for MDH, and when MDH is limiting, this preference is expressed, since racemization under such conditions is fast ($t_{1/2} \sim 7$ min) compared to reduction. Under conditions of excess MDH and NADH, racemization is not fast compared to reduction, and both isomers are reduced.

Table III: $^{13}(V/K)$ Isotope Effects Measured for F-PEP[a]

ratio of (Z) to (E) in substrate	ratio of (2R,3R)-FMal[b] to (2R,3S)-FMal[c] in product	ratio of FMal[d] to FLac[e] in product	$^{13}(V/K)$
85.6/14.4	22.8/77.2	13.5/86.5	1.021 ± 0.001
87.8/12.2	24.7/75.3	13.9/85.3	1.017 ± 0.002
86.7/13.3	27.1/72.9	14.7/85.3	1.018 ± 0.002
			av 1.019 ± 0.001
79.1/20.9	12.1/87.9	22.3/77.7	1.012 ± 0.003
78.2/21.8	10.3/89.7	20.6/79.4	1.014 ± 0.002
			av 1.013 ± 0.003

[a] The first three reactions were performed with F-PEP that not been photoisomerized, whereas F-PEP used in the last two experiments had been photoisomerized to enrich the sample in (E) isomer. [b] (2R,3R)-3-Fluoromalate. [c] (2R,3S)-3-Fluoromalate. [d] Both isomers of 3-fluoromalate. [e] 3-Fluorolactate.

equilibrium of ~0.67. This corresponds to a ratio of exchange to chemical reaction of ~8.7.

Isotope Effect Studies with PEP as the Substrate. The $^{13}(V/K)$ isotope effect for the carbon arising from bicarbonate was measured by the method of successive substrate fractions for the reaction catalyzed by PEP carboxylase in which PEP was supplied as a substrate (Winkler et al., 1983; Arnelle & O'Leary, 1992). Three separate determinations of $^{13}(V/K)$ were made, yielding an average value of 1.0021 ± 0.0001 at pH 8.0, 25 °C. Glucose 6-phosphate present in these experiments as part of a nucleotide recycling system is known to be a K_m activator of PEP carboxylase (Coombs et al., 1973; Meyer et al., 1989). A determination of $^{13}(V/K)$ was made in which 1-propanol/alcohol dehydrogenase/aldehyde dehydrogenase was used to recycle NADH; under these conditions $^{13}(V/K)$ was 1.0021 ± 0.0002. These values are in good agreement with those reported previously by O'Leary et al. (1981) and Winkler et al. (1983). In a control experiment in which PEP carboxylase was omitted from the reaction mixture, the isotopic content of the source bicarbonate was constant for the length of the experiment.

Isotope Effect Studies with F-PEP as the Substrate. The $^{13}(V/K)$ isotope effect for the carbon arising from bicarbonate with F-PEP as substrate was determined in an identical fashion. Data from one experiment are shown in Figure 4. Three experiments were performed in which the average ratio of (Z)- to (E)-F-PEP was 87/13 and the average isotope effect measured was $^{13}(V/K)$ = 1.019 ± 0.001 (Table III). In two experiments with F-PEP that had been photoisomerized to give a (Z) to (E) ratio of 79/21, the average $^{13}(V/K)$ value was 1.013 ± 0.003.

The isotope effect measured with a mixture of (Z) and (E) isomers of F-PEP is an average of the isotope effects for the two isomers weighted by the contribution of each isomer to the overall amount of carboxylation observed. Due to the identity of the V/K values for the two isomers (see below), and the differences in partitioning of the (Z) (3% carboxylation) and (E) (86% carboxylation) isomers, the (E) isomers contributes approximately 75% of the isotope effect measured at an initial ratio of (Z)/(E) of 87/13. At a (Z)/(E) ratio of 79/21, 88% of the isotope effect is contributed by carboxylation of the (E) isomer and the remaining 12% by carboxylation of the (Z) isomer. From these values we can calculate that $^{13}(V/K)$ is 1.049 ± 0.003 for (Z)-F-PEP and 1.009 ± 0.006 for (E)-F-PEP.

Relative V/K Values for the F-PEP Isomers. The relative V/K values (carboxylation plus hydrolysis) for the isomers of F-PEP as substrates for PEP carboxylase were determined with eq 4 from the relative extent of conversion of each in the isotope effect experiments using the ^{19}F NMR data. From five separate determinations, the average value for the ratio of V/K for the (Z) isomer to that of the (E) isomer was 0.97 ± 0.05, or not significantly different from unity.

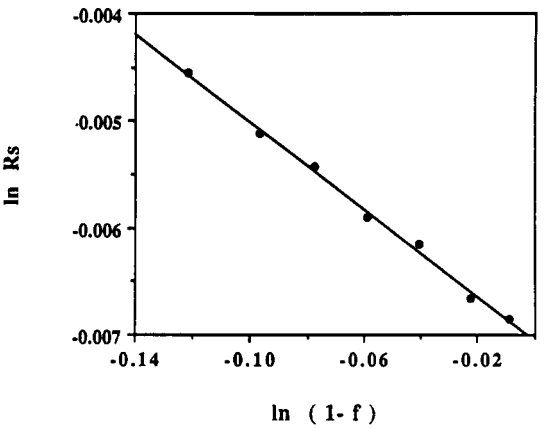


FIGURE 4: Representative plot for determination of the ^{13}C isotope effect on the PEP carboxylase catalyzed carboxylation of F-PEP. The reaction mixture contained 100 mM HEPES (pH 8.0), 26 mM F-PEP [86% (Z), 14% (E)], 5 mM $MgCl_2$, 1 mM NADH, 1 mM dithiothreitol, 100 mM glucose-6-P, 2400 units of MDH, 2400 units of LDH, 1100 units of carbonic anhydrase, and 2500 units of G6PDH in 12 mL. PEP carboxylase (15 units) was used to initiate the reaction. $^{13}(V/K)$ = 1.021 ● 0.001.

Kinetic Parameters for F-PEP Isomers. Previous determinations of the kinetic parameters for F-PEP have produced K_m values of 14 μM (Gonzales & Andreo, 1988) or 1.15 mM (Diaz et al., 1988) and V_{max} values relative to PEP of 10% or 50% for the two sets of authors. To clarify this situation, we determined the kinetic parameters of the mixture of F-PEP isomers relative to those of PEP with MDH and LDH both present to measure both carboxylation and hydrolysis. The mixture of F-PEP isomers showed a V_{max} for the sum of carboxylation and hydrolysis 5% that with PEP and a K_m of 7.6 μM (4% that with PEP). The V/K values are not significantly different between PEP and the two F-PEP isomers, which we have shown to have identical V/K values. This situation would result if the steps up to and including the first irreversible one went at the same rate for all three molecules, while any subsequent steps were slower for F-PEP.

Discussion

Consistent with previous reports by Diaz et al. (1988) and Gonzalez and Andreo (1988), we find that reaction of F-PEP with PEP carboxylase results in a mixture of F-OAA and F-pyruvate. The (E) isomer gives 86% (S)-3-F-OAA and 14% 3-fluoropyruvate. In contrast, only 3% of (Z)-F-PEP was carboxylated to (R)-3-F-OAA, and the remaining 97% was hydrolyzed to 3-fluoropyruvate.

The observation that (Z)- and (E)-F-PEP are carboxylated to produce (R)-3-F-OAA and (S)-3-F-OAA, respectively, indicates that carboxylation of the enolate of fluoropyruvate occurs on the 2-*re* face (Figure 5). This is the first report of the stereochemistry of carboxylation catalyzed by PEP carboxylase from a C_4 plant. Rose et al. (1969) have shown

that PEP carboxylase from peanut (a C_3 plant) and *Acetobacter xylinum* catalyze the carboxylation of PEP on the 2-*si* face, which corresponds to the 2-*re* face of F-PEP.

Previous investigations of the substrate activity of predominantly (*Z*)-F-PEP have reported that carboxylation accounts for 10% (Gonzalez & Andreo, 1988) and 14% (Diaz et al., 1988) of the consumption of F-PEP. The F-PEP used in the experiments of Diaz et al. (1988) contained 13% of the (*E*) isomer, and the majority of the 14% of F-OAA formed arose therefore from the carboxylation of the (*E*) isomer. Gonzalez and Andreo (1988) did not comment on the isomeric purity of the F-PEP used in their experiments, but the results presented here allow us to estimate that it contained 8% of the (*E*) isomer, so that the carboxylation they report is again predominantly the result of carboxylation of the (*E*) isomer.

Like F-PEP, PEP itself partitions between carboxylation and hydrolysis (Ausenhus & O'Leary, 1992). In both cases, the partitioning is strongly dependent on the metal ion used, with Mg^{2+} giving the least hydrolysis and Ni^{2+} giving the most. Quantitative comparison of results for PEP with stability of bidentate complexes between metal ions and oxalate (an isoelectronic analogue of the enolate of pyruvate) suggests that the enolate forms a bidentate complex with the metal ion at the active site of PEP carboxylase. The same trend with metal ions is observed for F-PEP, suggesting that, in this case, too, a bidentate complex is formed.

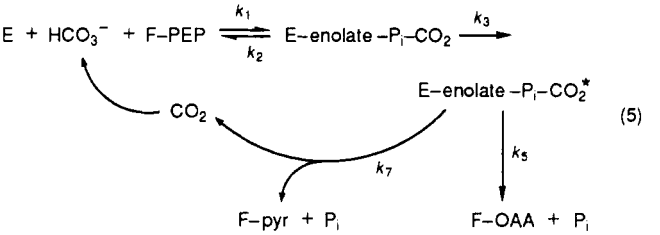
The different behavior of the two isomers of F-PEP is striking. Only 3% of the (*Z*) isomer is carboxylated, where 86% of the (*E*) isomer is carboxylated. This raises interesting questions as to what factors govern the ratio of carboxylation to hydrolysis. Previous studies of the substrate activity of F-PEP with avian phosphoenolpyruvate carboxykinase have demonstrated that (*Z*)-F-PEP is a slow alternate substrate while the (*E*) isomer shows no substrate activity (Duffy & Nowak, 1984; Hwang & Nowak, 1989). Proton relaxation rate studies and CD studies demonstrate that (*Z*)-F-PEP and PEP induce different structural changes around the Mn^{2+} binding site/active site than does (*E*)-F-PEP (the inactive isomer). The difference in conformation of the two geometric isomers may result from differences in H-bonding of the fluorine at the active site (Hwang & Nowak, 1989).

During normal catalysis, PEP carboxylase catalyzes the transfer of one atom of ^{18}O from bicarbonate to the phosphate group of PEP (Maruyama et al., 1966; O'Leary & Hermes, 1987). Our results demonstrate that more than one atom of ^{18}O is transferred from bicarbonate to phosphate during the reaction of F-PEP. In addition, analysis of residual starting material reveals that ^{18}O is incorporated into the phosphate groups of both (*Z*)- and (*E*)-F-PEP. In control experiments with carbonic anhydrase (present to accelerate the exchange of ^{18}O-labeled bicarbonate with $H_2{}^{16}O$), neither phosphate nor residual (*Z*)- or (*E*)-F-PEP was labeled with ^{18}O. Similar results have been reported for the substrate analogue Me-PEP, in which multiply ^{18}O-labeled phosphate (Fujita et al., 1984; O'Laughlin, 1988) as well as ^{18}O-labeled residual Me-PEP was observed (O'Laughlin, 1988). O'Laughlin (1988) postulated that carboxy phosphate reversibly collapses at the active site of PEP carboxylase to produce CO_2 and phosphate. A mechanism accounting for the labeling pattern observed with F-PEP is presented in Scheme I. This mechanism requires that both phosphate and bicarbonate can rotate in the active site to bring about the observed positional isotope exchange and that the carboxy phosphate intermediate (which must form for ^{18}O to be incorporated into phosphate) must reversibly collapse to CO_2 and phosphate during catalysis.

The earlier mechanism for the reaction catalyzed by PEP carboxylase involving direct attack of the enolate of pyruvate on carboxy phosphate (Walsh, 1979; O'Leary et al., 1981; Hanson & Knowles, 1982) is inconsistent with our isotope exchange data. Instead, reversible decomposition of carboxy phosphate in the active site must precede attack of the enolate on CO_2.

The observation of a relatively high level of ^{18}O incorporation into residual F-PEP during reaction requires that the steps involving phosphoryl transfer and decarboxylation of carboxy phosphate to CO_2 and phosphate be reversible and that F-PEP release from the enzyme be reasonably fast relative to the forward reaction rate. However, in order for the observed ^{13}C isotope effect to be suppressed to the extent observed for the (*E*) isomer, a commitment to catalysis of at least 5 must be present. The problem is that this is a mathematically impossible situation unless an irreversible step precedes the parititioning between carboxylation and hydrolysis. If the commitment is this large, no ^{18}O incorporation into residual (*E*)-F-PEP should be seen, and yet the ^{18}O incorporation appears to be the same for both isomers!

The dilemma is solved by assuming that an irreversible conformation change occurs after CO_2 formation in the active site that allows CO_2 to move to a position where it can react with the enolate. The active site stays closed only a finite time after this conformation change, and if carboxylation has not taken place when the active site opens up, CO_2 is lost and the net reaction is hydrolysis. This mechanism can be modeled simply as

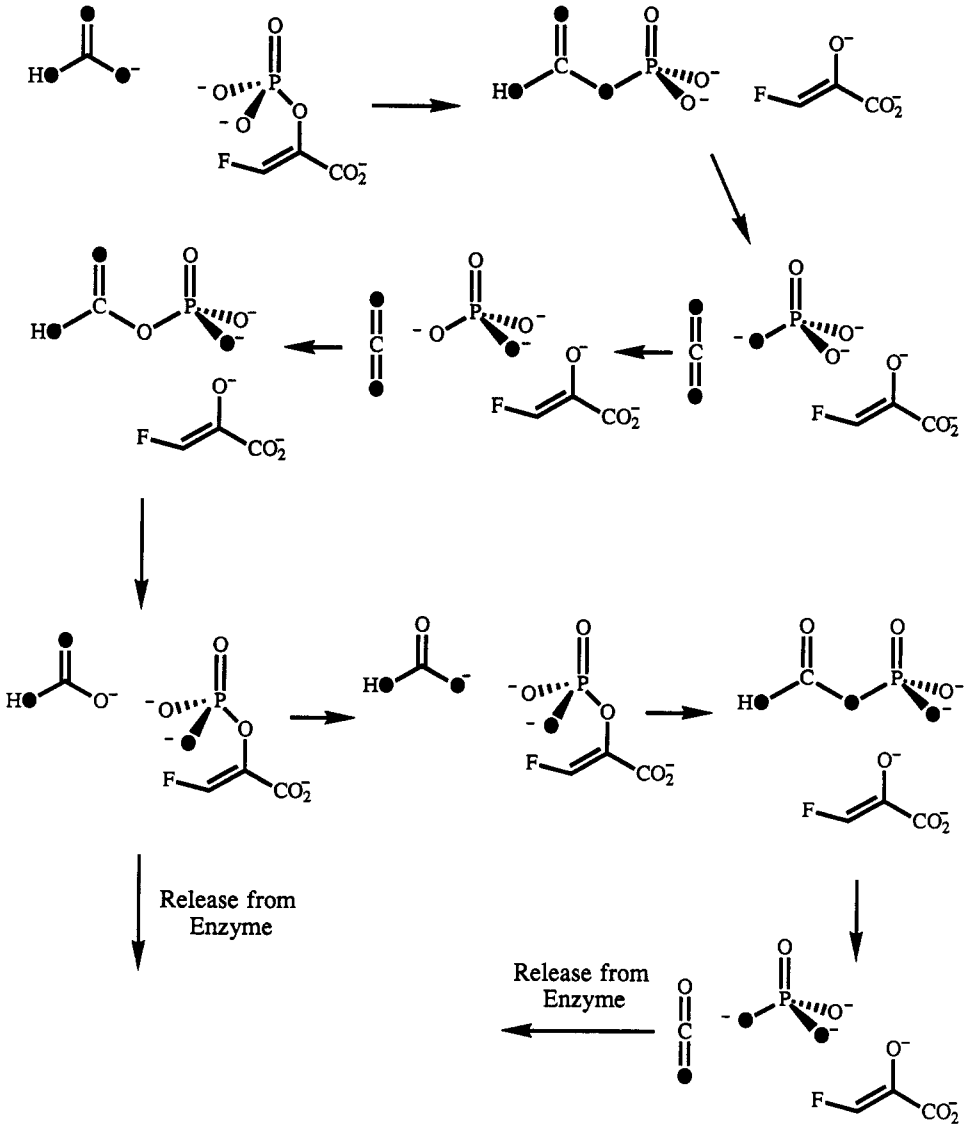


(5)

The step represented by k_1 includes addition of substrate to the enzyme, phosphoryl transfer from F-PEP to bicarbonate, and subsequent decarboxylation to CO_2 and phosphate. In the reverse direction, k_2 includes substrate release as well as reversal of the chemical steps. In this mechanism, the ratio of ^{18}O exchange into residual F-PEP to the rate of F-PEP disappearance is k_2/k_3 and is expected to be independent of the nature of the isomers, as observed. Since the V/K for disappearance of F-PEP is $k_1k_3/(k_2 + k_3)$, again presumably independent of isomer, it is logical for the V/K values of the two isomers to be identical, as is observed.

The ^{13}C isotope effects on k_1 and k_2 in mechanism 5 are small. Phosphoryl transfer should be accompanied by a secondary ^{13}C isotope effect with a $^{13}K_{eq}$ value of 0.9983 [by analogy with the value for phosphorylation of carbamate to carbamyl phosphate reported by Tipton and Cleland (1988)]. The decarboxylation of carboxy phosphate to CO_2 and phosphate should have a $^{13}K_{eq}$ value of 1.0096, since the overall $^{13}K_{eq}$ value for conversion of aqueous bicarbonate to gaseous CO_2 is 1.0079 (Mook et al., 1974). There is presumably no water left in the active site, so gaseous CO_2 is a better model than aqueous CO_2 for CO_2 in the active site. The kinetic isotope effect on decarboxylation of carboxy phosphate is likely to be similar to that for dehydration of bicarbonate to CO_2 (1.015; Marlier & O'Leary, 1984), but the magnitude of the observed ^{13}C isotope effect on k_1 will depend on the relative rates of phosphoryl transfer and carboxy phosphate decarboxylation. If the former is at equilibrium, $^{13}k_1 = 1.013$, while

Scheme I



if the latter is at equilibrium, $^{13}k_1$ would be in the range 0.9983–1.0000. The corresponding values of $^{13}k_2$ will be the values of $^{13}k_1$ divided by 1.0079.

The observed ^{13}C isotope effect on carboxylation in mechanism 5 will be

$$^{13}(V/K) = (^{13}k_5 + k_5/k_7)(^{13}k_1 + {}^{13}K_{eq\,1}k_2/k_3)/(1 + k_5/k_7)(1 + k_2/k_3) \quad (6)$$

where $^{13}K_{eq\,1}$ is 1.0079, and from the ^{18}O incorporation into residual F-PEP, $k_2/k_3 = 5.6$. The ratio of the second terms in eq 6 will depend on the value of $^{13}k_1$ and can range from 1.0087 to 1.0064 for the range of $^{13}k_1$ values discussed above. Since we see a value of only 1.009 for $^{13}(V/K)$ with the (*E*)-F-PEP isomer, and a value of 1.0021 with PEP (where, however, the absence of ^{18}O incorporation into residual PEP makes k_2/k_3 small so the ratio of the second terms in eq 6 is only $^{13}k_1$), it seems likely that carboxy phosphate decarboxylation is fast and close to equilibrium so that a value near or less than unity for $^{13}k_1$ is most probable. Thus 1.0064 seems the most reasonable value for the ratio of the second terms for the F-PEP isomers.

The value of k_5/k_7 for the F-PEP isomers is simply the ratio of carboxylation to hydrolysis [0.03 for the (*Z*) isomer and 6.1 for the (*E*) isomer]. The observed $^{13}(V/K)$ value of 1.049 for the (*Z*) isomer then corresponds to a $^{13}k_5$ value of 1.044, which is close to the measured values for the nonenzymatic decarboxylation of OAA of 1.049 (Grissom & Cleland, 1986) and of F-OAA of 1.052 (Urbauer and Cleland, unpublished results) and equal to the calculated intrinsic isotope effect on enzymatic decarboxylation of the OAA intermediate by malic enzyme of 1.044 (Grissom & Cleland, 1985). [Since $^{13}K_{eq}$ is near unity for these decarboxylations (O'Leary & Yapp, 1978), $^{13}(V/K)$ is identical for carboxylation and decarboxylation.]

With the value of 1.044 for $^{13}k_5$, when the value of 6.1 for k_5/k_7 for the (*E*) isomer is used in eq 6, the predicted $^{13}(V/K)$ value is 1.013, which is not significantly different from the observed value of 1.009.

To determine the expected isotope effect with PEP as substrate, we will assume the same isotope effect on carboxylation of the enolate of pyruvate and let k_2/k_3 be 0 because no ^{18}O incorporation into residual PEP has ever been observed. Taking $^{13}k_1$ as unity (see above), we can use the observed ratio between carboxylation and hydrolysis of ~19 (Ausenhus & O'Leary, 1992) as k_5/k_7 in eq 6 to calculate 1.0022 as the expected ^{13}C isotope effect on the reaction, in excellent agreement with the experimental value of 1.0021.

Thus the model in mechanism 5 correctly predicts all of the observed ^{13}C isotope effects for PEP and for both isomers of F-PEP. This model requires that CO_2 be an intermediate in

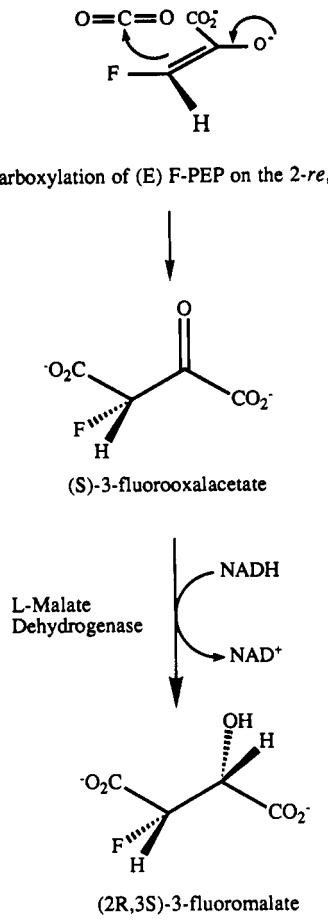


FIGURE 5: Stereochemistry of the carboxylation of (*E*)-F-PEP catalyzed by PEP carboxylase.

the carboxylation reaction, since if carboxylation occurred by attack of the enolate on carboxy phosphate, the partitioning between carboxylation and hydrolysis would not follow an irreversible step, and the isotope effect for the (*E*) isomer would not be suppressed to the extent observed.

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