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Binding of Carbon Dioxide to Phosphoenolpyruvate Carboxykinase Deduced from Carbon Kinetic Isotope Effects†

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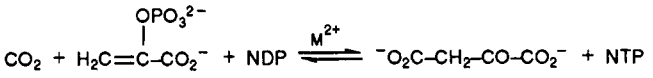
ABSTRACT: Phosphoenolpyruvate carboxykinase [ATP:oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.49] from *Chloris gayana* Kunth has been purified by a combination of ammonium sulfate fractionation, ion exchange, gel filtration, and affinity chromatography on agarose–hexane–ATP. In the direction of OAA formation, the specific activity of the enzyme was 33 μmol/(min·mg of protein). The carbon isotope effect on carboxylation was measured by successive analysis of remaining CO_2 over the course of the reaction. At 22 mM PEP and 1.3 mM MgADP, pH 7.5, the isotope effect is 1.024 ± 0.001. When the concentration of PEP was reduced to 1 mM, the isotope effect rose to 1.034 ± 0.004; when the concentration of MgADP was reduced to 60 μM, the value rose to 1.040 ± 0.006. The variation of the carbon isotope effect on carboxylation with both substrate concentrations indicates that the enzyme operates by a random kinetic mechanism. This in turn requires that the enzyme have a binding site for substrate CO_2; this is one of the first enzymes for which such a site has been demonstrated.

Phosphoenolpyruvate (PEP)[1] carboxykinase [ATP:oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.49] catalyzes the nucleoside diphosphate-dependent carboxylation of phosphoenolpyruvate to form oxalacetate and a nucleoside triphosphate (Utter & Kolenbrander, 1972):



PEP carboxykinase is ubiquitous in living things, but it serves different roles in plants and in animals. In vertebrates, the enzyme catalyzes the decarboxylation of OAA to PEP as the first committed step in gluconeogenesis. In PEP carboxykinase-dependent C_4 plants, the decarboxylation of OAA by PEP carboxykinase is coupled to photosynthetic carbon fixation by the Calvin cycle (Furbank & Hatch, 1987; Hatch, 1976).

Analogy with PEP carboxylase suggests that the mechanism of PEP carboxykinase should involve two distinct steps (Scheme I): The first step is transfer of phosphate from PEP to the nucleoside diphosphate substrate, a process which occurs with inversion of configuration at phosphorus (Konopka et al., 1986). The other product of this step is the enolate of pyruvate, which readily reacts with CO_2 to form OAA. CO_2, rather than HCO_3^-, is known to be the substrate (Utter & Kolenbrander, 1972). The stepwise mechanism is consistent with isotope exchange studies (Krebs & Bridger, 1980; Miller & Lane, 1968).[2]

From the point of view of kinetic mechanism, PEP carboxykinase is one of the best studied carboxylases. The preponderance of kinetic evidence for the enzyme from animal sources suggests that the order of substrate binding is random. Measurements of product inhibition and isotope exchange at equilibrium are consistent with random binding of substrates (Jomain-Baum & Schramm, 1978; Chang et al., 1966). Proton relaxation rate measurements on the avian enzyme reveal that all three substrates (PEP, CO_2, ITP) can bind to the E–Mn^{2+} complex (Hebda & Nowak, 1982); however, the kinetic competency of all these complexes has not been dem-

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[1] Abbreviations: NDP, nucleotide 5′-diphosphate; NTP, nucleotide 5′-triphosphate; OAA, oxalacetic acid; PEP, phosphoenolpyruvate; EPR, electron paramagnetic resonance; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; HEPES, *N*-(2-hydroxyethyl)-piperazine–*N*′-2-ethanesulfonic acid; HPC, hydroxypropylcellulose; BSA, bovine serum albumin; MOPS, 3-(*N*-morpholino)propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; EPPS, *N*-(2-hydroxyethyl)-piperazine-*N*′-3′-propanesulfonic acid; DTT, dithiothreitol.

[2] A referee has pointed out that convincing mechanistic evidence for the stepwise mechanism is not available, and a concerted mechanism (involving simultaneous carboxylation and phosphate transfer) is feasible. We agree that no specific evidence to eliminate a concerted mechanism exists, but we believe that the stepwise mechanism is correct. Our conclusions would not be altered if the mechanism were concerted.

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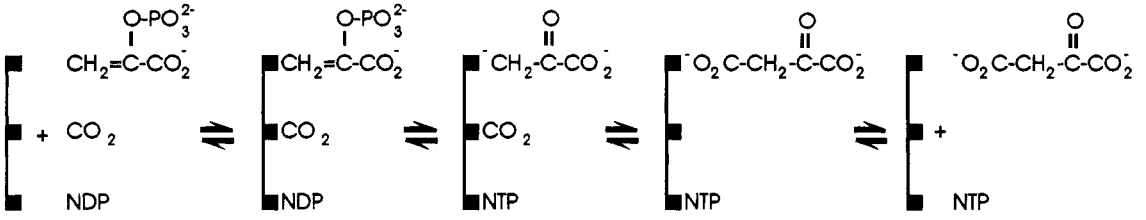
Scheme I



onstrated. Studies of porcine liver PEP carboxykinase by initial rates, EPR, and proton relaxation rates suggested a mixed ordered random mechanism in which Mn^{2+} and PEP bind first and either CO_2 or nucleotide may be the next substrate (Chang et al., 1966; Miller & Lane, 1968). Studies of the enzyme from sheep kidney mitochondria suggest that nucleotide preferentially binds before PEP (Barns et al., 1972); CO_2 can apparently bind either before or after PEP. Positional isotope exchange studies suggest that there may be differences in kinetic mechanism between the rat liver enzyme and the chicken liver enzyme (Chen et al., 1991). PEP carboxykinase from *Escherichia coli* also appears to operate by a random mechanism in which CO_2 is not the last substrate to bind (Krebs & Bridger, 1980).

PEP carboxykinase from plants is less thoroughly studied than the mammalian enzyme. The enzyme has been purified from Crassulacean acid metabolism plants, from C_4 plants, and from the photosynthetic bacterium *Rhodospirillum rubrum* (Burnell, 1986; Cooper & Benedict, 1968; Daley et al., 1977; Dittrich et al., 1973; Girolami & Cavalie, 1977; Hatch & Mau, 1977; Holdsworth & Bruck, 1977; Klemme, 1976; Ray & Black, 1976; Urbina & Avilan, 1989). PEP carboxykinases from three C_4 plants, *Panicum maximum*, *Chloris gayana*, and *Urochloa panicoides*, have similar physical and kinetic properties (Burnell, 1986). ADP and ATP are the best nucleotide substrates for the plant enzymes, in contrast to animal enzymes, which are most active with inosine or guanine nucleotides.

Unlike the mammalian enzyme, PEP carboxykinase from *P. maximum* has been suggested on the basis of steady-state kinetic studies to have a fully ordered kinetic mechanism, with ADP binding first, then PEP, and then CO_2 (Urbina & Avilan, 1989). OAA release was suggested to precede ATP release. However, product inhibition studies of the enzyme from *C. gayana* are inconsistent with a fully ordered mechanism (D. Jordan and M. H. O'Leary, unpublished results).

The variation of isotope effects with substrate concentration can be used to determine kinetic mechanism (Cook & Cleland, 1981; Hermes et al., 1982; Miller & Klinman, 1982). We have used the variation of the carbon isotope effect on carboxylation with substrate concentration to provide evidence that PEP carboxykinase from *C. gayana* operates by a random kinetic mechanism and so describe the first application of this technique to a ter-reactant mechanism. These same experiments provide evidence for the existence of a CO_2-binding site and thus for sequestration of CO_2 away from water at the active site.

Materials and Methods

Materials. Bio-Rad protein assay solution and Ultracent-30 tubes were obtained from Bio-Rad Biochemicals. Column materials were obtained from Pharmacia Fine Chemicals or Pharmacia P-L Biochemicals, Inc. Dowex AG1-X8 (200–400 mesh) anion exchange resin (analytical grade) was cleaned by the method of Dunaway-Mariano and Cleland (1980) prior to use. All other enzymes and biochemicals were obtained from Sigma Chemical Co. or Boehringer-Mannheim, USA. All other chemicals were commercially available reagent grade. Water was purified by a Millipore Super-Q filtration system. PEP carboxylase was purified by the method of O'Leary et al. (1981).

Enzyme assays were performed either on a Cary 118 recording spectrophotometer or on a Cary 2200 spectrophotometer. All assays were carried out in cuvettes of 1.0-cm path length at 25 °C. Isotope ratios were measured using a Finnigan Delta-E isotope ratio mass spectrometer.

Assays. PEP carboxykinase activity in crude preparations was determined by measuring the ATP-dependent enzyme-catalyzed decarboxylation of OAA at 280 nm (Hatch, 1973). A cuvette containing 0.980 mL of 50 mM HEPES/KOH, pH 7.6, 100 mM KCl, 2.5 mM $MgCl_2$, 2.5 mM $MnCl_2$, 0.9 mM OAA, 0.02% HPC or 0.1% BSA, 4 units/mL pyruvate kinase, and PEP carboxykinase was equilibrated to 25 °C in the cell compartment of the spectrophotometer. The initial rate of the absorbance change at 280 nm due to the background decarboxylation of OAA was recorded. ATP was then added (final concentration 0.25 mM), and the total rate was observed. Subtraction of the background rate gave the rate of the catalyzed decarboxylation.

Enzyme activity in the carboxylation direction was used to measure PEP carboxykinase activity in later stages of the purification. The decrease in absorbance at 340 nm was measured by coupling OAA formation to the oxidation of NADH by malate dehydrogenase. The reaction mixture contained enzyme, 75 mM MOPS/KOH, pH 6.8, 2.5 mM $MgCl_2$, 2.5 mM $MnCl_2$, 0.01% HPC or 0.1% BSA, 0.14 mM NADH, 0.6 mM ADP, 33 mM glucose, 22 mM $KHCO_3$, 15 units/mL hexokinase, and 4 units/mL malate dehydrogenase. Reactions were initiated by the addition of sufficient PEP to bring the final concentration to 2.2 mM PEP in a total volume of 2.24 mL.

PEP carboxykinase activity of purified preparations could also be measured in the decarboxylation direction by coupling PEP formation to the oxidation of NADH through pyruvate kinase and lactate dehydrogenase. Reactions contained enzyme, 50 mM HEPES/KOH, pH 7.6, 2.5 mM $MgCl_2$, 2.5 mM $MnCl_2$, 0.5 mM ATP, 0.14 mM NADH, 0.5 mM OAA, 0.02% HPC or 0.1% BSA, 4 units/mL of pyruvate kinase, and 2 units/mL of lactate dehydrogenase in a total volume of 1 mL. Blank rates due to the decarboxylation of OAA and traces of malate dehydrogenase in PEP carboxykinase and the coupling enzymes were determined prior to adding ATP.

Endpoint assays of malate concentration were performed using reaction mixtures containing sample, 100 mM triethanolamine hydrochloride/KOH, pH 7.5, 0.1 mM EDTA, 0.8 M $MnCl_2$, 0.05 mM NADP, and 0.1 unit/mL malic enzyme in a total volume of 2.06 mL.

Endpoint assays for ATP concentration were performed using a hexokinase/glucose-6-phosphate dehydrogenase coupled system (Truautschold et al., 1985). The reaction mixture contained sample, 38 mM triethanolamine hydrochloride/KOH, pH 7.5, 50 mM glucose, 6.66 mM $MgCl_2$, 0.33 mM

NADP, 1.8 units/mL hexokinase, and 0.5 unit/mL glucose-6-phosphate dehydrogenase in a total volume of 3 mL.

Endpoint assays for ADP and AMP concentration were performed using an adenylate kinase/pyruvate kinase/lactate dehydrogenase coupled system (Jaworek & Welsch, 1986). The reaction mixture contained sample, 100 mM triethanolamine hydrochloride/KOH, pH 7.5, 0.94 mM PEP, 33.4 mM $MgSO_4$, 0.12 M KCl, 0.36 mM NADH, 24 units/mL lactate dehydrogenase, 18 units/mL pyruvate kinase, and 16 units/mL adenylate kinase in a total volume of 3 mL.

Endpoint assays for bicarbonate concentration were performed using a PEP carboxylase/malate dehydrogenase coupled system (Peled, 1986). The reaction mixture contained sample, 20 mM EPPS/KOH, pH 8.0, 6.4 mM PEP, 3 mM $Mg(C_2H_3O_2)_2$, 20 mM oxamate, 1.2 mM NADH, 0.5 unit/mL PEP carboxylase, and 3.5 units/mL malate dehydrogenase in a total volume of 2.06 mL.

Endpoint assays for PEP concentration were performed using a pyruvate kinase/lactate dehydrogenase coupled system (Lamprecht & Heinz, 1986). The reaction mixture contained sample, 150 mM triethanolamine hydrochloride/KOH, pH 7.5, 86 mM ADP, 12 mM $MgSO_4$, 0.1 mM Na_2EDTA, 44 mM KCl, 0.17 mM NADH, 2.7 units/mL lactate dehydrogenase, and 1.2 units/mL pyruvate kinase in a total volume of 2.02 mL.

Protein concentrations were determined using the Bio-Rad protein assay system, which is based on the Bradford protein assay (Read & Northcote, 1978).

SDS–polyacrylamide gel electrophoresis was performed using a Bio-Rad Protean Dual Slab Cell with 9% or 12% polyacrylamide gels prepared according to the system of Laemmli (Hames, 1981; Laemmli, 1970).

Purification of PEP carboxykinase from C. gayana. The enzyme was extracted from 4–6 week old *C. gayana* Kunth grown either in a greenhouse maintained between 22 and 30 °C or in a growth chamber at 25–32 °C. All of the following operations were performed at 4 °C.

Leaves (100–120 g) were rinsed in distilled water and cut into 0.5–1-cm sections. The sliced tissue (in two portions) was added to a Waring blender containing 5 mL of extraction buffer [50 mM HEPES-KOH, 2 mM $MnCl_2$, 2 mM $MgCl_2$, 5 mM DTT, and 1% (w/v) poly(vinylpyrrolidone)-40, pH 7.5] and 0.5 g of sand per gram of leaf. The mixture was blended at low speed for 1.5–2 min, then filtered through four layers of cheesecloth, and the filtrate was set aside. The remaining solid material was extracted a second time as above. After straining through cheesecloth, the second filtrate was combined with the first, centrifuged for 20 min at 10000*g*, and decanted.

Protamine sulfate (0.1 mg/mg of protein in the crude extract) was added to the extract. The suspension was allowed to stand for 20 min with stirring and then was centrifuged for 20 min at 10000*g* and decanted. Sufficient solid ammonium sulfate was added to the supernatant to bring the solution to 45% saturation. After 20 min, the suspension was centrifuged for 20 min at 10000*g* and decanted. Sufficient solid ammonium sulfate was added to the supernatant to bring the solution to 65% saturation. After 30 min, the suspension was centrifuged for 20 min at 10000*g* and the supernatant was discarded. The protein pellet was dissolved in a minimal volume of general column buffer (50 mM HEPES-KOH, 2 mM $MnCl_2$, 2 mM $MgCl_2$, 3 mM DTT, pH 7.5) and centrifuged at 12000*g* for 10 min to remove any insoluble matter.

The solution was desalted on Sephadex G-25 Fine (35 × 2.6 cm) equilibrated with column buffer. Active fractions were pooled and immediately applied to a DEAE-Sepharose CL 6B column (36 × 2.6 cm) equilibrated with column buffer at a flow rate of 1.0 mL/min. The column was washed with 90 mL of column buffer and then eluted with a 500-mL linear gradient of 0–0.3 M KCl in column buffer. PEP carboxykinase activity, as measured by the carboxylation assay, eluted at about 0.15 M KCl. Sufficient saturated ammonium sulfate solution was added to the collected fractions to give a 66% saturated solution.

The protein pellet was recovered by centrifugation at 12000*g* for 10 min, dissolved in a minimal volume of column buffer, and applied to a Sephacryl S-300 column (36 × 2.6 cm) equilibrated with column buffer containing 50 mM KCl at a flow rate of 1.0 mL/min and eluted with the same buffer. PEP carboxykinase activity emerged after about 70 mL of eluant.

The combined active fractions from the S-300 column were concentrated by ultrafiltration on an Amicon PM-30 membrane. The concentrated enzyme solution was passed through a Blue Sepharose CL-6B column equilibrated with general column buffer. PEP carboxykinase activity elutes in the void volume of the column. Active fractions were combined and concentrated by ultrafiltration and then dialyzed for storage as below or for affinity column chromatography.

Sufficient 1 M $MgCl_2$ solution in general column buffer was added to the enzyme solution to raise the Mg^{2+} concentration to 42 mM. The solution was applied to an ATP–hexane–agarose type 4 column (8 × 0.6 cm) equilibrated with column buffer containing 42 mM $MgCl_2$. The column was washed with 10 column volumes of buffer and then eluted with 5 mL of 3 mM Na_2ATP in column buffer at a flow rate of 0.4 mL/min. 0.5-mL fractions were collected, and the fractions with the highest specific activity were pooled. Occasionally, it was necessary to dialyze the collected fractions versus the affinity column buffer and treat the recovered PEP carboxykinase a second time in order to obtain enzyme which was pure by SDS–PAGE.

This affinity column purification step replaces a second ion exchange step used by Burnell (1986). The presence of Mg^{2+} was essential for the binding of PEP carboxykinase to the affinity column; Mn^{2+} in concentrations up to 20 mM could not replace Mg^{2+}. PEP carboxykinase does not bind to Blue Sepharose CL-6B, Blue Dextran Agarose, ATP- or ADP–hexane–agarose type 3 or type 2 resins, or to agarose–hexane–diadenosine pentaphosphate resin.

The enzyme was dialyzed against a nitrogen-sparged buffer containing 100 mM HEPES-KOH, 100 mM KCl, 3 mM DTT, 0.1 mM EDTA, and 20% glycerol, pH 7.5, and sealed in airtight vials for storage at −20 °C. PEP carboxykinase activity is stable for several months if stored in this manner.

Isotope Effect Determinations. A 20-mL solution containing 225 mM HEPES–KOH, pH 7.5, ADP, PEP, 33.3 mM glucose, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 1.3 mM NADH, 1% (w/v) BSA, 6 mM DTT, and $NaHCO_3$ was filtered through a syringe equipped with a 0.45-μm Millipore filter. The solution was taken up in a sterile 30-mL plastic syringe equipped with an Anspec two-way four-port valve as shown in Figure 1. A solution containing 250–400 units of glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides*, 400 units of hexokinase from Bakers' yeast, 230 units of malate dehydrogenase (bovine heart), and about 500 units of carbonic anhydrase was similarly filtered and mixed with the reaction solution. A sample of the reaction mixture was expelled into a sealed, nitrogen-flushed vial and assayed for bicarbonate in triplicate. The reaction solution was allowed to stand at 24 °C for several min before the addition of 0.9–0.15 units of PEP carboxykinase. The solution was mixed by inversion of the

Table I: Purification of PEP Carboxykinase from *C. gayana*

purification step	vol (mL)	protein conc (mg/mL)	total protein (mg)	act. (units/mL)[a]	total units	sp act. (units/mg)	yield (%)
combined extracts	1800	1.0	1840	1.21	2169	1.18	100
protamine and $(NH_4)_2SO_4$ fractionation	38	19.7	750	51	1935	2.6	89
Sephadex G-25	58	11	630	32.8	1886	3	87
DEAE-Sepharose 6B CL	63	1.0	65	29.8	1877	29.8	86
Sephacryl S-300	23	0.68	15.6	18.5	425	27	36
ATP–hexane–agarose[b]	9	0.76	6.8	25	226	33	10

[a] Micromoles of OAA formed per minute. [b] Combined results from several ATP–hexane–agarose chromatographies.

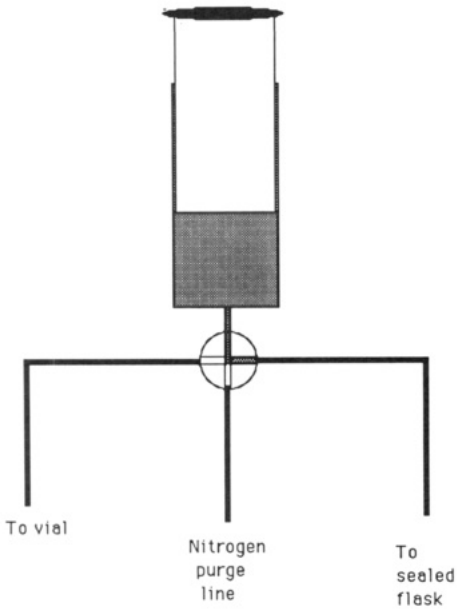


FIGURE 1: Flow system for determination of isotope effects.

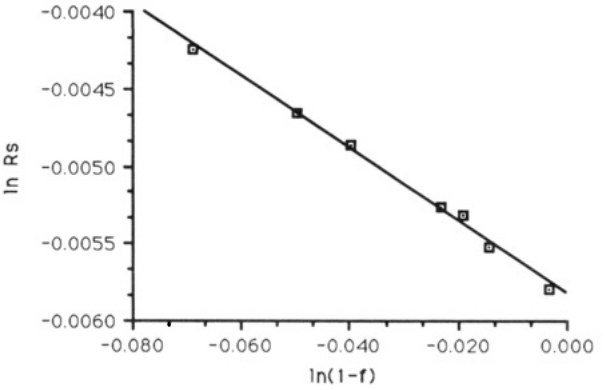


FIGURE 2: Time dependence of CO_2 isotope ratio in the carboxylation of PEP by PEP carboxykinase at pH 7.5. The reaction mixture contained 223 mM HEPES/KOH, 33 mM glucose, 1.3 mM ADP, 21 mM PEP, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 1.2 mM NADH, 0.1% BSA, 8 mM DTT, 57.9 mM $NaHCO_3$, 450 units of glucose-6-phosphate dehydrogenase, 400 units of hexokinase, 231 units of malate dehydrogenase, 500 units of carbonic anhydrase, and 0.9 units of PEP carboxykinase. The reaction was initiated by addition of PEP carboxykinase. $^{13}(V/K_{CO_2}) = 1.0243$.

syringe for solutions at pH 7.5 and above and by a small magnetic stirring bar at pH 7 or lower. At this point, any air bubble trapped in the syringe was expelled.

At intervals after the addition of PEP carboxykinase, an aliquot of the solution was expelled into a small plastic vial. 200 μL of this solution was immediately added to a vial containing 200 μL of 1 M $HClO_4$ and the time of the quench noted. A 1-mL sample of the reaction mixture was then expelled into a sealed flask containing 1 mL of 9 M H_2SO_4. CO_2 from this sample was recovered by trap-to-trap distillation and analyzed by isotope ratio mass spectrometry.

The bicarbonate concentration at the beginning of the reaction represented the total amount of source CO_2 in the reaction. Assayed malate concentration versus time data were fit to a quadratic equation by nonlinear least-squares regression. The calculated malate concentrations were used to establish the extent of the reaction, f, at time t. The isotopic content of the residual substrate at time t was corrected for the aqueous CO_2/HCO_3^- isotopic equilibrium and converted to a mass ratio, R_s (Mook et al., 1974).[3] The ^{13}C isotope effect on the carboxylation, ^{13}k, was calculated from the slope of a plot of ln R_s vs ln $(1-f)$ (Figure 2) (Melander & Saunders, 1980) according to

$$\ln R_s = \ln R_0 + \frac{1 - {}^{13}k}{{}^{13}k} \ln (1-f)$$

where R_s is the isotopic ratio, $^{13}CO_2/^{12}CO_2$, of the substrate CO_2 at some fraction of reaction, f; R_0 is the isotopic ratio of the substrate before any reaction has occurred.

[3] The measured isotopic content of the one-carbon substrate is the total isotopic content of CO_2 plus HCO_3^-, whereas the isotope effect reported must refer to the isotope fractionation relative to CO_2. Thus, the correction for isotopic equilibration is required.

Results

PEP carboxykinase from *C. gayana* was purified by the scheme summarized in Table I. This procedure is based on a partial purification of PEP carboxykinase reported by Hatch and Mau (1977) and a later report by Burnell (1986). The activity of PEP carboxykinase in the crude extract varied between 5 and 12 units per gram of leaf; 4–6 week old plants generally yielded 9–12 units per gram of leaf (Girolami & Cavalie, 1977). The purified enzyme appeared as a single band in an SDS–PAGE gel stained with Coomassie Blue, and the enzyme was more than 95% pure by a silver-stained SDS–PAGE gel. In the direction of OAA formation, the specific activity of the enzyme was 33 μmol/(min·mg of protein).

Isotope effects on the carboxylation of PEP by PEP carboxykinase were determined by measurement of changes in the isotopic composition of remaining CO_2 substrate with time (Schmidt et al., 1978). A representative plot of ln R_s vs ln $(1-f)$ is shown in Figure 2. The intercept of this plot is a function of R_0, the isotope ratio of HCO_3^- at t_0. The isotopic content of $NaHCO_3$ used in these experiments was virtually identical with that of air; under these conditions, minor contamination by CO_2 from air should make no difference in the isotopic content of the substrate. Control experiments indicated that no significant change in the isotopic content of CO_2 occurs over the course of at least 9 h in the absence of PEP carboxykinase. Likewise, no malate was produced in the reaction over this time period in the absence of PEP carboxykinase.

The results of carbon isotope effect measurements under a variety of conditions are summarized in Table II. The

Table II: Concentration Dependence of $^{13}(V/K_{CO_2})$ Isotope Effects on Carboxylation of PEP by PEP Carboxykinase[a]

substrate concentrations	$^{13}(V/K_{CO_2})$
22 mM PEP, 1.3 mM ADP	1.024 ± 0.001
22 mM PEP, 1.3 mM ADP	1.024 ± 0.001
22 mM PEP, 1.3 mM ADP	1.024 ± 0.002
22 mM PEP, 1.3 mM ADP	1.024 ± 0.002
22 mM PEP, 1.3 mM ADP	1.024 ± 0.001
mean	1.024 ± 0.001
1 mM PEP, 1.3 mM ADP	1.036 ± 0.003
1 mM PEP, 1.3 mM ADP	1.035 ± 0.006
1 mM PEP, 1.3 mM ADP	1.031 ± 0.004
1 mM PEP, 1.3 mM ADP	1.036 ± 0.006
mean	1.034 ± 0.004
22 mM PEP, 60 μM ADP	1.044 ± 0.015
22 mM PEP, 60 μM ADP	1.040 ± 0.005
22 mM PEP, 83 μM ADP	1.035 ± 0.007
mean	1.040 ± 0.006

[a] The reaction mixture contained HEPES/KOH buffer, pH 7.5, glucose, substrates, 5 mM $MgCl_2$, 1 mM $MnCl_2$, NADH, 0.1% BSA, DTT, glucose-6-phosphate dehydrogenase, hexokinase, malate dehydrogenase, and carbonic anhydrase as described under Materials and Methods and in the legend to Figure 2. PEP carboxykinase was used to initiate the reaction.

carbon isotope effect on carboxylation at pH 7.5 increased from 1.024 to 1.035 when the concentration of PEP was reduced from 22 to 1 mM at fixed ADP concentration. An increase from 1.024 to 1.040 was observed when the concentration of ADP was decreased from 1.2 mM to 60 μM at fixed PEP concentration.

Discussion

A variety of enzymes catalyzing carboxylations and decarboxylations have been studied by use of carbon isotope effects (O'Leary, 1989, 1992). Intrinsic isotope effects for decarboxylases are generally near 1.06 (Grissom & Cleland, 1985; O'Leary, 1988; Cleland, 1980). Equilibrium isotope effects for decarboxylations are near unity (O'Leary & Yapp, 1978), so intrinsic isotope effects for carboxylases are also expected to be near 1.06. The carbon isotope effects seen with PEP carboxykinase are large and indicate that the carboxylation step is principally rate-determining. The variation of the isotope effect with substrate concentration has important consequences for the kinetic mechanism, as we will see below.

Isotope Effects and Kinetic Mechanism. PEP carboxykinase appears to operate by a sequential mechanism (see the introduction). The observed isotope effect for PEP carboxykinase is influenced by steps beginning with CO_2 binding and ending with release of OAA.[4] The isotope effect is independent of steps prior to CO_2 binding and subsequent to OAA release. In a random or partially random pathway, the isotope effect may change as the predominant pathway changes.

The variation of isotope effects with substrate concentration can be used to determine the kinetic mechanism (Cook & Cleland, 1981; Hermes et al., 1982; Miller & Klinman, 1982; O'Leary & Paneth, 1986). If the mechanism is ordered and CO_2 is the last substrate to bind, then the concentrations of the other substrates do not affect the isotope effect. Alternatively, if CO_2 binds second in an ordered mechanism, the isotope effect will vary with the concentration of the third substrate, but not the first; at high concentrations of the third substrate, the isotope effect will go to unity.[5] If CO_2 is the first substrate to bind in an ordered mechanism, then the isotope effect will go to unity at high concentration of either of the other substrates.

Predictions for random mechanisms must take account of all possible pathways with respect to CO_2, as well as the relative contributions of these pathways. A substrate which always binds before CO_2 does not affect the isotope effect. A substrate which always binds after CO_2 will cause the isotope effect to go to unity at high concentrations of that substrate. A substrate whose binding is random with regard to CO_2 will cause the isotope effect to decrease at high concentrations of that substrate, but the limit will be greater than unity and will depend on the ease of dissociation of CO_2 from that complex.

In the case of PEP carboxykinase, sequential ordered mechanisms are inconsistent with the isotope effect data. Neither PEP nor MgADP can add to the enzyme before CO_2 in an ordered fashion, since the observed isotope effect would be independent of the concentration of the substrate which added before CO_2.

Conversely, neither PEP nor MgADP can add in an ordered fashion after CO_2. At saturating concentrations of either PEP or MgADP, ordered addition of either substrate would effectively trap CO_2 in the active site of the enzyme, and the observed isotope fractionation would approach unity at high concentrations of that substrate. Instead, raising the concentration of PEP by 30-fold over K_m (K_m = 0.78 mM) decreased the isotope effect from 1.034 to 1.024, while raising the MgADP concentration from $2K_m$ to $50K_m$ (K_m = 25 μM) decreased the observed isotope effect from 1.040 to 1.024. Thus, it appears that although concentrations of both ATP and PEP can diminish the isotope effect, the isotope effect does not go to unity at high substrate concentration, and some pathway(s) must exist which allows CO_2 to escape from the central complex even in the presence of bound ATP and/or PEP.

Thus, we are led to the conclusion that PEP carboxykinase operates by a random sequential mechanism. The dependence of the isotope effect on ADP concentration indicates that the addition of CO_2 may take place either before or after ADP; that is, addition of CO_2 and of ADP is random. Similarly, the concentration dependence of the isotope effect in the presence of variable concentrations of PEP requires that addition of CO_2 and of PEP is also random. Thus, the mechanism is fully random.

CO_2-Binding Site. CO_2 is the substrate for a variety of enzymatic reactions. By analogy with other enzymatic processes, it is natural to assume that CO_2 binds to enzymes (i.e., forms Michaelis complexes) prior to reaction. However, evidence for such binding is slim (O'Leary, 1992). The occurrence of saturation kinetics is not a sufficient condition to conclude that the substrate binds in the case of a multisubstrate mechanism. Searches for inhibitors competitive against CO_2 (which would be expected to bind to the same site) have generally failed. On the basis of concentration dependence of hydrogen isotope effects, Van Dyk and Schloss (1986) suggested that there is no Michaelis complex with CO_2 in the reaction catalyzed by ribulose bisphosphate carboxylase/oxygenase. Mallick et al. (1991) suggest that there may be no

[4] This is true provided that release of OAA is irreversible. In the present case, release of OAA was made irreversible by reducing this product with NADH and malate dehydrogenase. If product release were not irreversible, then additional complications would be introduced into the consideration of the isotope effect.

[5] Isotope effects such as those reported here represent a competition in reactivity between the different isotopic species. In an ordered mechanism, high concentrations of a later substrate will "lock" the isotopic substrate on the enzyme, thus reducing the isotope effect to unity.

binding site for CO_2 in the reverse reaction catalyzed by malic enzyme.

In most carboxylations and decarboxylations, CO_2 is bound last or released first, and in such cases the kinetic mechanism provides no evidence either for or against CO_2 binding. The random kinetic mechanism for PEP carboxykinase means that CO_2 can bind prior to the other substrates; thus, the enzyme must have a binding site for CO_2.

A variety of other lines of evidence also suggest that PEP carboxykinase has a binding site for CO_2. Cheng and Nowak (1989) showed that PEP carboxykinase has an arginine at the active site, and CO_2 protects against arginine modification. Proton relaxation rate measurements suggest that an enzyme–Mn^{2+}–CO_2 complex exists (although these measurements do not address the kinetic competency of this complex) (Hebda & Nowak, 1982).

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