Retention of the Oxygens at C-2 and C-3 of D-Ribulose 1,5-Bisphosphate in the Reaction Catalyzed by Ribulose-1,5-bisphosphate Carboxylase†

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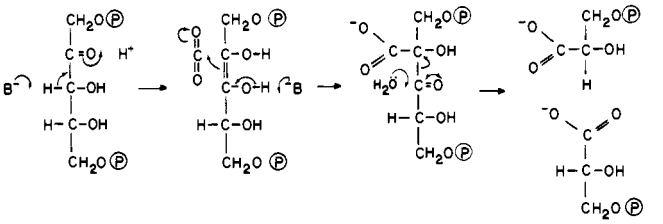
ABSTRACT: Ribulose-1,5-bisphosphate carboxylase catalyzes the conversion of D-ribulose 1,5-bisphosphate and CO_2 to 3-phospho-D-glycerate, with retention of the oxygen atoms at both C-2 and C-3 of the substrate. This observation is consistent with mechanistic pathways involving an enediol intermediate and eliminates suggested mechanisms that involve covalent intermediates between the enzyme and ribulose 1,5-bisphosphate in which the substrate oxygen at C-2 or C-3 is compulsorily lost.

The enzyme ribulose-1,5-bisphosphate carboxylase (EC 4.1.1.39) is responsible for the primary act of carbon fixation in photosynthetic organisms. Although the mechanism proposed by Calvin (1954) (Scheme I) is most frequently invoked, other pathways have been proposed. First, Rabin and Trown (1964a,b; Trown and Rabin, 1964) have suggested that a thio hemiketal forms at C-2 by addition of an enzymatic thiol group to the carbonyl group of ribulose 1,5-bisphosphate (RuBP); the thio hemiketal then suffers dehydration across C-2/C-3 to give a species (Scheme II, X) that subsequently condenses with CO_2. Although it has since been shown that the essential thiol groups of the enzyme are probably not at the active site (Argyroudi-Akoyunoglow and Akoyunoglow, 1967; Takabe and Akazawa, 1975; Schloss and Hartman, 1977; Sujiyama et al., 1968), the evidence does not exclude the Rabin and Trown pathway. Secondly, ketimine formation at either C-2 or C-3 with an active-site amino group has been suggested as a possible mode of substrate binding and activation. On the basis of the known essentiality of lysine residues (Schloss and Hartman, 1977; Norton et al., 1975; Whitman and Tabita, 1976, 1978; Paech et al., 1977), it has been proposed that the binding of substrate may involve ketimine formation between a lysine ϵ-amino group and the substrate carbonyl group at C-2 (McCurry and Tolbert, 1977). Alternatively, a protonated ketimine at C-3 has been suggested as a way of stabilizing the carbanion at C-2 thought to be required for the CO_2 addition step. Although attempts have been made to trap ketimine intermediates involving substrate (Wishnick and Lane, 1969), the failure of these experiments does not, of course, rule out the intermediacy of such species.

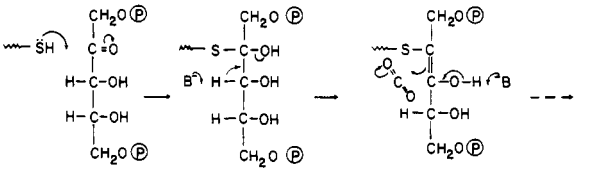
To limit the mechanistic possibilities for the carboxylase reaction, therefore, the fates of the oxygens at C-2 and C-3 of RuBP have been investigated. The Rabin and Trown mechanism requires that the oxygen at C-2 of RuBP be lost in the course of the carboxylase reaction, and ketimine formation at either C-2 or C-3 similarly requires that the oxygen at that position be lost. In contrast, the Calvin pathway predicts the retention of both the C-2 and C-3 oxygen atoms of the RuBP in the product 3-phospho-D-glycerate.

In this study, the enzyme from *Rhodospirillum rubrum* has been used. This enzyme is a dimer of identical subunits of 56 000 daltons (Tabita and McFadden, 1974b), which is simpler than the enzyme from higher plants [e.g., the spinach carboxylase contains eight copies of each of two kinds of subunit, of 56 000 and of 12 000 daltons (Rutner, 1970)]. Although some minor functional differences are known (McFadden and Tabita, 1974), the bacterial and plant enzymes do appear to have similar active sites (Schloss and Hartman, 1977).

SCHEME I: Mechanism of the Ribulose-1,5-bisphosphate Carboxylase Reaction According to Calvin (1954).



SCHEME II: Mechanism of Ribulose-1,5-bisphosphate Carboxylase Reaction According to Rabin and Trown (1964a,b).



X

Experimental Procedures

Materials

Enzyme. Rhodospirillum rubrum was grown anaerobically on butyrate in a growth medium essentially that of Kornberg and Lascelles (1960). RuBP carboxylase was purified as described by Tabita and McFadden (1974a) with minor modifications. The enzyme was >80% homogeneous as judged by polyacrylamide gel electrophoresis (Davis, 1964). Carboxylase activity was determined spectrophotometrically using the coupled enzyme assay of Racker (1962). The enzyme used had a specific activity of 1.4 units/mg, where 1 unit of enzyme activity is defined as that which catalyzes the reaction of 1 μmol of RuBP/min at 30 °C. The value for $E_{280nm}^{0.1\%,10mm}$ was taken to be 0.974 (Tabita and McFadden, 1974b). Concentrated solutions of enzyme were prepared by exhaustive

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dialysis at 4 °C against 200 mM triethanolamine hydrochloride buffer, pH 7.8, containing $MgCl_2$ (20 mM), $NaHCO_3$ (50 mM), and dithiothreitol (1 mM), followed by concentration by vacuum dialysis.

Substrate. RuBP was synthesized as described by Horecker et al. (1958). It was purified immediately before use by chromatography on DEAE-cellulose [equilibrated with 65 mM NH_4HCO_3 and eluted with a linear gradient (65–150 mM) of NH_4HCO_3 followed by 150 mM NH_4HCO_3]. RuBP was then converted into the disodium salt by treatment with Dowex-50 (Na^+ form). [2-^{18}O]RuBP was synthesized by dissolution of a freeze-dried sample of RuBP sodium salt (8 μmol) in $H_2{}^{18}O$ [16 μL of $H_2{}^{18}O$ (97.3% atom excess: Miles Laboratories)].

Methods

Assays. RuBP was assayed by adaption of the carboxylase assay (Racker, 1962). 3-Phospho-D-glycerate was estimated enzymatically (Orr and Knowles, 1974). Assays of pentitol 1,5-bisphosphates were carried out by treatment with alkaline phosphatase followed by estimation of the P_i according to Ames (1966).

Mass spectra were taken on an AEI MS-9 mass spectrometer. Spectra of phosphoglycerate were obtained after either methylation (Fisher et al., 1976) at room temperature or silylation (Fisher et al., 1976) at 110 °C for 10 min. The mass spectrum of methylated phosphoglycerate does not show a parent ion (at *m/z* 228), but the peak at $M^+ - 59$ (*m/z* 169) that comes from the loss of the carboxymethyl group from the *tri*methylated derivative conveniently monitors the ^{18}O content of the C-2 plus C-3 moiety of phosphoglycerate.

The ^{18}O content of RuBP was assessed by mass spectrometric examination of the pentitols derived from the RuBP sample. The borohydride reduction product (a mixture of D-ribitol 1,5-bisphosphate and D-arabitol 1,5-bisphosphate) was purified on DEAE-cellulose, treated with alkaline phosphatase, then with Dowex-50 (H^+ form), and finally filtered through Dowex-1 (OH^- form).

The mass spectrum of the trimethylsilylpentitols does not show a parent ion at *m/z* 512, and, although the $M^+ - 15$ fragment (at *m/z* 497) exists, the small amounts of sample handled in this experiment did not produce a signal strong enough to be quantitatively useful. Consequently, the more intense peak at $M^+ - 90$ (*m/z* 422) that corresponds to the loss of one Me_3SiOH group from the molecular ion was used to measure the ^{18}O content of the pentitols. A control mass spectrum taken on larger quantities of the silylated ^{18}O-pentitols showed that the $^{18}O/^{16}O$ ratio derived from the *m/z* 422 peak agreed with that ratio derived from the *m/z* 497 peak, to within 5%. This indicates that the Me_3SiOH group is preferentially lost from the two primary positions of the pentitols and confirms the validity of the $^{18}O/^{16}O$ ratio determined from the $M^+ - 90$ ion.

Reaction of [2-^{18}O]RuBP in $H_2{}^{16}O$. To a rapidly stirred solution of carboxylase [12 units in 2.0 mL of 200 mM triethanolamine hydrochloride buffer, pH 7.8, containing $MgCl_2$ (20 mM), $NaHCO_3$ (50 mM), and dithiothreitol (1 mM) at 0 °C] was added a solution of [2-^{18}O]RuBP (2 μmol, in 4 μL of $H_2{}^{18}O$). After 30 s at 0 °C, a solution of $NaBH_4$ [38 mg (i.e., 500-fold molar excess over RuBP) 500 μL of the above buffer] was rapidly added. After stirring for a further 4.5 min, excess borohydride was destroyed by the addition of acetic acid (1 mL). Under these conditions, there is negligible loss of ^{18}O from the RuBP following the addition of the borohydride. [This was checked by comparing the measured ^{18}O content (see below) of a sample of [2-^{18}O]RuBP injected directly onto solid borohydride with that of a sample that was dissolved in $H_2{}^{16}O$ buffer and quenched with borohydride immediately.] Borate and acetic acid were removed by repeated evaporation of added H_2O–methanol, and the sample was then subjected to chromatography on DEAE-cellulose [equilibrated with 65 mM NH_4HCO_3 and eluted with a shallow gradient (65–150 mM) of NH_4HCO_3, followed by 150 mM NH_4HCO_3]. Fractions containing phosphoglycerate and the pentitol bisphosphates were separately pooled, treated with Dowex-50 (H^+ form), and then freeze-dried. A control sample of [2-^{18}O]RuBP was treated identically, except that no carboxylase was added. The initial ^{18}O content of the labeled RuBP was determined by direct injection of a sample onto excess borohydride, followed by ion-exchange chromatography as above.

Reaction of RuBP in $H_2{}^{18}O$. All incubations involving enzyme were performed under nitrogen. A solution of carboxylase (100 μL, containing 1.3 units/mL) was dialyzed exhaustively against 100 mM triethanolamine hydrochloride buffer, pH 7.8, containing $MgCl_2$ (20 mM), $NaHCO_3$ (50 mM), and dithiothreitol (1 mM) at 4 °C, and freeze-dried. This was then dissolved in $H_2{}^{18}O$ (52 μL). A solution of RuBP (2 μmol) in $H_2{}^{18}O$ (15 μL) was prepared, and after 12 h at room temperature these two solutions were mixed and left at room temperature overnight. The mixture was then heated at 100 °C for 40 s, diluted with $H_2{}^{16}O$, and subjected to chromatography on DEAE-cellulose as described above. Fractions containing phosphoglycerate were pooled, treated with Dowex 50 (triethylammonium form) and freeze-dried prior to mass spectrometric analysis.

Results and Discussion

The Fate of the C-2 Oxygen of RuBP. To determine whether the carbonyl oxygen of RuBP is retained in the product phosphoglycerate (as is implied by the Calvin mechanism of Scheme I) or is lost to the solvent (as is required by Rabin's mechanism of Scheme II or by pathways involving a ketimine intermediate), we need to follow the enzyme-catalyzed reaction of [2-^{18}O]RuBP in $H_2{}^{16}O$. However, because the exchange rate of the C-2 oxygen of RuBP with water was expected to be fast [for comparison, the half-life of the carbonyl oxygen of dihydroxyacetone phosphate in water is less than 1 min at pH 7.0, 23 °C (Model et al., 1968)], it was necessary to ensure that the spontaneous rate of wash-out of ^{18}O label would not be faster than the carboxylase-catalyzed reaction. To measure the spontaneous rate of ^{18}O loss and to provide a method for establishing the ^{18}O content of the RuBP remaining after the incubation, samples of [2-^{18}O]RuBP were dissolved in $H_2{}^{16}O$ and quenched at various times by the addition of excess borohydride. In this way, it was established that in 200 mM triethanolamine buffer, pH 7.8, 0 °C, only about 7% of the ^{18}O label was lost after 10 s. (The rate of loss is at least tenfold faster in Tris-HCl or NH_4HCO_3 buffer. The acceleration observed in these buffers may derive from amine catalysis of the hydration–dehydration reaction, possibly via the ketimine.) Accordingly, [2-^{18}O]RuBP was allowed to react in $H_2{}^{16}O$ with a sufficient quantity of carboxylase to ensure approximately 50% conversion to phosphoglycerate within 30 s.

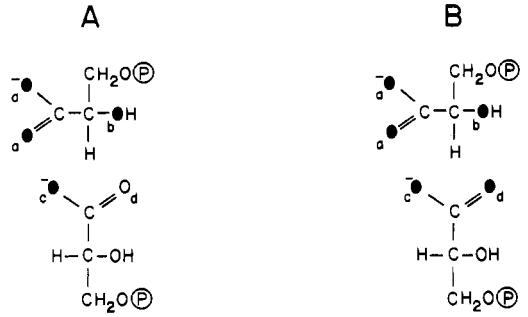
The results of these experiments are summarized in Table I, from which it is evident that (a) during the 30-s incubation of [2-^{18}O]RuBP in $H_2{}^{16}O$ the substrate ^{18}O content falls from about 87 to 68% in an exchange reaction that is independent of the presence of enzyme and (b) the ^{18}O content of the product phosphoglycerate corresponds to a substrate ^{18}O content of around 72% (36% × 2), which is close to that predicted on the basis that *in the enzyme-catalyzed reaction the*

TABLE I: ^{18}O Content of [2-^{18}O]Ribulose 1,5-Bisphosphate and of 3-Phosphoglycerate Derived from It by the Carboxylase Reaction.

sample	^{18}O content at C-2[a] (% atom excess)
[2-^{18}O]RuBP starting material	87 ± 5
[2-^{18}O]RuBP reisolated[b]	68 ± 3
3-phosphoglycerate product[c]	36 ± 1
[2-^{18}O]RuBP starting material	84 ± 3
[2-^{18}O]RuBP reisolated[d]	68 ± 6

[a] Averaged from at least ten scans of the mass spectrum. [b] After 30-s reaction in the presence of 12 units of carboxylase: ca. 33% of the starting RuBP was consumed during this time. [c] Two molecules of phosphoglycerate are formed, only one of which can contain label from C-2 of RuBP. [d] Control sample, after incubation in triethanolamine buffer in $H_2{}^{16}O$ in the *absence* of enzyme.

SCHEME III: Predicted ^{18}O-Labeling Patterns for the Two Molecules of 3-Phospho-D-glycerate If There Is (A) Exchange or (B) No Loss of the Oxygen at C-3 of RuBP during the Reaction.[a]



[a] For explanation of the letter subscripts, see the text.

RuBP oxygen at C-2 is completely retained in the product. Except in the event of the complete sequestration of the labeled substrate oxygen by enzyme and its return to the product, those mechanisms involving covalent attachment of C-2 of RuBP to the enzyme must be ruled out.

The Fate of the C-3 Oxygen of RuBP. Since in the course of the Calvin mechanism an intermediate is formed having a carbonyl group at C-3, it seemed opportune to test whether this oxygen too is (as drawn in Scheme I) retained during the carboxylase reaction. Accordingly, RuBP was allowed to react as substrate in $H_2{}^{18}O$. The predicted labeling patterns for the two molecules of 3-phospho-D-glycerate are shown in Scheme III. In both cases (complete loss of the C-3 oxygen or no loss of the C-3 oxygen), the two oxygen atoms (a) of the carboxyl group that derives from CO_2 are labeled, since the CO_2 has equilibrated with the $H_2{}^{18}O$ solvent via HCO_3^-. The C-2 oxygen (b) of the phosphoglycerate molecule that derives from C-1 and C-2 of RuBP is also labeled, since the C-2 carbonyl group of RuBP has been given enough time to equilibrate fully with the $H_2{}^{18}O$. Differences between the mechanistic pathways become manifest, however, in the phosphoglycerate that derives from C-3, C-4, and C-5 of RuBP. One of the carboxyl oxygens (c) of this phosphoglycerate is labeled, since it comes directly from the solvent $H_2{}^{18}O$. The other oxygen (d) will be labeled as well *only* if the C-3 oxygen of RuBP has been lost to the solvent during the course of the reaction.

Since in this experiment we need to analyze the carboxyl groups of phosphoglycerate, we require an ion in the mass spectrum which has not lost this part of the molecule. The pertrimethylsilyl derivative is suitable and gives a strong peak at $M^+ - 15$ (*m/z* 459). Figure 1 shows this region of the mass spectrum: (a) observed for the sample of 3-phosphoglycerate,

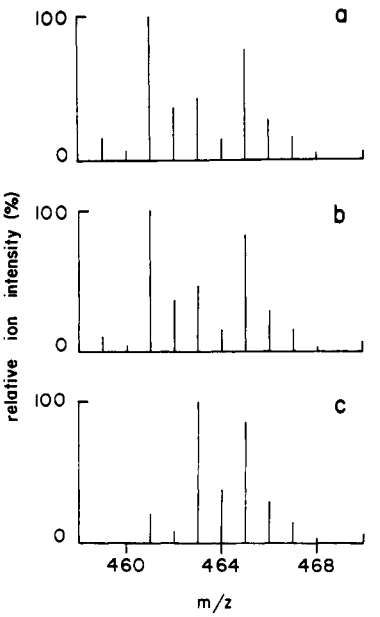


FIGURE 1: Mass spectra (from *m/z* 459 to 468) of pertrimethylsilyl 3-phospho-D-glycerate. (a) Observed spectrum; (b) spectrum predicted if there is no loss of the C-3 oxygen of RuBP; (c) spectrum predicted if there is complete loss of the C-3 oxygen of RuBP during the carboxylase reaction. Spectra b and c are calculated on the basis of an ^{18}O percentage atom excess of 89% in the solvent used. (This is derived from spectrum a and is predictably less than the 97% $H_2{}^{18}O$ used, because of the $H_2{}^{16}O$ content of the freeze-dried substrate and enzyme samples.) A control experiment demonstrated that the loss of ^{18}O from ^{18}O-labeled phosphoglycerate during the workup is negligible.

(b) predicted for no loss of the C-3 oxygen, and (c) predicted for complete loss of the C-3 oxygen. It is evident from these results that *no loss of the C-3 oxygen of RuBP occurs during the carboxylase reaction.* This result is analogous to the observation of Lorimer et al. (1973) that the phosphoglycerate molecule produced in the *oxygenase* reaction catalyzed by ribulose bisphosphate carboxylase contains only one carboxyl oxygen that is solvent derived.

The two experiments reported here show that the oxygen atoms on both C-2 and C-3 of ribulose 1,5-bisphosphate are retained during the course of the carboxylase reaction. Our results are consistent with the original mechanistic proposal of Calvin (1954) and exclude the pathway suggested by Rabin and Trown (1964a,b; Trown and Rabin, 1964), as well as any route that involves ketimine intermediates at C-2 or C-3 of RuBP.

Note Added in Proof

The results reported here are in complete agreement with those obtained by G. H. Lorimer [*Eur. J. Biochem.* (1978), (in press)] using the spinach enzyme.

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Denaturation of the Tryptic Fragments of the Calcium(II) Adenosine Triphosphatase from Sarcoplasmic Reticulum by Guanidinium Hydrochloride†

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ABSTRACT: Primary and secondary fragments of the Ca^{2+}-adenosine triphosphatase from sarcoplasmic reticulum are resistant to complete denaturation by guanidinium hydrochloride, a property characteristic of many intrinsic membrane proteins. None of the fragments display a single cooperative transition from ordered structure to random coil suggesting each fragment contains several domains of differing resistance to guanidinium hydrochloride denaturation. The data suggest that the native enzyme has at least three membrane-embedded domains, with an externally accessible link between each.

The structure and mechanism of ATP-dependent cation transport proteins are not fully understood. Considerable progress has been made with the Ca^{2+}-ATPase[1] of sarcotrypsin (Ikemoto et al., 1971; Inesi & Scales, 1974; Thorley-Lawson & Green, 1975; Stewart et al., 1976). The enzyme is composed of a polypeptide of 119 000 molecular weight (Rizzolo et al., 1976), which contains the ATP and Ca^{2+} binding sites (Degani & Boyer, 1973; Stewart et al., 1976). Enzyme preparations also contain a small polypeptide, termed proteolipid by MacLennan et al. (1972), which may be involved in enzyme activity (Racker & Eytan, 1975). Trypsin cleaves the ATPase into two equal size fragments, I and II, whose molecular weights determined by sedimentation equilibrium correspond to one half the weight of the intact chain (Rizzolo et al., 1976). Fragment I can be further cleaved by trypsin to form two fragments of approximately 35 000 (I_A) and 25 000 (I_B) molecular weight, determined by $NaDodSO_4$ gel electrophoresis (Stewart et al., 1976; Thorley-Lawson & Green, 1975). Neither cleavage has any affect on ATP hydrolysis or calcium translocation activities (Inesi & Scales, 1974; Stewart & MacLennan, 1974).

Thorley-Lawson & Green (1975) found that the amino acid composition of each fragment is very similar to that of the intact ATPase. Using the formulation of Capaldi & Vanderkooi (1972), the percentage of hydrophilic residues for each fragment is only slightly less than the average for water-soluble proteins. The use of antibodies against the various fragments suggests that fragment I is in large part exposed to solution, whereas fragments II and I_B are only slightly exposed (Stewart et al., 1976). This is consistent with the preferential iodination of fragment I by Thorley-Lawson & Green (1973). These data

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[1] Abbreviations used: ATPase, adenosine triphosphatase; ATP, adenosine triphosphate; $NaDodSO_4$, sodium dodecyl sulfate; GdmCl, guanidinium hydrochloride; CD, circular dichroism.

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