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Carbon Isotope Effects on the Enzyme-Catalyzed Carboxylation of Ribulose Bisphosphate†

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ABSTRACT: ^{13}C isotope effects associated with the carboxylation of ribulose bisphosphate by ribulosebisphosphate carboxylase from spinach have been measured by comparison of the isotopic composition of carbon 1 of the 3-phosphoglyceric acid formed with that of the CO_2 substrate. Correction was made for the 3-phosphoglyceric acid that is formed from carbons 3–5 of ribulose bisphosphate. The carbon isotope effect is $k^{12}/k^{13} = 1.029 \pm 0.001$ at pH 8.0, 25 °C. The same isotope effect is observed in the presence of either 1 or 0.2 mM ribulose bisphosphate. Deuteration of carbon 3 of the substrate decreases the carbon isotope effect to 1.021. At pH 9.0, the isotope effect is 1.026, whereas at pH 7.0 it is 1.030. These results are consistent with the generally accepted mechanism for the carboxylation involving enolization of ribulose bisphosphate followed by carboxylation, provided that CO_2 binds to the enzyme prior to the enolization. Carboxylation is principally, though not entirely, rate limiting. The enzyme probably catalyzes hydrogen exchange between solvent and substrate. The isotope effects do not provide an unambiguous choice between random and ordered mechanisms of substrate addition.

Ribulosebisphosphate carboxylase-oxygenase (EC 4.1.1.39), the major enzyme responsible for CO_2 fixation by plants (Miziorko & Lorimer, 1983; Lorimer, 1981a; Akazawa, 1979), catalyzes the carboxylation and oxygenation of RuBP (Figure 1). The accepted mechanism of carboxylation of RuBP,[1] first suggested by Calvin (1956), is shown in Figure 2. Carbon dioxide reacts with an enol or enolate derived from RuBP to form an enzyme-bound, six-carbon intermediate, which is cleaved by reaction with water to form two molecules of 3-PGA. When the carboxylation is conducted in labeled water, label is introduced at C-2 of one of the two 3-PGA molecules (Mullhofer & Rose, 1965; Hurwitz et al., 1956) and at C-3 of recovered RuBP (Saver & Knowles, 1982). 4-Carboxyarabinitol bisphosphate and 2-carboxyarabinitol bisphosphate, analogues of the putative six-carbon intermediate, are tight-binding inhibitors of the enzyme (Schloss & Lorimer, 1982; Pierce et al., 1980; Siegel & Lane, 1972). Recent studies of the six-carbon intermediate indicate that the enzyme-catalyzed cleavage of this intermediate to enzyme-bound 3-PGA is favored over cleavage to enolate and CO_2 by at least a factor of 10 (G. Lorimer and J. V. Schloss, personal communication). Isotope-labeling studies mitigate against formation of a covalent enzyme–RuBP complex (Lorimer, 1978; Sue & Knowles, 1978).

CO_2, rather than HCO_3^-, is the substrate (Cooper et al., 1969). Although the reaction shows saturation kinetics with respect to CO_2 (Badger & Andrews, 1974), this does not necessarily indicate that CO_2 binds to the enzyme prior to reaction with the enolate. Oxygenation of RuBP appears to occur by an analogous mechanism in which the enol or enolate reacts with O_2, rather than CO_2. Kinetically, the reaction also shows saturation kinetics with respect to O_2.

Both carboxylase (Lorimer et al., 1976) and oxygenase (Badger & Lorimer, 1976) require activation by CO_2 and a divalent metal. Kinetic studies of the activation have led to the two-step mechanism

$$E \xrightleftharpoons[\text{slow}]{CO_2} E\text{—}\underset{H}{N}\text{—}CO_2^- \xrightleftharpoons[\text{fast}]{M^{2+}} E\text{—}\underset{H}{N}\text{—}CO_2^-M^{2+} \quad (1)$$

This "activating" CO_2 is distinct from "substrate" CO_2 (Miziorko, 1979; Lorimer, 1979). Formation of a carbamate during activation has been demonstrated by ^{13}C NMR (O'Leary et al., 1979) and by isolation of a methyl ester derivative of the carbamate following treatment of the stable $^{14}CO_2$–Mg^{2+}–2-carboxyarabinitol bisphosphate complex with diazomethane (Lorimer & Miziorko, 1980). Peptides containing N^{ϵ}-(methoxycarbonyl)lysine have been sequenced (Lorimer, 1981b; Donnelly et al., 1983).

The enzyme can be assayed by a radiochemical procedure or by a complex coupled assay (Racker, 1974). Measured activities are often time dependent. The complexity of the assays and the activation requirement of the enzyme seem to have had a dampening effect on kinetic studies. One of the most serious difficulties encountered is the presence of inhibitors in the RuBP used. The best characterized of these is xylulose 1,5-bisphosphate, the C-3 epimer of RuBP (McCurry & Tolbert, 1977; Paech et al., 1978), which is a good inhibitor ($K_i \approx 3$ μM) and is generated in situ from RuBP. This inhibitor can be removed (with difficulty) by

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[1] Abbreviations: CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; di-PGA, 2,3-diphosphoglyceric acid; HEPPS, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; 3-PGA, 3-phosphoglyceric acid; RuBP, ribulose 1,5-bisphosphate; XuBP, xylulose 1,5-bisphosphate; EDTA, ethylenediaminetetraacetic acid; Bicine, *N,N*-bis(2-hydroxyethyl)glycine.

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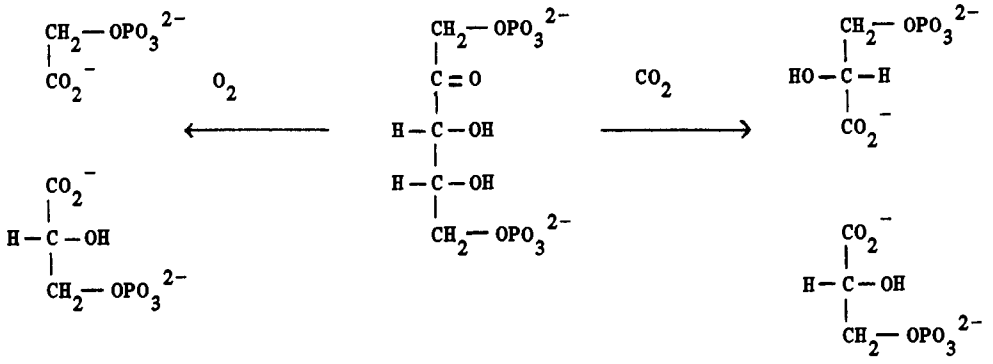

FIGURE 1: Carboxylation and oxygenation of ribulose bisphosphate.

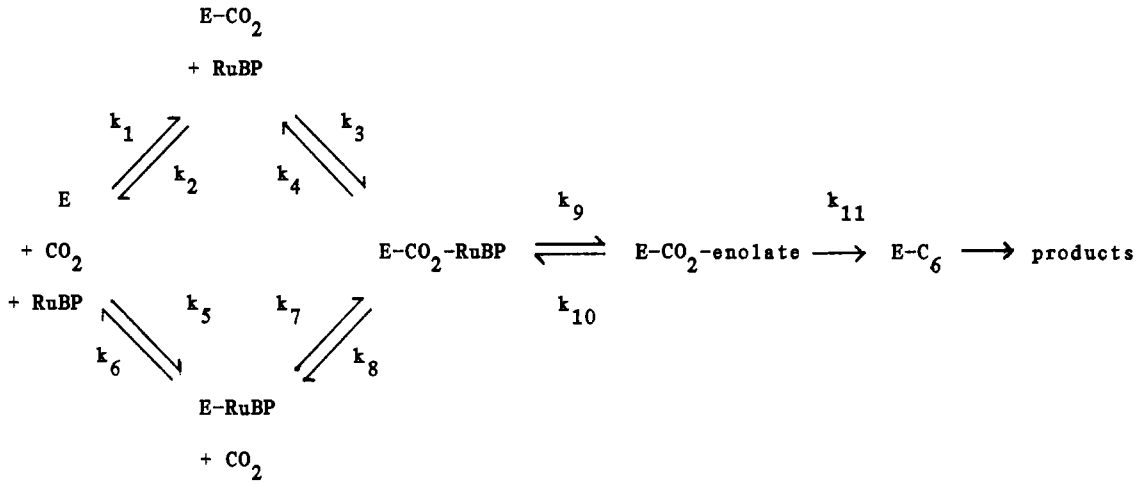

FIGURE 2: Mechanism of carboxylation of ribulose bisphosphate and definition of rate constants.

ion-exchange chromatography (Wong et al., 1982).

Kinetic studies have given only an incomplete picture of the order of binding of substrates to the enzyme. Carbon oxysulfide is a competitive inhibitor against CO_2 and is noncompetitive against RuBP (Laing & Christeller, 1980). This implies either random addition or ordered addition with CO_2 binding first. Fructose bisphosphate is competitive against RuBP and noncompetitive against CO_2 (Laing & Christeller, 1980), implying either random addition or ordered addition with RuBP binding first. Together, these results suggest that the kinetic mechanism involves random addition of substrates. Another possibility, however, is that carbon oxysulfide is an alternate substrate (Lorimer, 1981a), in which case either a random mechanism or an ordered one with RuBP binding first would be consistent with both sets of data.

H_2O_2 is competitive against O_2 in the oxygenase reaction, noncompetitive against CO_2 in the carboxylase reaction, and uncompetitive against RuBP in both reactions (Badger et al., 1980). These results imply a random mechanism for the carboxylation and an ordered mechanism for the oxygenation, with RuBP binding first. H_2O_2 is not an alternate substrate. Irreversible inhibition of the enzyme by H_2O_2 also occurs, with about 20% of the initial activity being lost in 10 min. The H_2O_2 inhibition could be complicated by the formation of 2-peroxypentitol 1,5-bisphosphate, which would serve as a transition-state analogue in the oxygenase reaction (Pierce et al., 1980).

Deuterium and tritium isotope effects have also been used to study RuBP carboxylase. For the spinach enzyme $(V/K)^H/(V/K)^D = 2.5 \pm 0.2$ from pH 6 to 9 (Schloss, 1983). The isotope effect is independent of the concentration of CO_2 (J. V. Schloss, personal communication). $V_{max}{}^H/V_{max}{}^D$ increases from 2 at pH 9 to 8 ± 2 at pH 6. For the *Rhodospirillum rubrum* enzyme, $V_{max}{}^H/V_{max}{}^D$ increases from 1.5 to 2.4 as the pH decreases from 8.4 to 6.4, and $(V/K)^H/(V/K)^D$ increases from 1.0 to 3.4 in the same interval (Sue & Knowles, 1982; J. V. Schloss, personal communication). These results indicate that the hydrogen-abstraction step is more nearly rate determining with the spinach enzyme than with the *R. rubrum* enzyme. However, in both cases significant carbon isotope effects are observed (see below), indicating that carboxylation is also at least partially rate determining.

Carbon isotope effects are finding increasing use as probes of mechanisms of enzyme-catalyzed reactions (O'Leary, 1978; Cleland, 1982). The general phenomenology underlying such isotope effects is similar to that for hydrogen isotope effects, with a couple of important differences. In the first place, carbon isotope effects are almost always measured by competitive methods, with the result that they reflect an isotope fractionation in V_{max}/K_m rather than in V_{max}. Thus, although the term "rate-determining step" is often used with regard to carbon isotope effects, the term is of somewhat questionable accuracy. In the second place, whereas hydrogen isotope effects are measured for initial rates, carbon isotope effects invariably integrate over some portion of the reaction. For decarboxylations, such integration is most often carried out over the first 10% or so of the reaction (O'Leary, 1981a). In most cases that have been studied to date, carbon isotope effects are concentration independent. However, in multisubstrate reactions carbon isotope effects may be concentration dependent. In such cases the measured effects may not be precisely comparable to hydrogen isotope effects on initial rates.

Carbon isotope fractionation associated with the carboxylation of RuBP has been of interest for many years because of the observation that plants are depleted in ^{13}C compared

to atmospheric CO_2 (O'Leary, 1981b). The potential role of RuBP carboxylase in causing this depletion has long been recognized (Craig, 1954), and a number of measurements of the carbon isotope fractionation associated with RuBP carboxylase have been reported (Park & Epstein, 1960; Deleens et al., 1974; Whelan et al., 1973; Wong et al., 1979; Christeller et al., 1976; Estep et al., 1978). For the most part, these measurements have been made by combustion methods, in which the isotopic composition of the 3-PGA produced is compared to that of the source CO_2 and RuBP. However, such methods have numerous problems, including isotopic contributions from impurities, possible isotope fractionations at carbons other than CO_2, and the necessity to multiply the observed isotopic difference by 6 in order to obtain the isotope fractionation. A more quantitative approach making use of changes in the isotopic composition of CO_2 over the course of the reaction has recently been published (Winkler et al., 1982). Most measurements of this isotope fractionation have given values in the range $k^{12}/k^{13} = 1.02–1.04$.

In this paper we describe studies of carbon isotope effects on the carboxylation of RuBP intended to clarify a number of aspects of mechanism and to provide an accurate value of the isotope fractionation for use in plant isotope fractionation studies.

Experimental Procedures

Materials. RuBP·$2Ba^{2+}$ was a gift from Dr. George Whitesides. [3-^{2}H]RuBP·$4Li^+$ was a gift of Dr. John Schloss. The $NaHCO_3$ (lot BKL) used in all the isotope effect experiments was from Mallinckrodt. $Ba^{13}CO_3$ (97% ^{13}C) was from Mound Laboratory (Miamisburg, OH). DE-52 ion-exchange cellulose was from Whatman; other ion-exchange resins were from Bio-Rad. EDTA disodium salt was obtained from Aldrich Chemical Co. and dithiothreitol from Chemalog. All other organic chemicals and enzymes except RuBP carboxylase were obtained from Sigma Chemical Co. Water was purified with a Millipore Super-Q filtration system.

RuBP carboxylase from *Rhodospirillum rubrum* was a gift from Dr. John Schloss or Dr. Fred Hartman. RuBP carboxylase from spinach was purified by the method of Hall & Tolbert (1978) except that $Mg(OAc)_2$ was used instead of $MgCl_2$ and the DE-52 column was approximately 300 mL instead of 500 mL. The specific activity was 2.8–2.9 μmol of CO_2 min^{-1} (mg of protein)$^{-1}$ when measured with freshly purified RuBP. (Care was taken that dithiothreitol not be present when the absorbance at 280 nm was measured.) The purified enzyme showed less than 0.1% phosphatase activity at pH 8.0, 25 °C (Torriani, 1968), as determined with 4-nitrophenyl phosphate. The enzyme was stored at −20 °C in a 30% glycerol solution containing 50 mM Bicine, pH 8.0, 1 mM EDTA, and 10 mM β-mercaptoethanol.

RuBP was purified just prior to use by anion-exchange chromatography at 4 °C. Typically, 450 mg of RuBP·$2Ba^{2+}$ was dissolved with 3 mL of Dowex 50W-X8 (H^+ form, 200–400 mesh). The filtered, neutralized solution was applied to a 1.3 × 45 cm Dowex 1-X8 column (Cl^- form, 200–400 mesh) and eluted with a 1-L HCl linear gradient, 30–150 mM. RuBP was precipitated from the pooled fractions as the dibarium salt (Wong et al., 1982). The solid was redissolved at 4 °C with Dowex 50W-X8 (H^+ form, 200–400 mesh), and the pH of the filtered solution was adjusted to 6.5 at 4 °C with 1 M LiOH.

[3-^{2}H]RuBP was synthesized in 98% D_2O from ribulose 5-phosphate with ribulose-5-phosphate 3-epimerase and phosphoribulokinase and was purified by ion-exchange chromatography prior to use. ^{1}H NMR showed no ^{1}H at C-3. The major contaminant was identified as ADP (J. V. Schloss, personal communication).

RuBP carboxylase-oxygenase was assayed for carboxylase activity by the spectrophotometric method of Racker (1974) on a Gilford 222 spectrophotometer kept at 25.0 ± 0.1 °C. RuBP carboxylase was activated (Pierce et al., 1982), and RuBP was purified just prior to the assays. A 2-mL mixture of glyceraldehyde-3 phosphate dehydrogenase (1000 units), triosephosphate isomerase (10 000 units), α-glycerophosphate dehydrogenase (1000 units), and 2 mg of bovine serum albumin was dialyzed against four 250-mL changes of a 30% glycerol solution containing 10 mM reduced glutathione, 0.1 mM EDTA, and 50 mM Bicine, pH 8.0, for at least 2 h per change. This mixture of coupling enzymes was stored at −20 °C in 100-μL aliquots in plastic vials. A total of 20 μL was usually added to 1 mL of assay solution. The same method was used to determine concentrations of RuBP or 3-PGA except that 3-PGA assays were performed without RuBP and RuBP carboxylase.

RuBP carboxylase-oxygenase was assayed for oxygenase activity at 25.0 ± 0.1 °C (Pierce et al., 1982) with a Clark oxygen sensor (Hansatech Ltd., King's Lynn, Norfolk, England) on loan from Dr. Lawrence Schrader. XuBP concentrations were determined as described by Sue & Knowles (1982).

Decarboxylation of 3-PGA. 3-PGA was decarboxylated and CO_2 collected by the general method of O'Leary (1981a) except that the enzymes used in the decarboxylation were not degassed. A reaction was started by the addition of 50 μL of a solution containing PGA mutase (6 units), enolase (60 units), pyruvate kinase (3 units), hexokinase (6 units), and pyruvate decarboxylase (2 units) to a degassed 10-mL reaction solution consisting of up to 100 μmol of 3-PGA, 8 mM $MgCl_2$, 30 mM KCl, 60 mM ATP, 0.1 mM 2,3 di-PGA, 1 mM thiamin pyrophosphate, 35 mM glucose, and 0.1 mM EDTA in 100 mM MES, pH 6.2. Reaction solutions were incubated overnight at 25.0 ± 0.1 °C.

CO_2 Isotope Effects. A reaction solution (40 or 200 mL, making the final concentration of RuBP 1 or 0.2 mM) containing 50 mM buffer (Bicine at pH 8.0, CHES at pH 9.0, MOPS at pH 7.0) and 10 mM $MgCl_2$ was filtered through a 0.45-μm cellulose acetate filter to remove bacteria. Reaction solutions for experiments at low RuBP concentration were bubbled with CO_2-free N_2 overnight at pH 8 to remove CO_2. $NaHCO_3$, carbonic anhydrase (2000–4000 Wilbur–Anderson units), and activated RuBP carboxylase were added, and the flask was sealed with a rubber septum.[2] The solution was shaken at 25.0 ± 0.1 °C for 4–6 h to allow CO_2(aq), CO_2(g), and HCO_3^- to come to isotopic equilibrium. A total of 30–60 μmol of HCO_3^- was removed with a syringe and injected into a reaction vessel containing 0.5–1 mL of concentrated H_2SO_4 which had been flushed with N_2 for 0.5 h prior to the addition of HCO_3^-. CO_2 was collected from this sample in order to determine $\delta(CO_2(aq))$ as described below. Carboxylation was initiated by the addition of 35–40 μmol of RuBP that had been purified as described above. The reaction was quenched after at least 9 h with 0.6–2 mL of concentrated HCl. The solution was shaken to release CO_2, the pH was adjusted to 9.2–9.5 with saturated KOH, and the solution was filtered through a 0.45-μm cellulose acetate filter to remove denatured protein. 3-PGA was precipitated by the addition of 7–8 mmol of $BaCl_2 \cdot 2H_2O$ and an equal volume of cold ethanol. The white

[2] Note that not all of the reaction solutions were bubbled with N_2 to remove trace amounts of CO_2. Thus, measured isotopic composition of the substrate CO_2 varies slightly from experiment to experiment.

precipitate was collected by centrifugation. The barium salt was redissolved with approximately 0.5 mL of Dowex 50W-X8 (H^+ form, 200–400 mesh) and decarboxylated as described above.

Collection of CO_2 from HCO_3^- Samples. CO_2 for the determination of $\delta(CO_2(aq))$ was collected from the HCO_3^- samples described above by a continuous multiple-trap method rather than the slower freeze–thaw method (O'Leary, 1981a). The reaction vessel and adapter were attached to a three-way stopcock on the vacuum line. The stopcock was opened to the manifold, and the manifold and connection were pumped down to 10^{-4} Torr with the aid of an oil diffusion pump. The stopcock was then opened in the opposite direction, so that CO_2, N_2, and water vapor from the reaction vessel were pulled through a standard dry ice trap followed by a 15-cm helix (175 cm of 5 mm i.d. tubing) in dry ice/isopropyl alcohol, followed by a 20-cm double coil (80 cm of 10 mm i.d. tubing) in liquid N_2. After 20 min the stopcocks on each side of the liquid N_2 trap were shut. The coil was warmed and then placed in a dry ice/isopropyl alcohol slush bath. CO_2 was distilled from the coil into a cold finger in liquid N_2. The distillation was repeated to remove remaining traces of water, and then the CO_2 was distilled into a sample tube for isotope-ratio analysis. The amount of CO_2 present was determined to within 1 μmol on a calibrate manometer.

Determination of $\delta(^{13}C)$. Isotope ratio analyses were done on a Nuclide Associates RMS 6-60 isotope ratio mass spectrometer with a dual-inlet system. Data were corrected for ^{17}O and internal fractionation (Craig, 1957). Rather than examining the ratio of the m/e 46 and 44 peaks in every sample, the ratio was checked every few months and an average of several samples was used to calculate δ. The correction factor for internal fractionation was supplied by Drs. Margaret and Paul Bender. The $\delta(^{13}C)$ and $\delta(^{18}O)$ values for the reference tank were determined by Dr. Crayton Yapp.

Determination of δ(C-3(RuBP)). RuBP carboxylase from *R. rubrum* was activated by dialysis against at least 100 volumes of 1 mM $CoCl_2$, 20 mM $NaHCO_3$, and 50 mM HEPPS, pH 8.0. A 60-mL reaction solution containing 50 mM HEPPS, pH 7.8, and 1 mM $CoCl_2$ was bubbled with O_2 for 5 h. Activated RuBP carboxylase (18 units) and 1 mL of 66 mM $NaHCO_3$ were added. The reaction was started by the addition of RuBP to a final concentration of 0.8 mM. 3-PGA was collected and decarboxylated as described above. The degree of competing carboxylase activity was examined by carrying out the reaction with $NaH^{13}CO_3$.

Isotope Effect at C-3 of RuBP. A 170-mL reaction solution containing 50 mM Bicine, pH 8.0, 10 mM $NaHCO_3$, 10 mM Mg^{2+}, carbonic anhydrase (4000 units), and activated RuBP carboxylase (6 units) was incubated for 7 h in a 25.0 ± 0.1 °C gyrotory water bath (New Brunswick Scientific Co., Model G-76). A 9-mL aliquot was removed to determine $\delta(HCO_3^- + CO_2)$. The reaction was started by the addition of 9 mL of 18.9 mM RuBP (pH 6.5). Samples of <1 mL were removed and assayed for 3-PGA. Reactions were stopped after 40–60 min with 1 mL of concentrated HCl each. 3-PGA was precipitated and decarboxylated as described above.

Theory

Isotopic compositions are reported in terms of δ values (parts per thousand):

$$\delta(^{13}C)\ (‰) = (R_{sample}/R_{standard} - 1) \times 10^3 \qquad (2)$$

where the standard is PDB limestone. R is the corrected ratio $^{13}CO_2/^{12}CO_2$ determined on an isotope ratio mass spectrometer. Isotope discriminations are given as the difference in isotopic composition between starting material and product

$$\text{discrimination} = \frac{\delta(^{13}C(\text{source})) - \delta(^{13}C(\text{product}))}{1 + \delta(^{13}C(\text{source}))/1000} \qquad (3)$$

or, alternatively, as an isotope effect, k^{12}/k^{13}

$$\text{discrimination} = 1000 \times (1 - k^{13}/k^{12}) \qquad (4)$$

In the carboxylation of RuBP, the source is CO_2, and the product is C-1 of the 3-PGA from CO_2 and carbons 1 and 2 of RuBP (Figure 1). Experimentally, correction for 3-PGA from carbons 3–5 of RuBP must be made before the isotope effect is calculated. The various isotopic compostions required here are as follows: (1) δ(C-1(3-PGA)) is the δ value of carbon 1 of 3-PGA. No distinction is made between 3-PGA from various sources. (2) δ(u) is the δ value of C-1 of the upper 3-PGA molecule in Figure 1. This carbon came from CO_2. (3) δ(l) is the δ value of C-1 of the lower 3-PGA molecule in Figure 1. This carbon came from C-3 of RuBP. (4) $\delta(CO_2(aq))$ is the δ value of dissolved CO_2.

From the definition of $\delta(^{13}C)$ (eq 2), it can be shown that if the fraction of ^{13}C present is small (e.g., 1%), then the δ value of a group of atoms is a weighted average of the δ values of the components. Thus in the carboxylation of RuBP, C-1 of 3-PGA is composed of equal numbers of carbon atoms from the upper and lower molecules in Figure 1, so

$$\delta(\text{C-1(3-PGA)}) = (1/2)\delta(\text{u}) + (1/2)\delta(\text{l}) \qquad (5)$$

δ(C-1(3-PGA)) can be determined by degradation of 3-PGA following carboxylation of RuBP. δ(l) can be determined independently because the enzymic oxygenation of RuBP gives 3-PGA from C-3 through C-5 of RuBP and phosphoglycolate from C-1 and C-2. Thus, δ(u) can be calculated from eq 5.

When an isotope effect is measured by comparison of the δ value of the product with that of the starting material, the isotope effect is given by

$$\frac{k^{12}}{k^{13}} = \frac{\ln(1-f)}{\ln\left[1 - f\dfrac{1 + 10^{-3}\delta(P_t)}{1 + 10^{-3}\delta(S_0)}\right]} \qquad (6)$$

where f = fraction reaction, S = substrate, and P = product. Substituting the terms defined above into eq 6 gives the isotope effect at CO_2 in the RuBP carboxylase catalyzed reaction:

$$k^{12}/k^{13} = \ln(1 - f_{CO_2})/\ln\left[1 - f_{CO_2}\frac{2[1 + 10^{-3}\delta(\text{C-1(3-PGA)})] - [1 + 10^{-3}\delta(\text{l})]}{1 + 10^{-3}\delta(CO_2(aq))}\right] \qquad (7)$$

where f_{CO_2} is the fraction of the CO_2 pool that has been consumed. The value of $\delta(CO_2(aq))$ is determined from the δ value of the total aqueous "CO_2" pool, which is composed of $CO_2(aq)$, HCO_3^-, and CO_3^{2-}. The proportions of these components are calculated from known equilibrium constants. The δ value of the aqueous CO_2 pool can then be expressed as a weighted average of the δ values of its components by using known equilibrium isotope effects (Vogel et al., 1970; Thode et al., 1965; Mook et al., 1974). Thus, at pH 8

$$\delta(CO_2(aq)) = 0.991\delta(HCO_3^- + CO_2(aq)) - 8.82 \qquad (8)$$

The δ value of the carbon in 3-PGA from C-3 of RuBP, δ(l), is constant provided that all of the RuBP has been converted to product. There is an isotope effect at that position, so C-3 in RuBP becomes enriched in ^{13}C over the course of the reaction. The isotope effect at C-3 of RuBP can be determined

Table I: Determination of δ(l) by Oxygenation of Ribulose Bisphosphate[a]

reaction		δ(C-1(3-PGA))	reaction		δ(C-1(3-PGA))
RuBP			[^{2}H]RuBP		
1		−22.6	6		−11.6
2		−22.8	7		−10.9
3		−23.3	8		−11.1
4		−23.7	9		−11.6
5		−23.5	10		−10.9
	mean	−23.2 ± 0.5	11		−10.3
				mean	−11.1 ± 0.5

[a] Reactions were started by the addition of RuBP to a solution containing 1 mM $CoCl_2$, 1 mM $NaHCO_3$, 1.1 mM O_2, and 2 or 4 units of activated RuBP carboxylase-oxygenase (for reactions with RuBP or [3-^{2}H]RuBP, respectively) in 100 mM MOPS, pH 7.8. The final concentration of RuBP was 0.8 mM. Enzyme from *R. rubrum* was activated with 1 mM $CoCl_2$ and 20 mM $NaHCO_3$ in 50 mM HEPPS, pH 8.0.

from the general equation for isotope effects (eq 6).

Results

Isotopic Composition of C-1 of 3-PGA. δ(C-1(3-PGA)) was analyzed by conversion of 3-PGA successively to 2-PGA, then to PEP, then to pyruvate, and then to CO_2 + acetaldehyde in a single operation. The resulting CO_2 was isolated and analyzed. In a control experiment commercial 3-PGA was decarboxylated 6 times by this method, and the average δ value was −24.0 ± 0.2‰. Assays for 3-PGA showed that <0.05% of the initial 3-PGA remained after decarboxylation. Reactions run without 3-PGA or without enzymes gave no CO_2. The same method of decarboxylation was used on all subsequent 3-PGA samples.

Isotopic Composition of C-3 of RuBP. The δ value of C-3 of RuBP [δ(l)] was determined by measuring the δ value of C-1 of 3-PGA obtained by oxygenation of RuBP (see Figure 1). Determining δ(l) in this way requires that no carboxylation of RuBP occur and either that all of the RuBP is used up during the reaction or that there be no isotope effect at C-3. The isotope effect at C-3 is indeed small, as discussed below.

It has been reported that in the presence of Co^{2+}, RuBP carboxylase-oxygenase from *Rhodospirillum rubrum* catalyzes the oxygenation of RuBP without carboxylation (Christeller, 1981; Robinson et al., 1979; Martin & Tabita, 1981). In order to determine more exactly the extent of carboxylation, the enzyme was activated with $H^{13}CO_3^-$ in the presence of Co^{2+}, and a sample of RuBP was oxygenated.

The 3-PGA produced was purified and decarboxylated. The δ value of the resulting CO_2 was 910‰. Since $NaH^{13}CO_3$ was 97% ^{13}C, $\delta(HCO_3^- + CO_2(aq)) = 2.9 \times 10^6$‰ or $\delta(CO_2(aq)) = 2.9 \times 10^6$‰ at pH 8. The isotope effect at CO_2 will not significantly alter this, so the δ value of any carbon fixed by the carboxylation [δ(u)] will also be 2.9×10^6‰. When both carboxylation and oxygenation of RuBP occur, δ(C-1(3-PGA)) becomes a weighted average of δ(u) and δ(l). Thus, there was less than 1.1% carboxylation compared to total reaction, and the δ value of C-1 of 3-PGA obtained from the oxygenation of RuBP is the same as δ(l) when enzyme from *R. rubrum* is used in the presence of Co^{2+}.

Isotopic composition δ(l) was determined by oxygenation of RuBP with *R. rubrum* carboxylase in the presence of Co^{2+} and O_2. All RuBP was converted into products in order to avoid any isotope fractionation at carbon 3 of RuBP. The enzyme was activated with natural abundance CO_2. As noted above, under these reaction conditions at least 99% of the RuBP is converted by oxygenation into 3-PGA and phosphoglycolate. The isotopic composition of carbon 3 of RuBP thus obtained is given in Table I.

Table II: Isotope Effect at CO_2 in the Carboxylation of Ribulose Bisphosphate Catalyzed by RuBP Carboxylase from Spinach at pH 8.0, 25 °C[a]

reaction	δ(CO_2(aq))	δ(C-1(3-PGA))	fraction of CO_2 pool used		k^{12}/k^{13}[c]
		1 mM RuBP			
1	−15.3	−31.5	0.067		1.0266
2	−15.2	−32.3	0.062		1.0283
3	−15.1	−31.8	0.089		1.0283
4	−15.1	−33.1	0.067		1.0303
5	−15.3	−32.9	0.065		1.0296
6	−15.1[d]	−32.7	0.070		1.0294
7	−15.1	−33.0	0.061		1.0299
				mean	1.0288 ± 0.0013
		0.2 mM RuBP[e]			
1	−15.0	−31.9	0.091		1.0281
2	−14.3	−32.1	0.094		1.0294
3	−14.6	−32.1	0.103		1.0292
4	−14.3	−32.0	0.106		1.0291
5	−13.1	−31.4	0.106		1.0292
				mean	1.0290 ± 0.0005

[a] Reactions were started by addition of RuBP to a solution containing 10 mM $MgCl_2$, 10 mM $NaHCO_3$, carbonic anhydrase, and 6 units of activated RuBP carboxylase in 50 mM Bicine, pH 8.0. The carboxylase was activated in 1 mM DTT, 10 mM $MgCl_2$, 10 mM $NaHCO_3$, and 50 mM Bicine, pH 8.3. [b] Calculated from $\delta(HCO_3^- + CO_2)$. [c] Calculated on the basis of δ(l) = −23.2 (Table I). See eq 7. [d] Average of δ(CO_2(aq)) from reactions 4, 6, and 7, which were run on the same day from the same batch of reaction solution. [e] 1 mM $NaHCO_3$ was used in these studies.

Kinetic Isotope Effect at CO_2. The isotope effect at CO_2 for the carboxylation of RuBP was determined from δ(CO_2(aq)), δ(C-1(3-PGA)), and δ(l) (eq 7). The isotope effect was also corrected for changes in the isotopic content of the CO_2 pool over the course of the reaction. The fraction of the total CO_2 pool used was calculated from the amount of CO_2 collected from 3-PGA and the total CO_2 pool in the initial reaction solution. Experiments conducted at pH 8.0 with two diifferent concentrations of RuBP are summarized in Table II.

In order to avoid isotope fractionation at C-3 of RuBP, reactions were forced to completion with excess enzyme. RuBP was purified at most 1 day before it was used to minimize inhibition of the enzyme by XuBP and other degradation products.

To avoid isotopic fractionation in CO_2, the total CO_2 pool was carefully equilibrated, both in solution and between the solution and the gas phase. Excess carbonic anhydrase was added to ensure that not only chemical but also isotopic equilibrium was reached. The solution was stirred for at least 4 h to allow equilibration with the gas phase.

Variation of Isotope Effect with pH. The reaction described above was repeated at pH 7 and 9 with 0.2 mM RuBP, 10 mM $NaHCO_3$, and 10 mM $MgCl_2$. At pH 7, twice as much RuBP carboxylase was used as at pH 8 to compensate for the decrease in enzyme activity. At pH 9, 3 times as much RuBP carboxylase was used as at pH 8 to compensate for the increased rate of isomerization and degradation of RuBP. The amount of carbonic anhydrase was doubled at pH 9. The δ values of starting material and product and the calculated isotope effects are shown in Table III. The δ value for CO_2 at pH 9 was calculated from previous values for δ(CO_2(aq)), which were obtained from the same batch of $NaHCO_3$. The δ values of starting material and product and the calculated isotope effects at pH 7.0 are shown in Table IV.

Table III: Isotope Effect at CO_2 in the Carboxylation of Ribulose Bisphosphate Catalyzed by RuBP Carboxylase from Spinach at 25 °C, pH 9.0, with 0.2 mM RuBP[a]

reaction	δ(C-1(3-PGA))	fraction of CO_2 pool used		k^{12}/k^{13}[b]
1	−31.9	0.018		1.0267
2	−32.1	0.018		1.0272
3	−31.7	0.017		1.0261
4	−31.4	0.017		1.0256
5	−31.8	0.017		1.0266
6	−31.5	0.017		1.0260
			mean	1.0264 ± 0.0006

[a] Reaction conditions were as described in Table II. Amounts of enzymes are discussed in the text. [b] Based on $\delta(CO_2(aq)) = -15.2$ (see text) and $\delta(l) = -23.2$ (Table I). See eq 7.

Table IV: Isotope Effect at CO_2 in the Carboxylation of Ribulose Bisphosphate Catalyzed by RuBP Carboxylase from Spinach at pH 7.0, 25 °C, with 0.2 mM RuBP[a]

reaction	$\delta(CO_2(aq))$[b]	δ(C-1(3-PGA))	fraction of CO_2 pool used		k^{12}/k^{13}[c]
1	−12.9	−32.4	0.021		1.0304
2	−13.3	−32.7	0.019		1.0304
3	−13.2	−31.8	0.018		1.0286
4	−12.4	−31.9	0.018		1.0300
5	−12.8	−31.9	0.018		1.0293
				mean	1.0297 ± 0.0008

[a] Reaction conditions were as described in Table II. Amounts of enzymes are discussed in the text. [b] Calculated from $\delta(HCO_3^- + CO_2(aq))$. [c] Based on $\delta(l) = -23.2$ (Table I).

Table V: Isotope Effect at CO_2 in Carboxylation of Ribulose Bisphosphate Catalyzed by RuBP Carboxylase from Spinach at pH 8.0, 25 °C, with 0.2 mM [3-^{2}H]RuBP[a]

reaction	$\delta(CO_2(aq))$[b]	δ(C-1(3-PGA))	fraction of CO_2 pool used		k^{12}/k^{13}[c]
1	−15.3	−21.0	0.020		1.0164
2	−15.2	−23.0	0.016		1.0205
3	−14.9	−24.7	0.020		1.0245
4	−13.9	−21.8	0.015		1.0194
5	−14.6	−21.2	0.016		1.0174
6	−13.3	−22.4	0.017		1.0213
7	−13.9	−23.3	0.012		1.0225
8	−15.3	−24.0	0.017		1.0226
9	−14.6	−23.2	0.017		1.0216
10	−14.9	−24.2	0.017		1.0235
11	−14.9	−25.4	0.017		1.0261
				mean	1.0214 ± 0.0029

[a] Reactions were started by addition of [3-^{2}H]RuBP to 10 mM $MgCl_2$, 10 mM $NaHCO_3$, carbonic anhydrase, and 12 units of activated RuBP carboxylase in 50 mM Bicine, pH 8.0. The carboxylase was activated in 0.5 mM DTT, 10 mM $NaHCO_3$, and 10 mM $MgCl_2$ in 50 mM Bicine, pH 8.3. [b] Calculated from $\delta(HCO_3^- + CO_2(aq))$. [c] Based on $\delta(l) = -11.1$ (Table I).

Isotope Effect at CO_2 with [3-^{2}H]RuBP. Isotope effects were measured at pH 8 with 0.2 mM [3-^{2}H]RuBP. The amount of RuBP carboxylase was increased by a factor of 2 to compensate for the deuterium isotope effect. The δ values of starting material and product and the calculated isotope effects are listed in Table V.

Isotope Effect at C-3 of RuBP. The isotope effect at C-3 of RuBP was determined by comparing δ(C-1(3-PGA)) at low fraction reaction to the value at complete reaction (see eq 6). The δ value of C-3 at complete reaction [δ(l)] has been determined (Table I). Samples were assayed spectrophotometrically for product so that the reaction could be stopped before a large fraction of the RuBP was used up. The fraction of RuBP used was calculated from the initial amount of RuBP and the amount of CO_2 collected from 3-PGA. $\delta(CO_2(aq))$ was determined so that δ(C-1(3-PGA)) could be corrected for δ(u). The δ values and isotope effects are given in Table VI.

Discussion

Although the carbon isotope fractionation associated with the carboxylation of RuBP has already been measured numerous times by combustion methods, the values obtained have varied over a wide range. It appears that this variation comes about for two principal reasons. First, impurities in the RuBP and 3-PGA contribute to the isotope signal. Commercially available RuBP is quite impure, and purified RuBP decomposes quite readily. The 3-PGA obtained from this reaction is often contaminated with a variety of other phosphate esters, and these have not always been removed. Second, isotopic compositions can vary with extent of conversion. Because a small isotope effect (1.008) is observed at C-3 of RuBP, the isotopic composition of the 3-PGA produced will reflect both the isotope effect at CO_2 and the isotope effect at C-3 of RuBP unless the reaction is carried to completion. Further, there might also be an isotope fractionation at C-2 of RuBP that could cause similar problems. None of these problems has been directly addressed in most previous studies.

The measurement of the carbon isotope effect on RuBP carboxylase by the specific-atom method used in this study represents a significant improvement over combustion methods. We measured isotopic abundances for carbon 1 of purified 3-PGA. We have been able to determine δ(u) and δ(l) separately, and thus, we have avoided the problems inherent in the combustion studies. The determination of the isotope effect at CO_2 could also be affected by incomplete reaction because of changes in the isotopic composition δ(l). However, we have avoided this by carrying all carboxylations to completion except those used to determine the isotope effect at C-3 of RuBP.

The isotope effect at CO_2 in the carboxylation of RuBP at pH 8, 25 °C, with Mg^{2+} and RuBP carboxylase from spinach is 1.029. This compares favorably with the value of 1.029 obtained by the combustion method by Christeller et al. (1976) with RuBP carboxylase from soybean with Mg^{2+} at pH 8, 25 °C, and the value of 1.029 obtained by Wong et al. (1979).

Table VI: Isotope Effect at C-3 of RuBP in Carboxylation of Ribulose Bisphosphate by RuBP Carboxylase from Spinach at pH 8.0, 25 °C[a]

reaction	$\delta(CO_2(aq))$[b]	δ(C-1(3-PGA))	fraction of CO_2 pool used	fraction of RuBP used		k^{12}/k^{13}[c]
1	−15.0	−35.8	0.038	0.36		1.0080
2	−15.0	−35.9	0.031	0.29		1.0077
3	−14.8	−36.4	0.027	0.24		1.0088
4	−15.2	−36.4	0.024	0.22		1.0082
					mean	1.0082 ± 0.0005

[a] Reactions were started by addition of RuBP to a solution containing 10 mM $NaHCO_3$, 10 mM $MgCl_2$, carbonic anhydrase, and 6 units of activated RuBP carboxylase in 50 mM Bicine, pH 8.0. The final concentration of RuBP was 1 mM. The carboxylase was activated in 0.5 mM DTT, 10 mM $NaHCO_3$, and 10 mM $MgCl_2$ in 50 mM Bicine, pH 8.3. [b] Calculated from $\delta(HCO_3^- + CO_2(aq))$. [c] Based on $\delta(l) = -11.1$ (Table I).

Qualitative Interpretation. The carbon isotope effect that is observed at CO_2 in the carboxylation of RuBP is larger than the carbon isotope effects observed in most enzymatic reactions involving CO_2 as a substrate or product (O'Leary, 1978). Qualitatively, this suggests that the carboxylation step is principally rate limiting. However, the occurrence of a significant hydrogen isotope effect for the hydrogen at C-3 of RuBP (Schloss, 1983) eliminates the possibility that carboxylation is solely rate limiting. Although it appears that this hydrogen isotope effect is significantly smaller than the intrinsic isotope effect at this site, the presence of a hydrogen isotope effect indicates that the enolization step must be only marginally faster than the carboxylation step. The fact that significant carbon and hydrogen isotope effects are observed indicates that neither CO_2 nor RuBP is "sticky"; i.e., neither dissociates slowly from the enzyme.

More detailed consideration of these isotope effects requires that we estimate the magnitudes of the intrinsic[3] carbon and hydrogen isotope effects. Few data are available for enzymatic or nonenzymatic carboxylations that might permit estimation of the intrinsic carbon isotope effect. Instead, we must rely on studies of decarboxylations. Intrinsic isotope effects in decarboxylations fall in the range 1.03–1.07 (Dunn, 1977). Equilibrium isotope effects for decarboxylations are small (O'Leary & Yapp, 1978). Provided that transition states for decarboxylations are similar to those for carboxylations (in terms of degree of bonding to the isotopic atom), then intrinsic isotope effects for carboxylations should also fall in this 1.03–1.07 range. The observed isotope effect of 1.029, together with the occurrence of a significant hydrogen isotope effect, rules out values near 1.03. Thus, we believe that a range of 1.04–1.07 is a reasonable starting point for considering the carboxylation of RuBP.

The intrinsic hydrogen isotope effect for the enolization step is somewhat easier to estimate. Schloss has shown that the observed hydrogen isotope effect is ~8 at low pH. This value provides a lower limit on the possible intrinsic isotope effect. Thus, we assume a range of 8–12 for the intrinsic hydrogen isotope effect.

Thus, a qualitative comparison of the observed carbon and hydrogen isotope effects with the estimated intrinsic values indicates that both enolization and carboxylation are among the slow steps in the reaction. Carboxylation is somewhat more limiting than enolization.

Reversibility of Carboxylation. Available data do not permit us to eliminate with certainty the possibility that the carboxylation step itself may be slightly reversible, although it is unlikely that the six-carbon intermediate reverts to CO_2 and enolate more than 5–10% of the time. The magnitudes of the isotope effects observed in this study are inconsistent with a substantial extent of reversal of the carboxylation step. However, we cannot eliminate the possibility that the six-carbon intermediate (Figure 2) might revert to CO_2 and enolate as much as 5–10% of the time. Such reversion would have at most a minor effect on the analysis given below.

Timing of CO_2 Binding to Enzyme. We have previously shown (Hermes et al., 1982) that multiple-isotope effect can be used to determine the order of steps in an enzymatic reaction. The fact that in the present case deuteration of carbon 3 of RuBP decreases the carbon isotope effect indicates that enolization precedes carboxylation. Concerted enolization–carboxylation mechanisms and (chemically unlikely) mechanisms in which carbon–carbon bond formation precedes enolization are eliminated by this observation.

Likewise, the effect of deuteration of RuBP on the carbon isotope effect eliminates the possibility that CO_2 does not bind to the enzyme until after enolization has occurred. In such a case, the carbon isotope effect would not have been altered by deuteration of the substrate.

Mathematical Modeling of Kinetic Mechanisms. Further consideration of carboxylation mechanisms requires quantitative analysis of the experimental isotope effects. Available data are consistent with kinetic mechanisms for RuBP carboxylase involving either ordered addition of substrates with CO_2 binding first or random addition. The ordered mechanism with RuBP binding first has been ruled out because the deuterium isotope effect is constant over a wide range of CO_2 concentrations (J. V. Schloss, personal communication). The other two mechanisms are considered below. A model for the carboxylation of RuBP should include substrate binding, formation of an enol or enolate from RuBP, and reaction with CO_2 to form a six-carbon intermediate. The equation for the isotope effect contains steps up to and including the first irreversible step, which is assumed to be formation of the six-carbon intermediate.

The model used for the kinetic mechanism of this reaction is shown in Figure 2. Although the model is written as a random mechanism, it includes the ordered mechanisms as special cases. Assuming that only the bond-forming step is sensitive to the carbon isotope in CO_2, then the carbon isotope effect in the random mechanism is

$$\frac{k^{12}}{k^{13}} = \frac{\dfrac{k_{11}^{12}}{k_{11}^{13}} + \dfrac{k_{11}}{k_{10}}\left[1 + \dfrac{k_9}{k_8 + k_4k_2/(k_2 + k_3[\mathrm{RuBP}])}\right]}{1 + \dfrac{k_{11}}{k_{10}}\left[1 + \dfrac{k_9}{k_8 + k_4k_2/(k_2 + k_3[\mathrm{RuBP}])}\right]} \tag{9}$$

where the superscript denotes the carbon isotope. The observed isotope effect ranges from

$$\frac{\dfrac{k_{11}^{12}}{k_{11}^{13}} + \dfrac{k_{11}}{k_{10}}\left(1 + \dfrac{k_9}{k_8 + k_4}\right)}{1 + \dfrac{k_{11}}{k_{10}}\left(1 + \dfrac{k_9}{k_8 + k_4}\right)}$$

at very low [RuBP] to

$$\frac{\dfrac{k_{11}^{12}}{k_{11}^{13}} + \dfrac{k_{11}}{k_{10}}\left(1 + \dfrac{k_9}{k_8}\right)}{1 + \dfrac{k_{11}}{k_{10}}\left(1 + \dfrac{k_9}{k_8}\right)}$$

at very high [RuBP]. As one would expect, at very high RuBP concentrations the equation is the same as when the mechanism is ordered with RuBP adding first, in which case the isotope effect does not vary with the concentration of RuBP. k_{11}^{12}/k_{11}^{13} is the intrinsic isotope effect on k_{11}.

The deuterium isotope effect at C-3 of RuBP can be described by an equation of similar form:[4]

$$\frac{k^{H}}{k^{D}} = \frac{\dfrac{k_9^{H}}{k_9^{D}} + \dfrac{k_9}{k_4 + k_8k_6/(k_6 + k_7[CO_2])} + \dfrac{k_{10}K_{eq}^{H}}{k_{11}K_{eq}^{D}}}{1 + \dfrac{k_9}{k_4 + k_8k_6/(k_6 + k_7[CO_2])} + \dfrac{k_{10}}{k_{11}}} \tag{10}$$

[3] An intrinsic isotope effect is the isotope effect that would be observed for a single reaction step if that step could be observed in the absence of all other steps.

Table VII: Values That Satisfy the ^{13}C and ^{2}H Isotope Effect Equations If RuBP Carboxylase Kinetic Mechanism Is Ordered with CO_2 Binding First[a]

assumed		possible values			calcd
k_{11}^{12}/k_{11}^{13}	k_9^H/k_9^{D}[b]	$k_3[RuBP]/k_2$[c]	k_9/k_4	k_{11}/k_{10}	$(V/(K[CO_2]))^{12}/(V/(K[CO_2]))^{13}$ with [3-^{2}H]RuBP[d]
1.04	12	0–0.2	0.9–0.8	0.19	1.012
1.05	8	0–0.1	1–0.6	0.4–0.3	1.011–1.014
	12	0–0.1	1.9–1.7	0.25–0.24	1.011–1.012
1.06	8	0–0.05	1.2	0.4	1.013
	12	0–0.05	2.4–2.3	0.30–0.29	1.011–1.012
1.07	8	0–0.04	1.7–1.6	0.5–0.42	1.012–1.014
	12	0–0.04	0.5–0.4	0.35–0.33	1.011–1.012

[a] Rate constants are defined in Figure 2. The isotope effect at CO_2 ranged from 1.0275 to 1.0295 and the deuterium isotope effect from 2.3 to 2.7. [b] Assume $k_{10}^H/k_{10}^D = k_9^H/k_9^D$. [c] Maximum values are for 0.2 mM RuBP. If RuBP = 1 mM, the maximum value increases by a factor of 5. [d] Assumes no deuterium exchange.

The equations for the ^{13}C and ^{2}H isotope effects were used over a range of intrinsic isotope effects to identify possible values for the ratios of rate constants (also called partition factors) that are consistent with the experimental results. All calculations were done by assuming that the carboxylation is irreversible and that the ^{2}H equilibrium isotope effect is unity. The similarity between the predicted and observed isotope effects (or lack thereof) was used to distinguish between the kinetic mechanisms.

Ordered Mechanism. If the kinetic mechanism for the carboxylation is ordered with CO_2 binding first, then the isotope effect at CO_2 should decrease with increasing concentration of RuBP. The isotope effects of 1.0290 and 1.0288 at 0.2 and 1 mM RuBP are essentially the same, suggesting that the mechanism is not ordered.

This analysis, however, neglects any variation that might be hidden in the experimental error of the measurements. A more stringent analysis was done in which expeirmental errors were included. The isotope effect was taken to be 1.0295–1.0290 at 0.2 mM RuBP and 1.0288–1.0275 at 1 mM RuBP. Partitioning factors were chosen to give calculated isotope effects that match the experimental values of the ^{13}C and ^{2}H isotope effects.

Values for k_{11}/k_{10}, k_9/k_4, and $k_3[RuBP]/k_2$ that satisfy the isotope effect equations were determined from a computer program that generates values for the observed isotope effect given any chosen intrinsic isotope effect and a value of $k_3[RuBP]/k_2$. The latter term, of course, changes with the concentration of RuBP. Since we see little or no variation in the observed isotope effect with RuBP concentration, this term must be small compared to the other rate constant ratio terms in eq 9. Numerical modeling of the isotope effect indicates that this term can be no larger than about 0.1.

If we take this upper limit of $k_3[RuBP]/k_2$ together with a range of values for the intrinsic carbon isotope effect (1.04–1.07) and the intrinsic deuterium isotope effect (8–12), then we can calculate values of k_9/k_4 and k_{11}/k_{10} which are consistent with the observed carbon and deuterium isotope effects (Table VII). We find the best fit when k_9/k_4 is in the range 1–2 and k_{11}/k_{10} is 0.2–0.4. These results fit the qualitative picture that the carboxylation step is principally rate determining, but not entirely so.

If the kinetic mechanism is ordered with CO_2 binding first, then the partitioning factors derived above should also fit the ^{13}C isotope effect with [3-^{2}H]RuBP (with an 8–12-fold decrease in k_9 and k_{10}). The calculated values for the parameters in Table VII are 1.011–1.014, significantly less than the experimental value of 1.0214. However, we must also consider the possibility that the enzyme catalyzes the loss of ^{2}H from [3-^{2}H]RuBP. Carbon isotope effects are measured over the entire course of the carboxylation, rather than simply during the initial portion. RuBP carboxylase from *R. rubrum* is known to catalyze a slow hydrogen exchange between the hydrogen at carbon 3 of RuBP and the medium (Saver & Knowles, 1982). If this same exchange occurs during the reaction catalyzed by the spinach enzyme, then the observed carbon isotope effect will reflect in part the isotope effect for [3-^{2}H]RuBP and in part that for RuBP, with the balance of the two being governed by the rate of hydrogen exchange compared to the rate of carboxylation. Inclusion of this phenomenon in eq 9 and 10 permits calculation of isotope effects that are in good agreement with the experimental value. Unfortunately, it is not possible to estimate the rate of the hydrogen exchange from such an exercise.

The ordered mechanism as described above contains a term $k_3[RuBP]/k_2$ that must tend toward infinity as the concentration of RuBP becomes very large. It is surprising that at concentrations of RuBP which are 2 orders of magnitude in excess of K_m this term is estimated to be no larger than 0.1. A more satisfactory version of the ordered mechanism might consider RuBP binding to consist of a pair of steps, the first being the actual binding of the substrate to the enzyme and the second a structure change of the initial enzyme–RuBP complex that places enzyme and substrate in the proper conformation for reaction. When these two processes are combined, the overall binding process will show a first-order dependence on the concentration of RuBP only at low concentrations. At the concentrations being used here, the rate would be expected to be independent of RuBP concentration. Thus, the carbon isotope effects would also be independent of RuBP concentration.

To summarize what can be concluded about the ordered mechanism, the kinetic mechanism for RuBP carboxylase can be ordered with CO_2 binding first provided that formation of the enolate is accompanied by loss of ^{2}H to the solvent upon enolization. The fit to the experimental data is improved if we include a conformation change associated with the binding of RuBP to the enzyme.

Random Mechanism. A variety of partition factors and the intrinsic isotope effects described above were tried in eq 9 and 10 in order to determine the range of partition factors that would fit the experimental hydrogen and carbon isotope effects for a random mechanism. Unfortunately, the number of adjusted parameters in the random mechanism is too large to permit a unique solution. However, we can limit the range of possibilities, as described below.

As in the ordered mechanism, we would expect that the observed isotope effects would vary with substrate concentration. Experimentally, no such variation is seen. This indicates that even in the presence of a high concentration of

[4] This is the isotope effect on V_{max}/K_m.

one substrate the other substrate can dissociate relatively freely from the enzyme. This, of course, is the essence of the random mechanism. The individual ratios k_9/k_4 and k_9/k_8 cannot be estimated with certainty, but the ratio of these two ratios, which represents the partitioning between the two pathways (RuBP binding first or CO_2 binding first), is not far from 1:1 at the substrate concentrations used for the carbon isotope effect studies. This partitioning will change with substrate concentrations.

In general, the chemical steps are significantly slower than the binding and dissociation steps. In the case of k_{11}/k_{10}, the rate of carboxylation compared to the rate of proton transfer, it is possible to make a relatively accurate estimate of the partition factor. Independent of the values of the other factors, this ratio must be approximately 0.2–0.4. That is, carboxylation is 2–5 times slower than proton transfer. Thus, carboxylation is the principal rate-limiting step.

When the isotope effect calculations using the appropriate ranges of partition factors described above are repeated for deuterated RuBP, the predicted carbon isotope effect is less than half as large as the isotope effect for undeuterated RuBP. In order to match the experimental value of this isotope effect, it is again necessary to assume that deuterium exchange between RuBP and the solvent accompanies the carboxylation process.

Isotope Effect at C-3 of RuBP. The partition factors derived above for both the ordered and the random mechanisms and the observed isotope effect at carbon 3 of RuBP can be used to calculate a value for the intrinsic carbon isotope effect at carbon 3 of RuBP. This value is probably near 1.06 and thus is within the range that might be expected for such an isotope effect. Unfortunately, no appropriate models exist that would enable us to make a more accurate estimate of the expected intrinsic effect.

pH Dependence. The pH dependence of the carbon isotope effect on the carboxylation of RuBP is only marginally significant over a range of 3 pH units. This small variation in isotope effect may be the result of a slight pH dependence of the partitioning between the two binding pathways. The data simply are not adequate at present to permit unambiguous interpretation. However, it should be noted that the pH dependence is so slight that it is unlikely to be an important contributing factor for the isotope fractionation in vivo.

Summary of Mechanism. The kinetic mechanism of ribulosebisphosphate carboxylase could be either random or ordered with CO_2 binding first on the basis of the $^{13}CO_2$ and [3-^{2}H]RuBP isotope effects. If the ordered mechanism is correct, then the rate of formation of the enzyme–RuBP complex is at least 20 times slower than the release of CO_2 from the enzyme. Although this mechanism is not ruled out by the isotope effect studies, it is unlikely on the basis of inhibition studies.

The isotope effect at CO_2 with [3-^{2}H]RuBP is consistent with either mechanism provided that the enzyme catalyzes a partial loss of ^{2}H; i.e., exchange of ^{2}H with the solvent competes with reprotonation of the enolate. The decrease of the isotope effect at CO_2 with [3-^{2}H]RuBP compared to that with RuBP implies that enolization of RuBP occurs largely after CO_2 binds to RuBP carboxylase.

Implications for Plant Physiology. The carbon isotope fractionation associated with carboxylation of RuBP is one of the principal factors responsible for the carbon isotope fractionation in C-3 plants (O'Leary, 1981b). However, it should be noted that the enzyme isotope fractionation (1.029, or 29‰) is significantly larger than the fractionation shown by C-3 plants (near 20‰). Most quantitative modeling of isotope fractionation in C-3 plants suggests that this discrepancy is principally due to the effects of gas-phase diffusion (O'Leary, 1981b; Farquhar et al., 1982).

Previous studies have given a variety of values for the carbon isotope fractionation associated with RuBP carboxylase, and numerical modeling of isotopic processes in C-3 plants has been rendered somewhat uncertain by the uncertainty in this fractionation. Our studies show that the proper value to be use for the fractionation process is 29‰. It is unlikely that variations in chloroplastic pH are an important source of variation in the fractionation. Although under certain conditions it is known that there can be large variations in the chloroplastic concentration of RuBP, it is unlikely that such variations play any significant role in changing the carbon isotope fractionation in vivo. From previous studies (Estep et al., 1978) it is unlikely that temperature effects have a significant influence on the fractionation.

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