Partition Kinetics of Ribulose-1,5-bisphosphate Carboxylase from
Rhodospirillum rubrum*

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When the enzymatically generated intermediate 2-carboxy-3-keto-D-arabinitol-1,5-bisphosphate (II) was used as a substrate with fresh enzyme, 70% reacted to produce 3-phosphoglycerate (3PGA). When a reaction mixture of enzyme plus [1-32P]ribulose 1,5-bisphosphate (RuBP) was quenched in the steady state with the tightly bound inhibitor 2-carboxyarabinitol-1,5-bisphosphate, 30% of the enzyme-bound species was released as 3PGA and 70% as RuBP. The major source for this partition was the ternary substrates Michaelis complex. The level of carboxylated intermediate in the steady state was determined to be 8% of active sites under the conditions of substrate saturation. No burst was seen in the appearance of product when 6.5 eq of [1-32P]RuBP was mixed with enzyme plus saturating CO₂ and the reaction followed in the steady state.

From these data plus the steady-state Vₘₚₚ and Kᵣₚₚ of RuBP it is possible to derive the five bulk rate constants represented in the scheme

\[ E_{CO₂} + RuBP \rightarrow E_{CO₂}RuBP \rightarrow E \cdot II \rightarrow E + 2(3PGA). \]

The carboxylation of RuBP catalyzed by ribulosebiphosphate carboxylase (EC 4.1.1.38) is a slow reaction \((kₘₚ \approx 5 \text{ s}^{-1})\) involving an unusually large number of catalyzed steps which in the original proposal of Bassham et al. (1) is seen in Fig. 1. Abstraction of the C-3 proton of RuBP, presumably by a base on the enzyme, generates the enediol (I), a potential C-2 or C-3 carbanion. The carbanion is carboxylated by CO₂ to produce the intermediate 2-carboxy-3-keto-D-arabinitol-1,5-bisphosphate (II) which, following hydration to the gem-diol form, cleaves between what were carbons 2 and 3 of the substrate, generating one molecule of 3PGA (originating from carbons 3-5 of RuBP) and the carbanion of 3PGA (originating from carbons 1 and 2 of RuBP and CO₂). The 3PGA carbanion is subsequently stereospecifically protonated by the enzyme. Additional properties of all carboxylases are (a) a poorly understood oxygenase reaction in which phosphoglycolate is formed instead of the CO₂-containing 3PGA and (b) the requirement for CO₂ and Mg²⁺ as cofactors in both the carboxylation and oxygenation reactions. The enzyme has been reviewed extensively (2-4).

This long sequence of chemical steps (involving an enolization, carboxylation, hydration, carbon-carbon bond cleavage, and protonation) is catalyzed by a single enzyme in all photosynthetic organisms which in the bacterium Rhodospirillum rubrum is a homodimer of molecular weight 1.0 × 10⁶ (5, 6). Consistent with the mechanism of Fig. 1 are the observations that the carbonyl carbon of the substrate is the one to which both the CO₂ and the solvent-derived proton become attached in forming C-2 of 3PGA (7). The oxygen atoms at carbons 2 and 3 of RuBP are retained in the products (8, 9). The intermediates postulated in Fig. 1 have only recently received direct chemical confirmation although the discovery of strong inhibition by 2-carboxy-3-keto-1,5-P₂ provided early evidence for intermediate II (10). The existence and stereochemistry at C-3 of the carboxylated intermediates, II, liberated by acid from the functioning enzyme, have been established after NaBH₄ treatment of the reaction mixture at neutral pH (11). Reduction of tetranitromethane to nitro form dependent upon the carboxylation of RuBP catalyzed by the spinach enzyme has been cited as evidence for the presence of a substrate carbanion during the reaction (12). Comparable experiments with the enzyme from R. rubrum have failed to show a ferricyanide reduction (13). More recently, evidence for the enediol intermediate (I) was obtained with the bacterial enzyme by liberation with acid of a compound unreactive or too labile to react with NaBH₄ that decomposed to give P₂, derived from the 1-position of RuBP (13). This P₂ formation was prevented when iodine was included in the acid-quench medium as expected if it originated from an enol such as I. The amount of this intermediate was found to vary inversely in relation to II depending on the CO₂ concentration as expected for I. An intermediate with the properties of the 3PGA carbanion (III) was below the level of detection, probably less than 5% of II (13).

A comprehensive study of the kinetic behavior of the various intermediates in the steady-state carboxylation reaction has not been reported for any RuBP carboxylase enzyme although extensive consideration has been given to the overall steady state and activation kinetics of many (14). Studies directed to determining the sequence of addition of CO₂ and RuBP appear to favor a random mechanism for the spinach enzyme (15). Discrimination against ³¹CO₂ is decreased with [2-²H]RuBP (15) implying that CO₂ reacts prior to enolization and may be required for enolization. Although their reaction pathways are probably identical, the different carboxylases show some important kinetic differences (16, 17). Of particular interest are the high isotope effects with [3-²H]- and [3-¹H]RuBP seen with the spinach enzyme (18, 19) compared with much lower values with the enzyme from R. rubrum (19, 20).
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MATERIALS AND METHODS

RuBP carboxylase from *R. rubrum* was the generous gift of Dr. F. C. Hartman, Oak Ridge National Laboratory. Enzymatic activity and concentration were measured as described previously (13). The enzyme was stored at -80°C in 50 mM Na bicarbonate, pH 8.0, 10 mM MgCl₂, 1 mM EDTA, 66 mM KHCO₃, 5 mM β-mercaptoethanol, 20% glycerol and incubated at room temperature (22°C) for at least 16 min prior to each experiment.

[1-32P]Ribulose-1,5-P₂ was synthesized from 6-phosphogluconate and [γ-32P]ATP and purified on a 1 x 8 cm column of DE52 cellulose (Whatman) in the cold as described (13). By using [γ-32P]ATP free of label in the β-position, purified on the same column pre-equilibrated with 20 mM triethylamine/carbonate buffer, pH 7.5, and eluted in the cold with a linear gradient (200 ml) of 20-500 mM of the same buffer at pH 7.5, it was possible to obtain labeled substrate that was >99% converted to [32P]P, with mild alkali. [γ-32P]ATP was prepared as follows. [32P]Pi (5 mCi, carrier-free from New England Nuclear) and ADP were coupled to the conversion of glyceraldehyde-3-P to 3PGA. Pyruvate and l-lactate dehydrogenase were added to regenerate NAD⁺. Triethanolamine/HCl, pH 8.0 (50 μmol), ADP (1 μmol), glyceraldehyde-3-P (2.3 μmol), sodium pyruvate (5 μmol), NAD⁺ (1 μmol), EDTA (0.5 μmol), diithiothreitol (1 μmol), MgCl₂ (4 μmol), KH₂PO₄ (5.0 x 10⁻³ M), and [32P]inorganic phosphate (5 μCi) were mixed in a final volume of 1.3 ml. 50 μl of phosphoglycerate kinase, l-lactate dehydrogenase, and glyceraldehyde-2-P dehydrogenase (100 μg each) were added. After 10 min the level of inorganic [32P]phosphate dropped to <3% as measured by the molybdate extraction assay (see below). The reaction was stopped by addition of 600 μmol of trichloroacetic acid. Precipitated protein was removed by centrifugation and trichloroacetic acid was extracted with 3 x 1 ml volumes of anhydrous diethyl ether. The aqueous phase was adjusted to pH 7.5 with triethanolamine, and [32P]ATP was purified using the salt gradient on DE52. The [32P]ATP prepared in this manner contained 0.1% inorganic [32P]phosphate and >99% of the radioactivity in the y position (estimated as acid-stable [32P]P after complete reaction with glucose and hexokinase).

Synthesis of Carbosynthetic Bisphosphates—40 μmol of cara-hydroxymethyl bisphosphate (a mixture of 2-carboxyribosinolyl bisphosphate and 2-carboxyribosinolyl bisphosphate) were synthesized by cyanide addition to RuBP (containing 5000 cpm of [1-32P]RuBP as tracer) followed by spontaneous hydrolysis of the resulting cyano acetone products (10, 21). The products were purified by chromatography on DE52 cellulose (see above) and eluted principally as a single peak of radioactivity corresponding to the unlaconitzed carboxynthetic bisphosphates.

Other Assays—Inorganic [32P]phosphate was extracted into butan-2-ol (13) as the acid molybdate complex according to Berenblum and Chien (22). Alkaline [32P]P (which includes 3PGA, phosphoglycerate, and NaBH₄ reduced intermediate II) was determined after 15 min in 0.5 N NaOH at 37°C by counting the aqueous phase after extraction of P (13). 3PGA was assayed using the coupled enzyme system: phosphoglycerate mutase, enolase, pyruvate kinase, and lactate dehydrogenase. RuBP was assayed after conversion of RuBP carboxylase to 3PGA in 50 mM Bicine, pH 8.2, 5 mM MgCl₂, 1 mM EDTA, and 66 mM KHCO₃ (Buffer B) and correcting for the amount of phosphoglycerate produced (13). 32P in column effluents was determined by Cherenkov counting.

Rapid Mixing and Quenching—Experiments were conducted with a system 1500 rapid mixing device with a Model 1501/1502 controller from Update Instrument, Inc. (Madison, WI). The procedure used in which the samples to be mixed were contained in the delivery hoses rather than in syringes made it possible to obtain reproducible results with microliter quantities of solutions (19).

RESULTS

Absence of a Burst in 3PGA Formation—With a turnover rate of ~5 s⁻¹, it was feasible to observe the rate of appearance of 3PGA containing label from [1-32P]RuBP during the approach to steady state using rapid mixing and quenching. With saturating levels of CO₂ and RuBP such a study could place the rate-determining step (a) prior to the formation of the 3PGA derived from CO₂ if a lag was observed in product formation and if the accumulation of intermediates was significant and rate-limiting or (b) after 3PGA formation if for example there were rate-limiting release of either product, in which case one would expect to see a burst in product formation.

With [1-32P]RuBP as substrate the rate of 3PGA formation was determined by following the appearance of radioactivity that was not extractable as [32P]P, after treatment with alkali. As seen from Fig. 2, product formation occurs with the expected turnover rate from the earliest time sampled (50 ms). The absence of any significant burst in 3PGA formation rules out product release as a rate-determining step. The results of Fig. 2 differ markedly from those found in a similar study with spinach enzyme in which an unexplained lag, t = 200 ms, was seen in the approach to a constant rate of CO₂ fixation (11).

Determination of Carbosynthesized Intermediate in the Steady State—6.2 pmol of enzyme subunits were mixed in the rapid quench machine with 62 nmol of RuBP in Buffer B containing KH₂CO₃ (specific activity = 1.94 x 10¹³ dpm mol⁻¹) in a final volume of 360 μl then quenched after 20 ms in 1 ml of 0.2 M trichloroacetic acid containing 0.95 μmol of 3PGA added from a third hose. The quenched reaction stream was directed into a closed vial through an air-tight rubber septum. Excess ¹⁴CO₂ was removed by gentle vortexing of the open vial for 1 min in a ventilated hood. Previous experiments had shown that loss of the carboxylated intermediate under these conditions is minimal within this time (13). The quenched solution was measured with 270 μmol of NaBH₄ in 1 ml 0.2 M NaOH (final pH ~8). After 2 min the excess NaBH₄ was destroyed with 1500 μmol of trichloroacetic acid. The products were chromatographed on a DE52 cellulose column (1 x 8 cm) as described above. Fractions (3.0 ml each) were assayed for radioactivity and 3PGA. Losses during the work-up were calculated from the recovery of the carrier 3PGA. After correction for the loss of carrier 3PGA (25%) a total of 430 pmol of ¹⁴C-labeled, NaBH₄ reduced II was calculated. This is equivalent to 7% of the enzyme subunits. Since at the [HCO₃⁻] used, approximately 9% of the RuBP is oxidized to phosphoglycerate (13, 23), approximately 0.08 eq of enzyme is present as the E-II complex in the carboxylation pathway.
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This value rests on the assumption that the recovery of the NaBH₄-reduced intermediate during chromatography is the same as that of 3PGA. The level of II is similar to 0.07 eq reported for the spinach enzyme (11).

Partition of [¹⁴C]Carboxy-labeled Intermediate II—Free intermediate II was produced by mixing RuBP, [¹⁴C]CO₂, and RuBP carboxylase followed by acid (see legend to Fig. 3 for details). This quenched mixture contained [¹⁴C] label in CO₂, II, and 3PGA. Most of the excess [¹⁴C]CO₂ was removed by briefly vortexing the acid reaction mixture in a fume hood. An aliquot of the degassed mixture was reduced with NaBH₄ to determine the amount of II and [¹⁴C]PGA present. A second aliquot was mixed with fresh enzyme and unlabeled CO₂. The partition ratio is determined from the increase in [¹⁴C]PGA compared with the amount of II available. The final RuBP carboxylase concentration in the trapping solution (14 μM) was sufficient to fully trap the intermediate before it decomposed in the buffered medium, t₁/₂ ~ 5 min (13), even if it reacts only at the same rate as RuBP (kₐ/Kₐ = 3 × 10⁵ M⁻¹ s⁻¹) (17). Since NaBH₄ reduces CO₂ to formate and there is a vast excess of H¹⁴CO₃⁻ relative to other labeled species, most of the CO₂ was removed prior to reduction. (The half-time for the escape of CO₂ under the conditions used was 5-6 s.) The tight-binding inhibitor 2-carboxyarabinitol bisphosphate was included in the NaBH₄-reduced incubation to prevent any reaction of II with enzyme that might have renatured after neutralization. It also served as a chromatographic marker for the reduced intermediate. Chromatography of the reaction product resolved four [¹⁴C]-labeled peaks in the NaBH₄-reduced material (labeled peaks 1-4, respectively) and two (peaks 1 and 2) in the enzyme reaction (Fig. 3). The large peak at the void volume (peak 1) is presumably [¹⁴C]formate. Peak 2 of radioactivity co-chromatographs with the 3PGA added as carrier. Peaks 3 and 4 are assigned to the NaBH₄-reduced intermediate and its γ-lactone, respectively (11). These peaks are absent in the column profile of the enzyme-partitioned material as expected for derivatives of intermediates. The assignment of peaks 3 and 4 to reduced-carboxylated intermediate was verified by showing that both [¹⁴C] peaks contain very strong inhibitory activity derived from the added mixture of 2-carboxyarabinitol bisphosphate and its lactone.

The specific radioactivity of the 3PGA was constant across its peak for both columns. The fractions containing 3PGA were pooled, rotary evaporated to remove volatile buffer salts, and taken up in 1 ml of H²O and the specific radioactivities were determined. The amount of [¹⁴C]3PGA in each sample was calculated from the amount of carrier added and the ratio

![Figure 2: Presteady-state product formation](image1)

**FIG. 2.** Presteady-state product formation. RuBP carboxylase (2.0 nmol of subunits) was mixed with [1-³²P]RuBP (13 nmol, 3300 dpm/nmol) in Buffer B (50 mM Bicine, pH 8.2, 5 mM MgCl₂, 1 mM EDTA, and 66 mM KHCO₃) in a final volume of 100 μl with rapid mixing in the model 1500 apparatus of Update Instruments (Madison, WI) (13). The reactions at 22 °C were quenched at the indicated times in 0.9 ml 0.2 M trichloroacetic acid. Samples were assayed for alkali-stable ³²P as described under "Materials and Methods" after centrifugation to remove precipitated protein.

![Figure 3: Partition of intermediate II](image2)

**FIG. 3.** Partition of intermediate II. RuBP carboxylase (18 nmol of subunits) was mixed with RuBP (21 nmol) in 400 μl of Buffer B with KH¹⁴CO₃ (66 mM and 2 × 10⁶ cpm nmol⁻¹), quenched after 12.5 ms in 1 ml of 0.1 M HClO₄ containing 0.31 pmol of 3PGA, and agitated for 2 min to remove [¹⁴C]CO₂. Two aliquots of 0.5 ml each were simultaneously brought to pH 8.2 by addition to 1 ml of Buffer B containing 76 μmol of triethanolamine and either RuBP carboxylase (14 nmol of subunits) or 1 μmol of carboxypentitol bisphosphate plus 3.7 mg of solid NaBH₄. After 2 min, both reactions were terminated with HClO₄ to pH 1.4 and 0.31 pmol of 3PGA added. The neutralized samples were resolved on DE52-cellulose with a salt gradient as shown (see "Materials and Methods"). Fractions (3.0 ml) were assayed for radioactivity, 3PGA, and inhibitor of RuBP carboxylase activity. Column profiles: O—O reacted with RuBP carboxylase; —— reduced with NaBH₄. Results are summarized in Table I.
of the specific radioactivities of 3PGA to KH\(^{14}\)CO\(_3\). The level of \(^{14}\)C-labeled intermediate was estimated from the total recovered counts in the reduced intermediate peaks corrected to 100% recovery of carrier 3PGA divided by the specific activity of the \(^{14}\)CO\(_3\). The results of these determinations show that in the reaction of RuBP with \(^{14}\)CO\(_3\) used to generate the \(^{14}\)C-labeled intermediate, 0.41 nmol of \([1-^{14}\)C]3PGA was produced (Table I). After reaction with fresh RuBP carboxylase, a total of 0.96 nmol was recovered. Therefore 0.55 nmol of II was converted to 3PGA in the second enzyme incubation. Since the partition reaction was carried out in unlabeled CO\(_3\) any extra \([^{14}\)C]3PGA seen must originate from the partition of II and not from the carboxylation of unreacted RuBP. From the total amount of II recovered after NaBH\(_4\) reduction (0.79 nmol), 70% of intermediate II partitioned forward to 3PGA.

**Partition of Total Enzyme-bound Species between Conversion to 3PGA and Return to RuBP**—A complete reaction mixture containing \([1-^{32}\)P]RuBP and 1.4 nmol enzyme sites was allowed to reach steady state and was either quenched with acid or "chased" by the addition of 10 mM carboxypentitol bisphosphate (a potent competitive inhibitor of enzymic activity). The chased reaction was quenched in acid after various times long enough to complete or incomplete the partition of counts bound at the time of mixing with inhibitor and as a check for the effectiveness of inhibition. The amount of product was determined. The results (Fig. 4) show that after 31 ms 0.52 nmol of product was formed (equivalent to 0.37 turnovers). As opposed to an acid-quench, the use of a competitive inhibitor for quenching allows all enzyme-bound species to partition until they are released as either substrate or product. The amount that partitions forward to product is given by the difference in the amount of product found in the chase and the acid quench. From the data of Fig. 4, the chase solution contained 0.87 ± 0.05 nmol of product, and therefore the amount of bound species which partitioned forward is 0.35 nmol, equivalent to 25% of enzyme active sites. That complete inhibition of free enzyme was achieved under these conditions is shown by the lack of any additional product formation within 5 s.

At 62 \(\mu\)M RuBP, the enzyme is approximately 80–85% saturated (20). Correcting for this, one finds that 30% of the label that is bound to enzyme in the steady state partitions forward to product of which 91% is \([^{32}\)P]3PGA, and that 70% returns to free RuBP.

**DISCUSSION**

With the steady-state kinetic constants already available for the *R. rubrum* enzyme and the additional observations reported here, one is able to provide a much more detailed picture of the dynamics of the catalytic pathway. In this analysis the distribution patterns of enzyme-bound species are derived from the earlier observation that under the conditions employed intermediate II is present in far greater concentration than III, which was undetectable, and I, the enediol, which was <20% of II (13). The absence of a burst in 3PGA formation (Fig. 1) rules out any significant accumulation of enzyme-bound product prior to a limiting dissociation rate or of free enzyme that must undergo a kinetically important change before re-entering the catalytic cycle. Therefore the only complexes that can be entered into the partition analysis at this time are intermediate II, 0.08 of ET, and the complex \(E-\text{CO}_2\text{-RuBP}\), which at saturation would make up more than 95% of the remaining 0.92. Under the reaction conditions, about 9% of the RuBP goes to phosphoglycolate making use of \(O_2\) (1 atm) instead of \(\text{CO}_2\) (1 mM). The remaining 91% goes to 3PGA. This distribution of products was observed at all extents of substrate consumption. Correcting for this and the contribution to the partition of \(^{32}\)P of \(E-I\) (8% of ET with 70% going to PGA), one obtains the true partition of \(E^{32}\)P, \(P_n\), from the equation

\[
P_n = 0.91 \times 0.92 \times P - 0.91 \times 3/7 - 0.08 \times 7/3
\]

giving \(P_n = 0.20\). These new data allow one to use the existing steady-state kinetic constants, \(k_{\text{cat}}\) and \(k_{\text{cat}}/K_m\), to determine all of the bulk rate constants of this minimal reaction sequence,

\[
E^{32}\text{CO}_2 + \text{RuBP} \rightleftharpoons \frac{3}{4} E-I \rightarrow E + 2(3\text{PGA})
\]

where \(E-I\) is meant to include both the keto and diol forms of the borohydride-trapped carboxylated intermediate. To be sure, all determined rate constants must be considered to be potentially complex until proven otherwise in specific ways. In the present case when sufficient information is not available to resolve a constant into its known individual steps, the simplifying use of bulk constants provides the kinetic connections between significant known intermediates. For example, in Equation 1, \(k_3\) represents the combination \(k_{\text{cat}}(k_{-1} + k_0)^{-1}\) of the expanded form

\[
E^{32}\text{CO}_2 \rightleftharpoons E-I \rightleftharpoons E-II.
\]
The partition equations with the values determined are

\[ E \text{--II} \rightleftharpoons \frac{\text{PGA}}{\text{CO}_2} = \frac{k_3(k_5 + k_6)}{k_3k_4} = 2.3 \]  

and

\[ \frac{E_{\text{III}}^{\text{RuBP}}}{E_{\text{II}}^{\text{RuBP}}} \rightleftharpoons \frac{\text{PGA}}{\text{RuBP}} = \frac{k_3k_6}{k_3(k_4 + k_6)} = 0.20. \]  

The additional equations used in these calculations are

\[ k_3(E \text{--II}) = k_{\text{II}}(E_{\text{II}}) = 5 \times 10^{-4} \text{ s}^{-1} \]  

\[ k_{\text{II}}/K_{\text{m}}^{\text{RuBP}} = k_4k_5/k_3(k_4 + k_6) = 3 \times 10^8 \text{ M}^{-1} \text{ s}^{-1} \]  

\[ k_{\text{II}}/K_{\text{m}}^{\text{RuBP}} = 1.5 \times 10^4 \text{ M}^{-1} \text{ s}^{-1} \]

and in the steady state

\[ \frac{E_{\text{III}}^{\text{RuBP}}/E_{\text{II}}^{\text{RuBP}}}{E_{\text{I}}^{\text{RuBP}}/E_{\text{II}}^{\text{RuBP}}} = \frac{k_4 + k_6}{k_3} = 11.5. \]

From these five equations the five unknown bulk-rate constants are easily evaluated (Table II).

The value calculated for \( k_1 \) depends on which substrate is established to add last in forming the full Michaelis complex. Since \( k_1 \) is directly proportional to \( k_{\text{II}}/K_{\text{m}} \), its value will reflect the \( K_m \) of the second substrate, 300 \( \mu \text{M} \) for \( \text{CO}_2 \) and 15 \( \mu \text{M} \) for RuBP (17). Attempts to establish a function for \( E \text{--I}^{\text{13CO}_2} \) by isotope trapping with 30 \( \mu \text{M} \) RuBP (2000 \( \times \) \( K_m \)) in the chase gave negative results, as was previously reported for the spinach enzyme (24). Therefore, either the rate constants for dissociation of \( \text{CO}_2 \) from the binary and/or ternary complexes are too rapid to be determined by this method, \( k_3 > 2 \times 10^4 \) \text{ s}^{-1}, or RuBP does not form a functional binary complex. The value of \( k_3 > 2.6 \times 10^4 \) \text{ s}^{-1}, represents the bulk-rate constant for release of one of the substrates, \( \text{CO}_2 \) or RuBP, from the ternary complex. Knowing the rate constant may help determine which substrate adds last.

The forward partition determined for \( E \text{--II} \) and the fact that bound \( \text{CO}_2 \) exchanges rapidly means that the net \( ^{13}\text{CO}_2/^{12}\text{CO}_2 \) discrimination value of 1.018 measured by Roeske and O'Leary with this enzyme can be significantly corrected based on the constants determined here to be 1.028. This value is intrinsic only to the extent that carboxylation is rate-determining for the bulk-rate constant \( k_3 \). The absence of a primary deuterium isotope effect with [3-\( ^2\text{H} \)]RuBP reported by Sue and Knowles (20) and the readily observed reverse partition of \( E \text{--II} \) suggest that the enediol intermediate and the ternary substrates complex are at equilibrium in the \( R. \ rubrum \) catalyzed reaction so that the corrected \( ^3\text{C} \) isotope effect may be the intrinsic value.

The \( E \text{--II}/E_{\text{III}}^{\text{RuBP}} \) ratio in the steady state is about one-third of what could be expected from true equilibrium. Therefore the catalytic rate is about 64% rate-limited by the carboxylation step and 34% limited by utilization of the carboxylated intermediate. No attempt had been made to distinguish the keto and hydrated keto forms of II. Very likely, their interconversion is enzyme-catalyzed. Not enough information is available to analyze \( k_5 \) in terms of its four elements, i.e., hydration, C=O bond cleavage, protonation of a not-yet-detected intermediate III, and release of products. There are a few clues to the origin of the C-2 proton that quenches intermediate III. No transfer has been detected from C-3 of \( \text{CO}_2 \) to the substrate (18, 25). Both the spinach and \( R. \ rubrum \) enzymes discriminate against tritium of \( ^3\text{H}_2\text{O} \) in the formation of \( 3\text{PGA} \) (18, 25). Therefore the last chemical step must be slow compared to product release.

A further discussion of this intriguing and biologically important reaction, revealing additional intermediates and their partition kinetics, can be expected from studies at other than the pH of optimal activity and at other temperatures.

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REFERENCES