Ribulose-1,5-bisphosphate Carboxylase: Fate of the Tritium Label in [3-^{3}H]Ribulose 1,5-Bisphosphate during the Enzyme-Catalyzed Reaction†

Julia M. Sue and Jeremy R. Knowles*

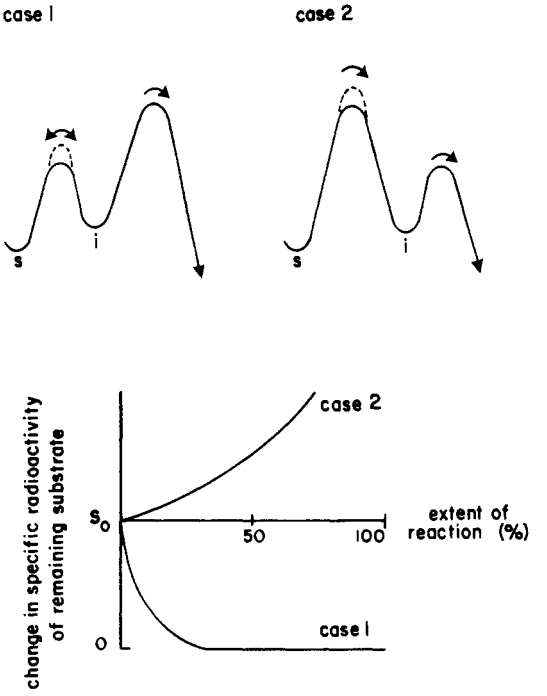
ABSTRACT: The reaction of [3-^{3}H]ribulose 1,5-bisphosphate and CO_2 with ribulose-1,5-bisphosphate carboxylase has been investigated in order to provide information about the early steps of the enzyme-catalyzed reaction. The specific radioactivity of ribulose 1,5-bisphosphate reisolated after partial reaction rises as the reaction proceeds, demonstrating that the isotopic discrimination (which results in the preferential consumption of unlabeled substrate) is more important than the equilibration of the hydrogen on C-3 with solvent protons. These data confirm the existence of the enediol intermediate and set limits on the range of permissible free-energy levels for the transition states in the catalyzed reaction. It is evident that there is a rather fine balance among the three critical transition states for this reaction (those of enolization, condensation of the enediol with CO_2, and solvent exchange of the C-3 proton).

In the preceding paper (Saver & Knowles, 1982), we have reported the results of an investigation of the appearance of solvent tritium in the product and in the remaining substrate of the reaction catalyzed by ribulose-1,5-bisphosphate carboxylase (RuBP carboxylase).[1] These experiments not only tested the validity of the original mechanistic formulation of Calvin (1954) but also provided some information about the relative rates of the early steps in the carboxylase-catalyzed reaction. As we have noted before in studies on the rather simple reaction catalyzed by triosephosphate isomerase (Albery & Knowles, 1976), evaluation of the fate of solvent tritium yields mechanistic information that is complementary to studies on the fate of the tritium of specifically labeled substrate. In the present work, we have followed the specific radioactivity of the specifically labeled substrate [3-^{3}H]ribulose 1,5-bisphosphate as a function of its conversion to product in the reaction catalyzed by RuBP carboxylase.

There are two factors that together determine the isotopic content of the remaining substrate pool in an enzyme-catalyzed reaction involving enolization. First, the partitioning of the enol intermediate between the "exchange" path back to substrate and the "conversion" path on to product determines the rate of influx of "exchanged" molecules back into the substrate pool. That is, whenever the intermediate species reverts back to reactant rather than proceeds to form product, the isotopic content of the remaining substrate will change if the intermediate picks up a solvent-derived proton on its way back. Whether the new proton is solvent derived will depend on the rate of equilibration of the enzyme's conjugate acid with the medium, from the enzyme–enol intermediate. Second, if there is a discrimination against tritium in the proton abstraction step, the proportion of tritiated molecules in the remaining substrate will increase during the course of the reaction. The balance between these two factors (the partition ratio and the isotope discrimination), along with the rate of proton equilibration of the intermediate, determines the net rate of change in the isotopic content of the remaining substrate.

Scheme I illustrates, for two idealized limiting cases, the fate of the tritium of a specifically labeled substrate sample during an enzyme-catalyzed reaction in unlabeled water. Let us assume that the rate of proton equilibration at i is very rapid. In case 1, in which the partition ratio for the intermediate i overwhelmingly favors the substrate, there will be a rapid washout of the label from substrate, and since the kinetic discrimination against tritiated reactant is insignificant, the specific radioactivity of the remaining substrate will fall rapidly until all the tritium label is washed out. In contrast,

Scheme I: Limiting Cases for the Fate of the ^{3}H Label in the Substrate (s) in an Enzyme-Catalyzed Reaction That Proceeds by Enolization of the Substrate to an Intermediate (i)[a]



[a] The substrate-derived proton is assumed to equilibrate rapidly with solvent protons at i. Solid lines in the free-energy profiles depict the paths for unlabeled molecules. Dotted lines show the paths for the transfer of a tritium. Isotope effects are represented as transition-state differences rather than ground-state differences for the sake of simplicity.

† From the Department of Chemistry, Harvard University, Cambridge, Massachusetts 02138. *Received March 3, 1982.* This work was supported by the National Science Foundation and the National Institutes of Health.

[1] Abbreviations: RuBP, D-ribulose 1,5-bisphosphate; RuBP carboxylase, ribulose-1,5-bisphosphate carboxylase; NAD^+, nicotinamide adenine dinucleotide; NADH, reduced NAD^+; NADP, nicotinamide adenine dinucleotide phosphate; EDTA, (ethylenedinitrilo)tetraacetic acid; DEAE, diethylaminoethyl.

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if the partition ratio of the intermediate i strongly favors conversion to product, as in case 2 (Scheme I), the kinetic discrimination against tritiated substrate molecules will be maximal, and, since only few molecules will return to reactant once they have overcome the first energy barrier, there will be an accumulation of labeled species in the remaining substrate. This is equivalent to an increase in the specific radioactivity of the remaining substrate during the course of the reaction. In intermediate cases in which the partitioning ratio and the isotope effect are more finely balanced, there may, of course, be little or no change in the specific radioactivity of the remaining substrate.

For further elucidation of the early steps of the reaction catalyzed by RuBP carboxylase, [3-^{3}H]RuBP has been incubated with active enzyme in unlabeled water, and the fate of tritium in the remaining RuBP has been followed as a function of the extent of the reaction.

Materials and Methods

Materials

Enzymes, substrates, and cofactors other than those specified below were obtained as outlined in the previous paper (Saver & Knowles, 1982). [2-^{3}H]Glycerol (200 mCi/mmol) was purchased from New England Nuclear.

D-[*3-^{3}H*]*Ribulose 1,5-Bisphosphate*. The preparation of [3-^{3}H]RuBP followed, with some modifications, the procedure described by Fiedler et al. (1967).

(*S*)-[*4-^{3}H*]*NADH*. [2-^{3}H]Glycerol ($\sim 4 \times 10^8$ cpm) was diluted with carrier to a specific radioactivity of $\sim 5 \times 10^6$ cpm/μmol. The reaction mixture comprised 250 mM hydrazine–500 mM glycine buffer, pH 9.0 (11 mL), containing $MgCl_2$ (2 mM), ATP (6.4 mM), NAD^+ (6.1 mM), [2-^{3}H]-glycerol (7.7 mM), glycerol-3-phosphate dehydrogenase (54 units), and glycerol kinase (15 units). Spectrophotometric monitoring at 340 nm indicated a 61% yield of NADH after 3.5 h at room temperature. The pH of the reaction mixture was then lowered by the addition of 0.1 N HCl (12 mL). The solution was diluted further to $\sim$160 mL, and the [4-^{3}H]-NADH was purified by chromatography on a column (2.6 × 34.5 cm) of DEAE-cellulose, equilibrated with 65 mM NH_4HCO_3, pH 8.0. The column was eluted with a linear gradient (800 mL plus 800 mL) of NH_4HCO_3 (65–250 mM). The fractions containing NADH were pooled. A specific radioactivity of $\sim 3 \times 10^6$ cpm/μmol was determined for the [4-^{3}H]NADH, and the isolated yield was estimated at 45%. The solution was concentrated nearly to dryness in vacuo at room temperature, and 2-propanol was added. After evaporation of the solvent, more 2-propanol was added. Repeated evaporations of added 2-propanol gave [4-^{3}H]NADH free from bicarbonate. The product was stored in the dark at room temperature.

D-[*4-^{3}H*]*Fructose 1,6-Bisphosphate*. [4-^{3}H]NADH ($\sim$30 μmol, 3×10^6 cpm/μmol) was incubated in 100 mM triethanolamine hydrochloride buffer, pH 8.0 (13.8 mL), containing $MgCl_2$ (20 mM), EDTA (4 mM), 2-mercaptoethanol (1 mM), 3-phosphoglycerate (10.9 mM), ATP (11.3 mM), dihydroxyacetone phosphate (7.7 mM), 3-phosphoglycerate kinase ($\sim$140 units), and glyceraldehyde-3-phosphate dehydrogenase ($\sim$50 units). The enzymes had been freed from triosephosphate isomerase (de la Mare et al., 1972). After 1.5 h, aldolase ($\sim$3 units, freed from isomerase) was added, and the incubation was continued for another 40 min. The mixture was then treated with acid-washed charcoal (1.5 g) and left for 10 min. The mixture was centrifuged (3200 rpm, 5 min), and the supernatant was filtered through paper. The charcoal was washed twice with water ($\sim$6 mL), and the combined filtrates (20.5 mL) were loaded onto a column (1.5 × 26.5 cm) of DEAE-cellulose, equilibrated with 100 mM NH_4CO_3, pH 8.0. The column was eluted with a linear gradient (235 mL plus 235 mL) of NH_4HCO_3 (100–250 mM). The fractions containing [4-^{3}H]fructose 1,6-bisphosphate were pooled, and the solution was treated with Dowex-50 (H^+ form) ($\sim$5 mL) and then passed through a column of Dowex-50 (cyclohexylammonium form) ($\sim$5 mL). The eluate was concentrated in vacuo and subjected to repeated evaporations of added 2-propanol. The yield of [4-^{3}H]fructose 1,6-bisphosphate was 27%, and the product had a specific radioactivity of $\sim 2.5 \times 10^6$ cpm/μmol.

6-Phospho-D-[*4-^{3}H*]*gluconate* was synthesized in an incubation mixture of 100 mM triethanolamine hydrochloride buffer, pH 8.0 (3.0 mL), containing $MgCl_2$ (20 mM), EDTA (4 mM), 2-mercaptoethanol (1 mM), $NADP^+$ (7.9 mM), [4-^{3}H]fructose 1,6-bisphosphate (8 μmol, $\sim 2.5 \times 10^6$ cpm/μmol), fructose 1,6-bisphosphatase ($\sim$1 unit), glucose-6-phosphate dehydrogenase ($\sim$3 units), and phosphoglucose isomerase ($\sim$21 units). The reaction was complete after 30 min at room temperature. The solution was diluted with water (15 mL) and applied to a column (1.6 × 10.2 cm) of DEAE-cellulose equilibrated with 65 mM NH_4HCO_3, pH 8.0. The column was eluted with a linear gradient (100 mL plus 100 mL) of NH_4HCO_3 (65–250 mM). The solution of 6-phospho[4-^{3}H]gluconate from the appropriate fractions was treated with Dowex-50 (H^+ form) (2 mL) and then passed down a column (2 mL) of Dowex-50 (cyclohexylammonium form). The yield of 6-phospho-D-[4-^{3}H]gluconate was $\sim$90%, and it was diluted approximately 10-fold with unlabeled material before use in the synthesis of ribulose 1,5-bisphosphate (see below).

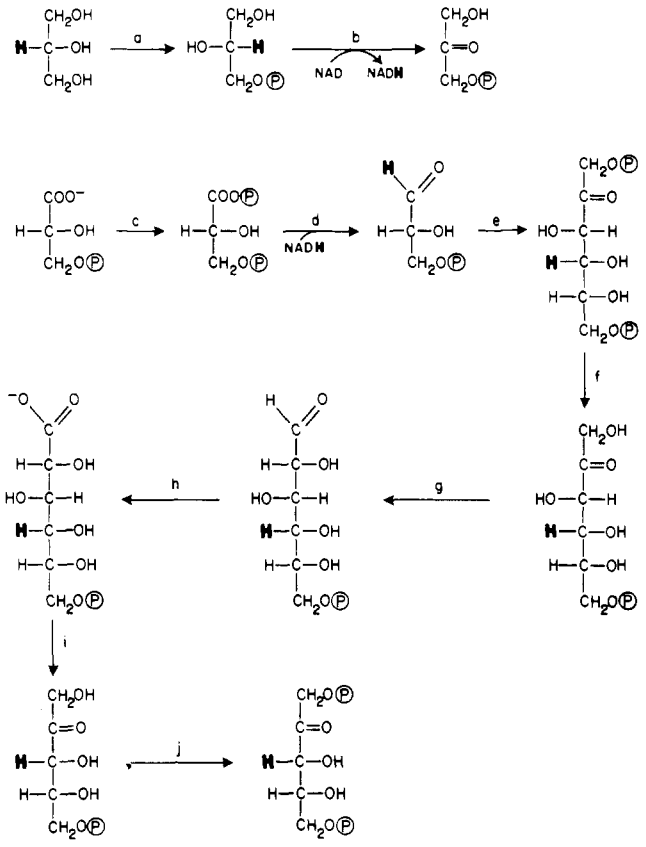
D-[*3-^{3}H*]*Ribulose 1,5-bisphosphate* was prepared according to the method described for the conversion of 6-phosphogluconate to ribulose 1,5-bisphosphate (Saver & Knowles, 1982). The specific radioactivity of the product [3-^{3}H]RuBP was $\sim 1.9 \times 10^5$ cpm/μmol, and the yield after conversion to the sodium salt was $\sim$54%.

Methods

Assays and radioactivity measurements were carried out as described in the previous paper (Saver & Knowles, 1982).

Reaction of [*3-^{3}H*]*RuBP in Unlabeled Water*. All operations leading to and including the carboxylase-catalyzed reaction were performed under nitrogen. For preparation of the reaction mixture, a nitrogen-flushed buffer solution of 100 mM triethanolamine hydrochloride, pH 8.0 (10 mL), containing $MgCl_2$ (20 mM), EDTA (4 mM), and dithioerythritol (1 mM) was added to $NaHCO_3$ (48.4 mg). A portion (6.5 mL) of the resulting solution was then transferred by syringe to a solution (760 μL) of [3-^{3}H]RuBP (13 μmol, sodium salt). The mixture, at 30 °C, was divided into two parts. To the part serving as the control sample (2.79 mL) was added water (37 μL). The other part was treated with a solution (58 μL) of activated carboxylase (0.3 unit). At different times, portions (500 μL) were withdrawn from either the control or the reaction sample and added to solid $NaBH_4$ (19 mg). After 20 min, excess borohydride was destroyed by the addition of glacial acetic acid (10 drops) and the sample freeze-dried by bulb to bulb distillation in vacuo. Each sample was then subjected to two further evaporations of added water and three evaporations of added methanol. The remaining residue was dissolved in water (10 mL) and the solution subjected to chromatography on a column (1.0 × 7.1 cm) of DEAE-cellulose. Elution of the column was carried out either with a linear gradient (25 mL plus 25 mL) of NH_4HCO_3 (65–200 mM) or with a linear gradient (20 mL plus 20 mL) of NH_4HCO_3 (65–150 mM) followed by 150 mM NH_4HCO_3 (10 mL). Fractions from

Scheme II: Preparation of [3-^{3}H]RuBP[a]



[a] (a) Glycerol kinase; (b) glycerol-3-phosphate dehydrogenase; (c) 3-phosphoglycerate kinase; (d) glyceraldehyde-3-phosphate dehydrogenase; (e) aldolase; (f) fructose 1,6-bisphosphatase; (g) 6-phosphoglucose isomerase; (h) glucose-6-phosphate dehydrogenase; (i) 6-phosphogluconate dehydrogenase; (j) phosphoribulokinase.

the column were assayed for radioactivity and for phosphate ester content. The specific radioactivity of the sample was then calculated from the amount of radioactivity and the amount of phosphate measured in known volumes of the sample.

Results

Preparation of [3-^{3}H]RuBP. [3-^{3}H]RuBP was prepared by modification of the method of Fiedler et al. (1967), as outlined in Scheme II. The location of the isotopic label in fructose 1,6-bisphosphate and RuBP was checked by mass spectral analysis of the deuterated compounds, prepared analogously [see the following paper (Sue & Knowles, 1982)]. The intermediates (*S*)-[4-^{3}H]NADH, [4-^{3}H]fructose 1,6-bisphosphate, and 6-phospho[4-^{3}H]gluconate, and the final product [3-^{3}H]ribulose 1,5-bisphosphate, were isolated and purified from their respective reaction mixtures by ion-exchange chromatography. In each case, the desired compound eluted from the column as a single peak well separated from any other radioactive species, and the radioactivity of the column fractions corresponding to that peak was proportional to the amount of assayable substrate in the appropriate enzymic assay. This manner of characterizing the tritiated compounds at each step in Scheme II served as additional criteria for the purity of the final product [3-^{3}H]RuBP. In the conversion of 3-phospho-D-glycerate to fructose 1,6-bisphosphate (Scheme II), care was taken that the aldolase was free of triosephosphate isomerase activity. In the presence of isomerase, D-[1-^{3}H]glyceraldehyde 3-phosphate would isomerize to [1(*S*)-^{3}H]dihydroxyacetone phosphate, resulting in the placement of the tritium label into a position labilized by aldolase. To minimize loss of the relatively unstable RuBP

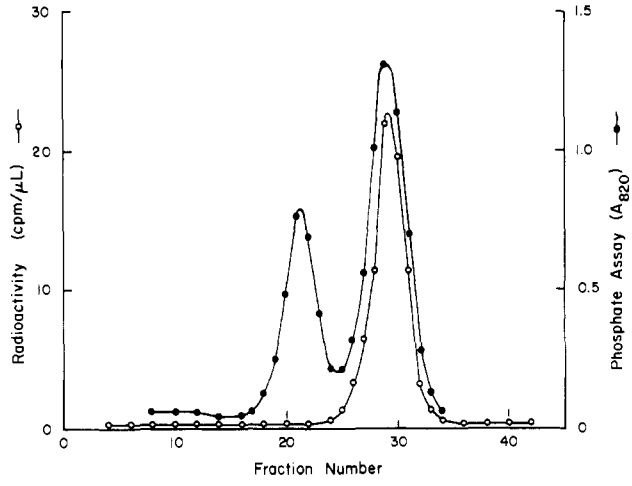


FIGURE 1: Separation of 3-phospho-D-glycerate and the pentitol bisphosphates on DEAE-cellulose.

via nonenzymatic pathways (Paech et al., 1978), we prepared [3-^{3}H]RuBP from 6-phospho[4-^{3}H]gluconate no earlier than 1 day before the carboxylase experiment.

Carboxylase-Catalyzed Reaction of [3-^{3}H]RuBP in Unlabeled Water. After preparation of the reaction mixture containing [3-^{3}H]RuBP but before the addition of carboxylase, the sample was divided into two parts. One part served as a control sample without added enzyme, and to the other part was added activated carboxylase. Samples were withdrawn by syringe from each mixture at different times during the incubation. Each sample was added to solid sodium borohydride (in >500-fold molar excess over RuBP) to reduce the RuBP. The quenched samples were treated as described under Methods, and the pentitol 1,5-bisphosphates were isolated by chromatography on DEAE-cellulose. The fractions from the column were assayed for radioactivity and phosphate ester content, and the specific radioactivity was determined for the product 3-phosphoglycerate and the pentitol bisphosphates derived from the reduction of remaining substrate. A typical column profile is shown in Figure 1. The extent of reaction was determined from the ratio of phosphoglycerate to pentitol bisphosphates as measured by the phosphate assays.

The results shown in Tables I and II describe the fate of the tritium label of [3-^{3}H]RuBP samples during incubations in unlabeled water with and without the carboxylase. It is evident that there is accumulation of tritium label in the substrate as the reaction approaches completion. When the reaction is 79% complete, the remaining RuBP has a specific radioactivity 134% of its original value.

Quantitative Treatment. The results presented in this and the preceding paper (Saver & Knowles, 1982) can be analyzed quantitatively by using the theory developed by Albery & Knowles (1976) for the reaction catalyzed by triosephosphate isomerase. The two studies are designated TS*s* and TS′*s* (T, tritium isotope; S, initially unlabeled substrate in tritiated solvent; S′, initially labeled substrate; *s*, analysis for isotope in the remaining substrate).

The TS′*s* experiment (this paper) is described by the function [eq 5.11 of Albery & Knowles (1976)]

$$\ln (s/s_0) = (A_6' - 1) \ln r \qquad (1)$$

where s is the specific radioactivity of the substrate of which fraction r remains and s_0 is the specific radioactivity of the substrate at the start of the reaction. The parameter A_6' describes the behavior of the substrate pool, and phenomenologically, for $A_6' < 1$, there is "accumulation" of the isotope in the remaining substrate, for $A_6' > 2$, there is "equilibration"

Table I: Specific Radioactivity of Pentitol Bisphosphates Derived from an Incubation of [3-^{3}H]RuBP in Unlabeled Water in the Absence of Enzyme[a]

time at quench (min)	specific radioactivity of bisphosphate[b] (cpm/μmol × 10^{-5})
6.5	1.31 ± 0.05
13.0	1.30 ± 0.01
24.5	1.23 ± 0.06
29.0	1.43 ± 0.08
	1.27 ± 0.13[c]

[a] The incubation was carried out in 100 mM triethanolamine hydrochloride buffer, pH 8.0, 30 °C. [b] Average values for the peak fractions from the column. [c] Extrapolated values for time zero.

Table II: Isotopic Content of Remaining [3-^{3}H]RuBP[a] during the Carboxylase-Catalyzed Reaction in Unlabeled Water

extent of reaction (%)	specific radioactivity[b] of remaining bisphosphate (cpm/μmol × 10^{-5})	fractional change[c] in specific radioactivity of remaining bisphosphate
33	1.37	0.080 ± 0.01
40	1.36	0.073 ± 0.01
59	1.70 ± 0.03	0.339 ± 0.03
72	1.58 ± 0.02	0.224 ± 0.02
75	1.62 ± 0.06	0.276 ± 0.07
79	1.70 ± 0.04	0.339 ± 0.04

[a] Analyzed as the pentitol bisphosphates. [b] Average value for the peak fractions from the column. [c] The specific radioactivity of the substrate at the start was 1.27 × 10^5 cpm/μmol (Table I). The errors for the first two points are estimates; the others are derived from the standard deviations in the determination of the specific radioactivities.

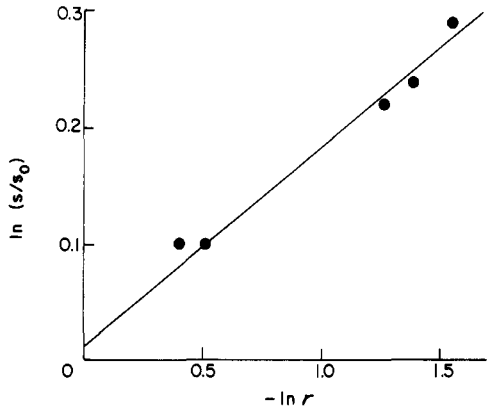


FIGURE 2: Determination of A_6'. Plot of the data from Tables I and II according to eq 1. The aberrant value at $r = 0.59$ has been omitted.

of the isotope with the medium (i.e., loss of isotope into solvent), and for $2 > A_6' > 1$, there is intermediate behavior. When the results from Tables I and II are plotted (Figure 2) according to eq 1, a value for A_6' of 0.83 ± 0.01 is obtained.

For the TS*s* experiment (Saver & Knowles, 1982), the relevant function is (Albery & Knowles, 1976)

$$\frac{s}{x} = \frac{A_7'(1 - r^{A_6'-1})}{A_6' - 1} \qquad (2)$$

where x is the specific radioactivity of the solvent, s, r, and A_6' are as previously defined, and A_7' is the kinetic parameter describing the state of the reactant. Phenomenologically, $A_7' \ll 1$, if there is accumulation of isotope in the remaining substrate, and $A_7' \gg 1$, if there is equilibration of isotope with the medium protons. When the value for A_6' of 0.83 (derived from the TS'*s* experiment) is used, the results of Table II of Saver & Knowles (1982) can be plotted according to eq 2, as shown in Figure 3. This gives a value for A_7' of 0.53 ± 0.01.

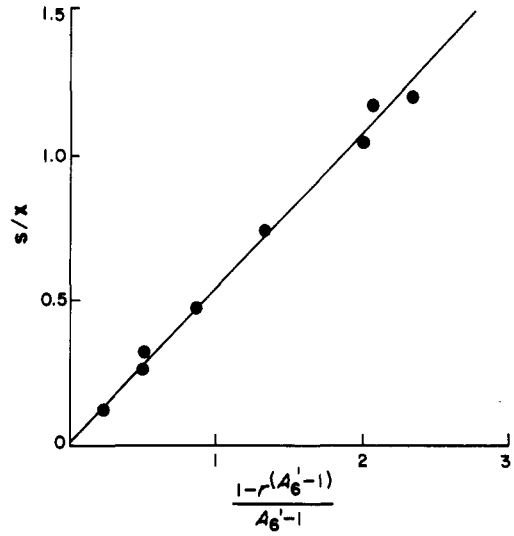


FIGURE 3: Determination of A_7'. Plot of the data from Table II of Saver & Knowles (1982) in which $A_6' - 1 = -0.17$. The result for 98% conversion is, of course, subject to large errors and is omitted from this plot.

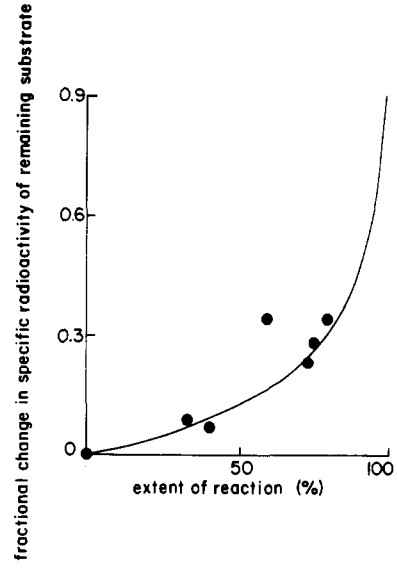


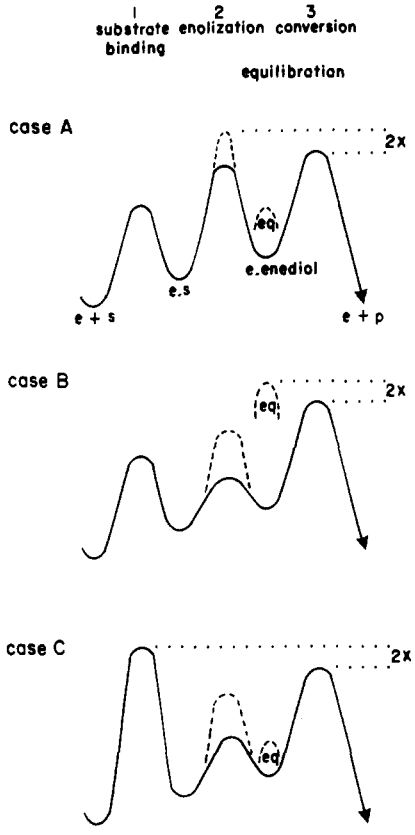
FIGURE 4: Fractional change in the specific radioactivity of remaining substrate RuBP as a function of the extent of the reaction. The curve is calculated from eq 1, assuming $A_6' = 0.83$ (see text).

Discussion

The results reported in this paper require the measurement of two variables: (i) the extent of reaction at the time of quenching, and (ii) the specific radioactivity of the remaining RuBP at that moment. The quench was accomplished by reducing the remaining RuBP with borohydride, which both destroys the substrate and locks the tritium label of [3-^{3}H]-RuBP into a position no longer exchangeable with solvent (that is, the hydrogen is no longer α to a carbonyl group). Furthermore, the reduced pentitol bisphosphate is stable to hydrolysis of the phosphoryl group, and the radioactivity representative of RuBP molecules could be recovered as a single peak from the ion-exchange column (Figure 1). No radioactivity is detected in the phosphoglycerate, which is consistent with our earlier results (Saver & Knowles, 1982) and with the conclusion of Fiedler et al. (1967) that the hydrogen abstracted from C-3 of RuBP is not delivered to the product.

The results, shown in Figure 4, demonstrate that the specific radioactivity of the remaining RuBP *rises* as the reaction proceeds. While there is a superficial resemblance between this curve and that observed earlier for the "tritium-in" ex-

Scheme III: Possible Free-Energy Profiles for the Early Steps of the Carboxylase-Catalyzed Reaction[a]



[a] e, s, and p denote the enzyme, substrate, and product species, respectively. The transition states are designated as follows: (1) association of e and s; (2) enolization of the substrate; (3) conversion of e·enediol to products; (eq) equilibration of the substrate-derived, enzyme-bound proton with a solvent proton. The dashed line in the enolization step describes the path for tritium transfer. For illustrative simplicity only, isotope effects are represented as transition-state differences.

periment [i.e., unlabeled RuBP reacting in tritiated water (Saver & Knowles, 1982)], the two experiments provide different information. While the substrate pool shows an increase in the concentration of tritiated molecules in both cases, the reasons for this behavior are rather different for the two experiments. In both experiments, the kinetic isotope effect in the proton abstraction process (the enolization step) has the same consequence in that it causes the specific radioactivity of the remaining RuBP to rise. However, the "washing-out" effect (deriving from loss of the C-3 proton and its exchange with the solvent) *raises* the isotopic content of the remaining substrate in the tritium-in experiment (Saver & Knowles, 1982) but *lowers* that quantity in the "tritium-out" experiment (this paper). The conclusions from both these tritium experiments are discussed below.

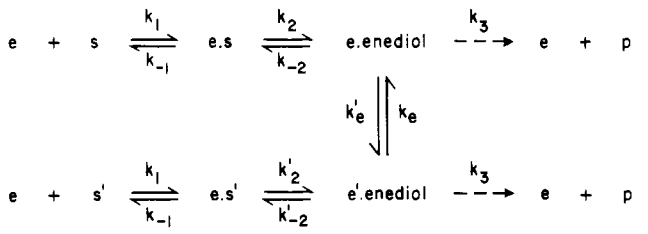
Enolization, Exchange, and Conversion. Two features characterize the experimental curve for the exchange vs. conversion experiment (Figure 1 of the preceding paper). First, at early extents of reaction, the slope of the curve is small and approximately linear, and second, toward the end of the reaction, the slope of the curve rises sharply, indicating that a second phenomenon has superseded the first. These facts place some constraints on the form of the free-energy profile for carboxylase. The initial slope of the curve at early times indicates that conversion [e·enediol going forward to enzyme (e) plus product (p)] is faster than exchange [e·enediol having equilibrated with tritiated solvent to e′·enediol, going back to e plus isotopically labeled substrate (s′)] by a factor of about 2. Three different situations are consistent with this partition ratio for e·enediol and are illustrated in Scheme III. There are three transition states for the interactions between enzyme and substrate: (i) substrate binding; (ii) enolization; (iii) conversion to products. There is also one transition state (eq) that represents the equilibration of the hydrogen on the conjugate acid of the enzyme's basic group, with solvent protons. The three free-energy profiles illustrated in Scheme III all accommodate the initial gradient of the exchange–conversion curve (Figure 1 of the preceding paper). In case A (Scheme III), the abstracted proton equilibrates rapidly with the medium (the "equilibration" barrier is very low), and at early times in the reaction of unlabeled s, the rate of conversion of the enediol to products (k_3, see Scheme IV) is twice as fast as the rate of return (picking up tritium, by the k_{-2}' step) to e + s′. In case B, the equilibration step is slow, and the enediol goes to products (k_3) twice as fast as equilibration occurs via k_e'. In case C, equilibration is fast, and the initial exchange/conversion ratio is satisfied by the more favorable partitioning forward (over transition state 3) than back (over transition state 1). Each of these situations accounts for the early part of Figure 1 of the preceding paper. Which of these, however, also accommodates the *accumulation* of tritiated RuBP at larger fractional extents of reaction?

In case C, the remaining substrate incorporates solvent tritium by enolization and equilibration of the substrate-derived proton. The protonated and tritiated substrates then compete for the enzyme, but in the absence of any discrimination against tritium in the binding step (1) and since the isotope effect in the enolization step (2) is not rate limiting, there is no selection against tritiated substrate in the enzyme-catalyzed consumption of starting material. Case C cannot, therefore, account for the upward curvature of the observed exchange–conversion curve (Figure 1 of the preceding paper) and must be rejected as a possible scheme for the carboxylase-catalyzed reaction.

In case B, enolization of the substrate takes place readily, but it is the equilibration step that governs the rate of tritium appearance into RuBP. Since the enolization step is relatively fast, case B can account for the late upward curvature of the observed exchange–conversion curve *only if* the equilibration process is subject to an isotope discrimination against tritium that results in the tritiated species of the substrate being "left behind". It is unlikely, however, that the equilibration step can show a significant isotope effect. Equilibration of the conjugate acid of an enzyme base with solvent protons requires two steps: (i) transfer of a hydrogen from the conjugate acid of the enzyme to the solvent and (ii) transfer of a new hydrogen from the solvent to the enzymic base. The overall equilibration path therefore always involves one proton transfer step and one tritium transfer step. The transition states encountered on washing tritium *into* the enzyme's conjugate acid are essentially the same transition states reversed when tritium is washed out from it. Because of this rough symmetry, there will be little or no discrimination against tritium in either direction. Case B, therefore, cannot account for the increase in specific radioactivity of remaining substrate at late times in the carboxylase-catalyzed reaction.

In case A of Scheme III, the enolization of substrate is partially rate determining for the conversion of substrate to product. We expect, therefore, an effective discrimination against tritiated substrate and thus predict that the tritium content of the remaining starting material will increase more rapidly than the (linear) "wash-in" rate. This prediction is consistent with the experimental findings, and the free-energy profile of case A can, therefore, account for the behavior of

Scheme IV: Pathway for the Early Steps of the Carboxylase-Catalyzed Reaction[a]



[a] Compare this with Scheme II of the preceding paper. e, s, and p are enzyme, substrate (RuBP), and products, respectively. Primed species are isotopically labeled.

the entire exchange–conversion curve (Figure 1 of the preceding paper).

Are the above conclusions, that favor case A of Scheme III, consistent with the results presented in this paper of the enzyme-catalyzed reaction of labeled substrate in unlabeled water? The isotopic content of the remaining substrate may (i) be depleted, (ii) remain unchanged, or (iii) be enriched. First, the process of enolization coupled with equilibration can result in the loss of tritium from the substrate, since the reaction is performed in unlabeled water. Second, the isotopic content of the starting material can rise *only if* there is a discrimination against tritiated molecules in the consumption of the substrate. In cases B and C of Scheme III, there can be no enrichment of the tritium label in the remaining substrate, because there is no discrimination against tritiated substrate in the rate-limiting transition states. Since we know from the results reported in this paper that there *is* enrichment of tritium in the remaining substrate, case B and case C must (again) be ruled out as possible descriptions of the carboxylase-catalyzed reaction. The free-energy diagram of case A can, however, accommodate the observed results. The actual extent of enrichment will depend, of course, on the balance between the wash-out rate (which depends upon the partitioning ratio of the e·enediol intermediate and the equilibration rate) and the discrimination against labeled substrate (which depends upon the partitioning ratio of the e·enediol intermediate and the intrinsic kinetic isotope effect on the enolization step).

The above discussion is only qualitative, though it is clear that case A of Scheme III properly describes the early steps of the carboxylase-catalyzed reaction. Quantitative constraints can be placed on the free-energy profile, however, and these are discussed in the following section.

Carboxylase-Catalyzed Reaction. The theory developed earlier (Albery & Knowles, 1976) for the quantitative treatment of proton exchange in enzyme-catalyzed reactions can be applied directly to the results of the tritium experiments described in this and the preceding paper. The equations were originally derived to evaluate the energetics of triosephosphate isomerase, an enzyme that catalyzes a freely reversible reaction. In the present work, however, the enzyme-catalyzed reaction is effectively irreversible, and the information derived from studying the reaction only in one direction is insufficient to allow a complete description of the free-energy profile for the reaction. Nonetheless, the range of permissible energy levels for the transition states in the free-energy profile can be set.

The observed kinetic parameters A_6' and A_7' obtained directly from the experiments described in this and the preceding paper are related (Albery & Knowles, 1976) to the rate constants and fractionation factors of Scheme IV as

$$A_6' = \Phi_{1,2}(1 + \theta)/[\Phi_s(1 + A_5'\theta')] \quad (3)$$

Table III: Values for the Parameters A_s', θ, and $\Phi_{1,2}$

A_s'	θ	$\Phi_{1,2}$
0	1.2	0.44
0.05	1.4	0.41
0.10	1.6	0.39
0.20	2.3	0.32
0.30	3.8	0.25
0.40	12	0.11

$$A_7' = \theta\Phi_{1,2}(1 - A_5')/(1 + A_5'\theta') \quad (4)$$

where $\Phi_{1,2}$ is the tritium mixed fractionation factor for the first two transition states of Scheme III (if $k_{-1} \gg k_2$, so the enolization step is clearly rate limiting, then $\Phi_{1,2}$ becomes Φ_2), θ is the partition ratio for the forward and reverse reactions of the e·enediol intermediate ($\theta > 1$ if the intermediate partitions preferentially back to substrate), Φ_s is the ground-state substrate fractionation factor [which is about 1.15 for unhydrated RuBP (Cleland, 1980)], A_5' describes the rate of equilibration of the isotope with the solvent at the e·enediol and ranges from fast equilibration ($A_5' = 0$) to no equilibration ($A_5' = 1$), and θ' is the partition ratio for the tritiated species, e'·enediol [$\theta' = \theta\Phi_{1,2}/\Phi_s$, on the basis that step 3 (Scheme III) is isotopically insensitive].

From eq 3 and 4, we may write

$$\theta = A_7'/[\Phi_s A_6'(1 - A_5') - A_7'] \quad (5)$$

and

$$\Phi_{1,2} = A_7'/[\theta(1 - A_5' - A_7'A_5'/\Phi_s)] \quad (6)$$

Since we know A_6' (=0.83), A_7' (=0.53), and Φ_s (~1.15), we may use eq 5 to evaluate the permissible range of values of θ and A_5'. From the denominator of eq 5, A_5' must lie between zero (very fast equilibration at k_e and k_e') and 0.44 (the equilibration rate is becoming kinetically significant), and the values of θ in this range of A_5' are shown in Table III. From eq 6, we may also derive the corresponding set of $\Phi_{1,2}$ values.

The results of Table III show that at one extreme (A_5' = 0) we may have fast equilibration, a θ of 1.2 (the e·enediol intermediate partitions marginally more favorably back to substrate), and a $\Phi_{1,2}$ of 0.44 (corresponding to an apparent tritium isotope effect of 2.6). As A_5' rises, and the equilibration rate slows, the data require that the e·enediol intermediate partition more and more toward substrate (this is clearly necessary to maintain a given amount of tritium "wash-in"), and the apparent tritium isotope effect (as indicated by $\Phi_{1,2}$) must rise (to maintain the observed discrimination against tritiated substrate).

In summary, the results reported in this and the preceding paper demonstrate that ribulose-1,5-bisphosphate carboxylase indeed involves the enolization of RuBP and the abstraction of the C-3 proton of the substrate. Quantitative limits on the relative levels of the three critical transition states [those for enolization, conversion, and equilibration (see Schemes III and IV)] can be set, and it is clear that case A (Scheme III) accommodates the results observed, as does a situation where barrier 2 is lower but the equilibration rate is slower.

If the results of Table II are used to calculate the apparent *overall* tritium isotope effect [using the equation $^T(V_{max}/K_m) = \log(1 - f)/\log\{r_f/[r_0(1 - f)]\}$, where f is the fractional extent of reaction, r_f is the specific radioactivity of the remaining substrate after fraction f has reacted, and r_0 is the specific radioactivity of the substrate before reaction], a value of 1.25 ± 0.08 is obtained. This is smaller than the range of 3–6 found by Fiedler et al. (1967) for the spinach enzyme and may imply some differences in the relative rate constants for the early steps of the carboxylase-catalyzed reaction for the enzymes

from spinach and from *Rhodospirillum rubrum*.

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Ribulose-1,5-bisphosphate Carboxylase: Primary Deuterium Kinetic Isotope Effect Using [3-^{2}H]Ribulose 1,5-Bisphosphate†

Julia M. Sue and Jeremy R. Knowles*

ABSTRACT: The primary deuterium kinetic isotope effect for the reaction of [3-^{2}H]ribulose 1,5-bisphosphate with CO_2 in the reaction catalyzed by ribulose-1,5-bisphosphate carboxylase has been determined. By use of highly purified substrates containing less than 0.13% of the C-3 epimer xylulose 1,5-bisphosphate (this material is known to be a potent competitive inhibitor), the kinetic isotope effect has been shown to be 1.2 ± 0.2 (V_{max}) and 1.3 ± 0.3 (V_{max}/K_m). These values are clearly too small to be intrinsic isotope effects for the rate-limiting removal of the C-3 proton from ribulose 1,5-bisphosphate. The results confirm the conclusions from the tritium experiments reported in the previous two papers and emphasize the fine balance between the forward and reverse reactions of the enediol intermediate.

In the original formulation of the mechanism of action of ribulose-1,5-bisphosphate carboxylase (RuBP carboxylase),[1] Calvin (1954) proposed that the first catalytic step involves the abstraction of the proton at C-3 of RuBP to form the 2,3-enediol. It was suggested that this enediol then attacks enzyme-bound CO_2 to give a six-carbon species, the hydrolysis of which leads to the products, two molecules of 3-phospho-D-glycerate. [The pathway is illustrated in Scheme I of the first paper of this series (Saver & Knowles, 1981).]

From experiments with unlabeled RuBP in tritiated water (Saver & Knowles, 1982) and with [3-^{3}H]RuBP in unlabeled water (Sue & Knowles, 1982), the reality of the enolization step of Calvin's mechanism was demonstrated. It was shown that the enolization step is reversible, and this reversibility can result in the incorporation of a solvent proton onto carbon 3 of the remaining RuBP. Significant discrimination against tritium in the consumption of C-3-labeled substrate was also observed, signifying an isotope effect in the proton abstraction step that occurs after the binding of RuBP but before the first irreversible step in the reaction. It was concluded that proton abstraction from RuBP is at least partially rate determining in the early steps of the carboxylase reaction. Identification of the rate-determining step of the *overall* catalyzed reaction, however, can be made only by examining all the steps in the enzymic cycle, catalytic or other. For that purpose, the steady-state rates of 3-phosphoglycerate formation from the enzyme-catalyzed reaction of unlabeled RuBP and of [3-^{2}H]RuBP have been measured. If the proton abstraction step is rate determining in catalytic turnover, deuterium substitution in the substrate will result in an observed primary kinetic isotope effect.

Since the proposed experiment involved the precise measurement of reaction rates, it was important that the RuBP samples used differed only in the hydrogen isotope at C-3. In particular, it was critical that the substrates not be variably contaminated with xylulose 1,5-bisphosphate (XuBP), which is the product of nonenzymatic epimerization at C-3 (Paech et al., 1978) and has been reported to be a potent competitive inhibitor of RuBP carboxylase [K_m(RuBP) ≈ 20 μM and K_i(XuBP) ≈ 3 μM for the enzyme from spinach (McCurry & Tolbert, 1977)]. Samples of labeled and unlabeled RuBP were therefore prepared by enzymatic synthesis from 6-phosphogluconate immediately prior to the velocity measurements, and a method was established to determine the extent of XuBP contamination in the preparations of RuBP. We report here the synthesis of RuBP and of [3-^{2}H]RuBP that are contaminated by XuBP to less than 0.13%, and the steady-state kinetic parameters for the consumption of these substrates by the carboxylase.

Materials and Methods

Materials

Enzymes, cofactors, and other materials were as specified in Saver & Knowles (1982) and Sue & Knowles (1982). Sodium borodeuteride was obtained from Ventron Corp. Dihydroxyacetone phosphate (as the dimethyl ketal dimonocyclohexylammonium salt) was from Sigma, and glycolaldehyde phosphate (as the diethyl acetal dimonocyclohexylammonium salt) was from Calbiochem.

(S)-[4-^{2}H]NADH. To a solution of dihydroxyacetone (~60 mg) in water (5 mL) was added sodium borodeuteride (140 mg), and the mixture was left for 30 min at room temperature. The solution was then treated with formic acid (500 μL) and the resulting solution concentrated by rotary evaporation. Water was added and the sample taken to dryness. The resulting precipitate was dissolved in water and the solution

† From the Department of Chemistry, Harvard University, Cambridge, Massachusetts 02138. *Received March 3, 1982.* This work was supported by the National Science Foundation and the National Institutes of Health.

[1] Abbreviations: RuBP, D-ribulose 1,5-bisphosphate; RuBP carboxylase, ribulose-1,5-bisphosphate carboxylase; XuBP, D-xylulose 1,5-bisphosphate; NAD^+, nicotinamide adenine dinucleotide; NADH, reduced NAD^+; $NADP^+$, nicotinamide adenine dinucleotide phosphate; EDTA, (ethylenedinitrilo)tetraacetic acid; DEAE, diethylaminoethyl.

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