Pyruvate is a minor product of the reaction catalyzed by ribulosebisphosphate carboxylase/oxygenase from spinach leaves. Labeled pyruvate was detected, in addition to the major labeled product, 3-phosphoglycerate, when 14CO₂ was the substrate. Pyruvate production was also measured spectrophotometrically in the presence of lactate dehydrogenase and NADH. The Vₘₐₓ for CO₂ of the pyruvate-producing activity was 12.5 μM, similar to the CO₂ affinity of the 3-phosphoglycerate-producing activity. No pyruvate was detected by the coupled assay when ribulose 1,5-bisphosphate was replaced by 3-phosphoglycerate or when the carboxylase was inhibited by the reaction-intermediate analog, 2'-carboxyarabinitol 1,5-bisphosphate. Therefore, pyruvate was not being produced from 3-phosphoglycerate by contaminant enzymes. The ratio of pyruvate produced to ribulose bisphosphate consumed at 25 °C was 0.7%, and this ratio was not altered by varying pH or CO₂ concentration or by substituting Mn²⁺ for Mg²⁺ as the catalytically essential metal. The ratio increased with increasing temperature. Ribulosebisphosphate carboxylases from the cyanobacterium *Synechococcus PCC 6301* and the bacterium *Rhodospirillum rubrum* also catalyzed pyruvate formation and to the same extent as the spinach enzyme. When the reaction was carried out in H₂O, the spinach carboxylase increased the proportion of its product partitioned to pyruvate to 2.2%. These observations provide evidence that the C-2 carbanion form of 3-phosphoglycerate is an intermediate in the catalytic sequence of ribulose-bisphosphate carboxylase. Pyruvate is formed by β elimination of a phosphate ion from a small portion of this intermediate.

The reaction catalyzed by ribulose-P₅ carboxylase-oxygenase (EC 4.1.1.39) is fundamental to almost all forms of photo- or chemolithotrophic life. However, despite presumably intense selective pressure to maximize its catalytic efficiency, this enzyme seems poorly adapted to its task. Its feeble Vₘₐₓ/Kₘ ratio necessitates that plants allocate as much as half of their soluble leaf protein to this one enzyme. Furthermore, its difficulty in distinguishing between CO₂ and O₂ as substrate encumbers photosynthetic organisms with the wasteful process of photorespiration (reviewed by Andrews and Lorimer, 1987).

The catalytic sequence of ribulose-P₅ carboxylase involves several enzyme-bound intermediates (Scheme 1). Calvin’s six-carbon, carboxylated intermediate (Calvin, 1956) (shown in Scheme 1 in both the free ketone and hydrated, gemdial forms as II and III) has proven stable enough to be isolated in quantity, and its reactions in solution and on the active site have been characterized (Schloss and Lorimer, 1982; Pierce et al., 1986; Lorimer et al., 1986; Andrews and Lorimer, 1987). The five-carbon enediol(ate) (I), the species to which the gaseous substrates add, has been revealed as a very unstable species which eliminates the C-1 phosphate moiety very rapidly when released from the active site (Jaworowski et al., 1984). The intermediacy of this species was further supported recently by observations that isolated ribulose-P₅ carboxylase slowly epimerizes ribulose-P₅ to produce the potent inhibitor, 3-keto-3-keto-3-arabinitol 1,5-bisphosphate, leading to progressive inhibition during catalysis. This epimerization results from stereoechemically incorrect reprotonation of the enediol (Edmondson et al., 1990a-d). The final intermediate shown in Scheme 1 (IV) is the C-2 carbanion (or aci-acid) of P-glycerate. Following Jaworowski et al. (1984), we use the term "aci-carbanion" to describe this resonant species. Evidence for the involvement of this species is less direct than for the earlier two intermediates. The strong isotope effect associated with the attachment of solvent tritium to the C-2 of P-glycerate (Hurwitz et al., 1956; Simon et al., 1964; Fiedler et al., 1967; Saver and Knowles, 1982) suggests the involvement of an intermediate whose protonation allows competition between H and H⁺. Jaworowski et al. (1984) were unable to detect the expected breakdown product of such an aci-carbanion (pyruvate) after acid quenching during turnover. However, their method did not have the sensitivity necessary to detect the small traces of such a breakdown product which might be expected if the aci-carbanion did not accumulate to high levels on the enzyme (see "Discussion").

The epimerization of ribulose-P₅ that ribulose-P₅ carboxylase catalyzes (Edmondson et al., 1990d) shows that the active site is unable to totally prevent unwanted side reactions associated with the highly reactive enediol intermediate (I). We reasoned that, if the aci-carbanion (IV) was a significant intermediate, it might also be subject to abortive side reactions. This intermediate would be expected to be subject to β
elimination of the phosphate moiety in a similar manner to endiol phosphates, and this was confirmed in studies of the behavior of the six-carbon intermediate, 3-keto-carboxyarabinitol-P₂ (II), in solution. Above pH 11, 3-keto-carboxyarabinitol-P₂ hydrolyzes to yield a molecule each of P-glycerate, pyruvate, and P₂ (Lorimer et al., 1986). The latter two species are the products expected for the β elimination reaction of the aci-carbanion (IV) formed, along with a molecule of P-glycerate, by heterolytic fission of the C2-C3 bond of the hydrated, gemdiol form of 3-keto-carboxyarabinitol-P₂ (III).

Here we report that pyruvate is, indeed, a significant product of ribulose-P₂ carboxylase’s catalytic process, and we document some of the properties of the pyruvate-producing activity. These observations establish the intermediacy of the aci-carbanion species and further emphasize the difficulties ribulose-P₂ carboxylase has in restraining such highly reactive intermediates.

**EXPERIMENTAL PROCEDURES**

**Materials**—Ribulose-P₂ carboxylase was purified from spinach leaves and ribulose-P₂ was synthesized as previously described (Edmundson et al., 1990a). *Synechococcus* PCC 6301 ribulose-P₂ carboxylase was purified from extracts of *Escherichia coli* coexpressing the rbcL and rbcS genes on plasmid pSH1 (Andrews, 1988) by the procedure of Andrews and Ballment (1983). *Rhodospirillum rubrum* ribulose-P₂ carboxylase was purified from extracts of *E. coli* expressing the rbcL gene on plasmid pSH1 (Andrews et al., 1989) by a combination of (NH₄)₂SO₄ fractionation (30–60% saturated fraction taken), anion-exchange chromatography on a Mono Q HR 10/10 column (Pharmacia LKB Biotechnology Inc.) with KCl gradient elution, and gel filtration on a Pharmacia Superose 12 HR 10/30 column. Carboxypentitol-P₂ was synthesized according to Collatz et al. (1979) and stored in 50 mM Bicine-NaOH buffer, pH 9.0. Other enzymes were obtained from Sigma or Boehringer Mannheim.

**Kₚ (CO₂) Measurement**—The rate of pyruvate production was measured at 25 °C spectrophotometrically at 340 nm. A solution containing 130 mM Hepes-NaOH, pH 8.3, 18 mM MgCl₂, 50 μM NADH, and 1 mM ribulose-P₂ was made CO₂- and O₂-free by exhaustive sparging with N₂. After transfer to a stopped cuvette and further sparging, sparging was discontinued and HCO₃⁻, bovine erythrocyte carbonic anhydrase, and rabbit muscle lactate dehydrogenase added to 0.05–20 mM, 100 μg/ml, and approximately 3 units/ml, respectively. Catalysis was then initiated by adding spinach ribulose-P₂ carboxylase (final concentration of catalytic sites, 2.1 μM) which had been preactivated in the presence of 19 mM MgCl₂ and 10 mM NaHCO₃.

**Other Methods**—Concentrations of spinach ribulose-P₂ carboxylase were estimated spectrophotometrically at 280 nm using the published absorption coefficient (Paulsen and Lane, 1986). Ribulose-P₂ concentrations were measured spectrophotometrically using an assay similar to that described for P-glycerate in the legend of Table I, except that 10 mM HCO₃⁻ and 20 μg/ml spinach ribulose-P₂ carboxylase were also present.

**RESULTS**

A Trace of Pyruvate Is Produced by Ribulose-P₂ Carboxylase—The products of complete carboxylation of ribulose-P₂ catalyzed by spinach ribulose-P₂ carboxylase in the presence of ¹⁴CO₂ were chromatographed on a Bio-Rad Aminex HPX-87H column (Fig. 1). In addition to the expected major radioactive peak of phosphoglyceric acid, a small peak of radioactivity was observed which coeluted with the UV peak due to the pyruvic acid internal standard. The identity of this peak as pyruvic acid was confirmed by the observation that it disappeared, and the radioactivity moved to coelute with lactic acid, when lactate dehydrogenase and NADH were present during the carboxylation reaction. A very small peak of radioactivity coeluted with glyceric acid under both conditions. Presumably it was the result of a trace of phosphatase present in the ribulose-P₂ carboxylase preparation.

**Pyruvate Production Is an Intrinsic Property of the Ribulose-P₂ Carboxylase Reaction**—Pyruvate production was observed spectrophotometrically in a continuous assay by coupling to the lactate dehydrogenase reaction (Fig. 2). Activity proceeded in an approximately linear fashion until ribulose-P₂ became limiting, and it eventually ceased when ribulose-P₂ was exhausted. The extent of the reaction corresponded to less than 1% of the ribulose-P₂ initially present (Table I) because most of the ribulose-P₂ was being converted to P-glycerate, which is silent in this assay. No pyruvate was produced when P-glycerate was substituted for ribulose-P₂, showing that the pyruvate was not being produced from P-glycerate by contaminating enzymes in the ribulose-P₂ carboxylase or lactate dehydrogenase preparations. Pretreatment of the ribulose-P₂ carboxylase with carboxypentitol-P₂, which contains the strongly inhibitory analog of the six-carbon reaction intermediate, 2'-carboxyarabinitol-P₂, completely inactivated the pyruvate-producing reaction and, even when the inhibitor was added after ribulose-P₂, pyruvate production stopped promptly well before the ribulose-P₂ was exhausted. When pyruvate production and total ¹⁴CO₂ fixation were measured in parallel identical reaction mixtures (Fig. 3), the time-courses for the two reactions were identical except for a brief lag in the oxidation of NADH caused by the coupling system used in the pyruvate assay. All of these data confirm that pyruvate must be produced as an integral part of the reaction mechanism of ribulose-P₂ carboxylase.

**Ratio of Pyruvate Production to Total Carboxylation**—This ratio may be calculated either from the extents of the two reactions (Table I) or from their initial rates (data not shown). Similar ratios were obtained using both methods. The ratio was approximately 0.7% and was remarkably unaffected by varying conditions (Table I). For spinach ribulose-P₂ carboxylase, varying pH between 6.4 and 9.1 was without effect, as was varying the HCO₃⁻ concentration between 0.5 and 10 mM at pH 8.3. Substituting Mn⁺⁺ for Mg⁺⁺ as the catalytically essential metal also did not alter the ratio. The enzymes from the cyanobacterium *Synechococcus* PCC 6301 and the bacterium *Rhodospirillum rubrum* also partitioned a similar portion of their product to pyruvate. All of these ratios were the same, within experimental error, and their mean was 0.68 ± 0.05
Pyruvate production by ribulose-P₂ carboxylase/oxygenase

Pyruvate production was measured spectrophotometrically as described for Fig. 2. Below pH 7, the buffer was Pipes-NaOH; between pH 7 and 8.3, it was Hepes-NaOH or Tris-HCl; for pH 8.6, it was Bicine-NaOH; for pH 9.1, it was Chex-NaOH. The divalent metal ion concentration was 15-20 mM, except for the experiment with Mn²⁺ (and its Mg²⁺ control) where the concentration was 4.5 mM and 0.9 mM EDTA was also present. When the calculated CO₂ concentration was lower than 50 μM, bovine erythrocyte carbonic anhydrase (0.10 mg/ml) was added and O₂-free conditions were maintained. The ribulose-P₂ concentration was 1.1 mM, except when the HCO₃⁻ concentration was 1 mM or lower, in which case the ribulose-P₂ concentration was adjusted to be half of the HCO₃⁻ concentration. When the solvent was predominantly H₂O, a concentrated solution of ribulose-P₂, carboxylyse, lactate dehydrogenase, and carbonic anhydrase was preactivated in H₂O containing 20 mM Hepes-NaOH, pH 8.0, 20 mM MgCl₂, 10 mM NaHCO₃, and 1 mM EDTA, and then a small aliquot (1.25% of the final assay volume) was added to the H₂O-based assay mixture to initiate catalysis. Because of lower enzyme activities in H₂O, the concentrations of ribulose-P₂, carboxylase, carboxylyse, and lactate dehydrogenase were increased to 22 μM (catalytic sites), 0.35 mg/ml, and approximately 55 units/ml, respectively. The reactions were allowed to proceed until all of the ribulose-P₂ was consumed and the ratio was calculated from the total extent of the absorbance change. Confirmation that cessation of the absorbance change corresponded to exhaustion of ribulose-P₂ was obtained by observing the decrease in absorbance at 340 nm following addition of an aliquot of the reaction mixture to a solution containing 90 mM Hepes-NaOH, pH 7.8, 17 mM MgCl₂, 150 μM NADH, 1 mM ATP, 5 mM phosphocreatine, and the following coupling enzymes (source and approximate units/ml in parentheses): creatine kinase (rabbit muscle, 1); phosphoglycerate kinase (yeast, 3); glyceraldehydesphosphate dehydrogenase (rabbit muscle, 1.3); triosephosphate isomerase (rabbit muscle, 3); glyceraldehydephosphate dehydrogenase (rabbit muscle, 1).

| Enzyme source | Solvent | Metal pH | [HCO₃⁻] (percent of ribulose-P₂ consumed) |
|---------------|---------|----------|------------------------------------------|
| Spinach       | H₂O     | Mg²⁺     | 6.0                                      |
| Spinach       | H₂O     | Mg²⁺     | 6.9                                      |
| Spinach       | H₂O     | Mg²⁺     | 7.3                                      |
| Spinach       | H₂O     | Mg²⁺     | 8.0                                      |
| Spinach       | H₂O     | Mg²⁺     | 8.3                                      |
| Spinach       | H₂O     | Mg²⁺     | 9.1                                      |
| Spinach       | H₂O     | Mn²⁺     | 8.3                                      |
| Rhodoporillium| H₂O     | Mg²⁺     | 7.8                                      |
| Synechococcus | H₂O     | Mg²⁺     | 7.8                                      |
| Synechococcus | H₂O     | Mn²⁺     | 7.8                                      |

[(S.E.)%]. However, when the experiment with the spinach enzyme was conducted in the presence of 98.5% H₂O, the partitioning toward pyruvate increased over 3-fold to a ratio of 2.2%.

The fraction of product partitioned toward pyruvate increased with temperature (Fig. 4). Over twice as much pyruvate was formed at 40 as at 10 °C. A plot of the logarithm of the percentage of pyruvate versus the inverse of the absolute temperature was linear (Fig. 4, inset). This plot is analogous to an Arrhenius plot, but its slope yields a value (4.8 Kcal/mol) which reflects the difference in activation energies between the pyruvate-producing and P-glycerate-producing activities.

Kₘ (CO₂) of the Pyruvate-producing Activity—The effect of

Fig. 1. Chromatographic separation of the ¹⁴C-labeled products of the ribulose-P₂ carboxylase reaction. Spinach ribulose-P₂ carboxylase (0.16 mg) was preincubated for 10 min at 25 °C in a 1-ml solution containing 60 mM Tris-HCl, pH 8.0, 15 mM MgCl₂, 10 mM NaHCO₃, 2000 cpm/nmol, and, where indicated, 0.15 mM NADH, and approximately 3 units of rabbit muscle lactate dehydrogenase. Ribulose-P₂ was then added to 2.2 mM and, after a further 60 min, the mixture was applied to a 0.5×5-cm column of Bio-Rad AG 50W-X8 cation exchange resin (100–200 mesh, H⁺ form) and washed through with 2 ml of H₂O. The elute plus washings was vortexed under a stream of N₂ to remove CO₂, neutralized to pH 6 with NaOH, and evaporated to dryness under a stream of N₂ at 40 °C. The residue was dissolved in 330 μl of 0.013 N H₃SO₄, containing 0.8 mM pyruvate, 4 mM DL-glycerate, and 4 mM DL-lactate and aliquots (50-150 μl) were chromatographed on a 0.78×30-cm Bio-Rad Aminex HPX-87H column with 0.013 N H₃SO₄ as the mobile phase at a flow rate of 0.6 ml/min. The absorbance of the eluate was monitored at 210 nm and fractions of 180 μl were collected for scintillation counting.

Fig. 2. Spectrophotometric measurement of pyruvate produced during ribulose-P₂ carboxylation. Spinach ribulose-P₂ carboxylase (1 μM catalytic sites) was preincubated at 25 °C in a solution containing 80 mM Tris-HCl, pH 8.0, 17 mM MgCl₂, 10 mM NaHCO₃, 50 μM NADH, and 5 μg/ml (approximately 3 units/ml) of rabbit muscle lactate dehydrogenase. Absorbance was monitored at 340 nm. Ribulose-P₂ (1.1 mM), P-glycerate (2 mM), and carboxyphosphatid-P₂ (40 μM) were added where indicated.
Fig. 3. Parallel measurement of \(^{14}\)CO\(_2\) fixation and pyruvate production. The procedure was the same as that described for Fig. 2, except that the ribulose-P\(_2\) carboxylase site concentration was 5 \(\mu\)M, the Tris buffer was replaced with 90 mM Hepes-NaOH, pH 8.3, and the solutions were sparged with \(N_2\). When \(^{14}\)CO\(_2\) fixation was being measured, 19.5 mM NaH\(^{14}\)CO\(_3\) (753 cpm/nmol) was substituted for unlabeled bicarbonate. Otherwise the solutions for the two assays were identical. When pyruvate production was being measured, the absorbance was monitored at 340 nm. When \(^{14}\)CO\(_2\) fixation was being measured, 29-\(\mu\)l aliquots were removed at the times shown and added to 0.5 ml of 20% (v/v) formic acid. After drying at 80°C, the acid-insoluble \(^{14}\)C was measured by scintillation counting.

Fig. 4. Effect of temperature on partitioning towards pyruvate. Pyruvate formation was measured spectrophotometrically as described for Fig. 2. The solution contained, in addition to ribulose-P\(_2\) carboxylase, 135 mM Hepes-NaOH buffer, pH 8.3, 18 mM MgCl\(_2\), 15 mM NaHCO\(_3\), 50 \(\mu\)M NADH, and approximately 10 units/ml of rabbit muscle lactate dehydrogenase. After preactivation for approximately 10 min, the reaction was started by adding ribulose-P\(_2\) to 0.95 mM. The stated temperature was maintained with a thermostated cuvette and measured using a calibrated thermocouple which dipped into the solution. The full extent of the absorbance change corresponding to complete consumption of the ribulose-P\(_2\) was recorded. P-glycerate was determined on aliquots of the solution after completion of the reaction as described for Table I, thus allowing calculation of the ratio of pyruvate produced to ribulose-P\(_2\) consumed. The inset shows a plot of the natural logarithm of the percentage of pyruvate versus the inverse of the absolute temperature. Its slope yields a value of 4.8 Kcal/mol for the difference in activation energies between pyruvate and P-glycerate production.

The coupled assay is intrinsically insensitive and an acceptable extent of absorbance change requires ribulose-P\(_2\) concentrations in excess of 200 \(\mu\)M. This limitation prevented study of the effect of varying the ribulose-P\(_2\) concentration, in the subsaturating range, on the rate of pyruvate production.

Discussion

Pyruvate was clearly identifiable among the products of carboxylation of ribulose-P\(_2\) catalyzed by ribulose-P\(_2\) carboxylase (Fig. 1). It was not produced when the carboxylase was specifically inhibited by carboxypentitol-P\(_2\) or when P-glycerate was substituted for ribulose-P\(_2\) (Fig. 2) and the time-course of pyruvate production exactly paralleled \(^{14}\)CO\(_2\) fixation and, by inference, P-glycerate production (Fig. 3). Therefore, production of pyruvate must be an integral part of the catalytic function of ribulose-P\(_2\) carboxylase. Since the pyruvate is labeled from \(^{14}\)CO\(_2\) (Fig. 1) and its production shows the same \(K_m\) (\(CO_2\)) as the overall carboxylation reaction, its precursor must be an intermediate in the carboxylase reaction sequence formed after the carboxylation event which produces 3-keto-carboxyarabinitol-P\(_2\). There seems little doubt, therefore, that the intermediate which decays to pyruvate is the three-carbon aci-carbanion formed following scission of the C2-C3 bond of the gemdiol form of 3-keto-carboxyarabinitol-P\(_2\) (Scheme 2). Apparently, the active site is not able to restrain this highly reactive three-carbon species exclusively to the productive catalytic pathway. Once in every ~150 turnovers (at 25°C) (Table I), this intermediate must \(\beta\) eliminate a phosphate ion to form pyruvate. Our data thus support those showing a strong isotope effect for the attachment of the proton at C-2 of P-glycerate (Hurwitz et al., 1956; Simon et al., 1964; Fiedler et al., 1967; Saver and Knowles, 1982), in establishing the intermediacy of this aci-carbanion species. The \(\beta\) elimination reaction of this intermediate is analogous to that of the endiolar intermediate involved in the triosephosphate isomerase reaction, where the \(\beta\) elimination prod-

Scheme 2. The final stages of the catalytic cycle of ribulose-P\(_2\) carboxylase. The following intermediates or transition states are shown complexed at the catalytic site: III, hydrated form of 3-keto-carboxyarabinitol-P\(_2\); IV, carbanion (aci-acid) form of P-glycerate; V, transition state for the final protonation.
uct is methyl glyoxal (Campbell et al., 1979). However, the triose-phosphate isomerase intermediate \( \beta \) eliminates much less frequently, relative to the total rate of catalytic throughput, than the ribulose-P\(_2\) carboxylase aci-carbanion apparently does.

The ease with which we have been able to detect pyruvate production contrasts with the failure of Jaworowski et al. (1984) to detect it by their rapid-quench method. However, the experiment of Jaworowski et al. (1984) involved the reaction of a small quantity of \([1,2-\text{\textsuperscript{14}}\text{C}]\text{ribulose-P}_2\) (250 pmol) with a larger quantity of enzyme active sites (15 nmol) for 16 ms in the presence of \(\text{\textsuperscript{14}}\text{CO}_2\) with a specific radioactivity of 2 mCi/mmol. Detection of any pyruvate formed must rely on the \(\text{\textsuperscript{14}}\text{C}\) label, because the phosphorus atom will have been eliminated. Release of \(\text{\textsuperscript{14}}\text{P}\) cannot be used to measure pyruvate formation because \(\text{\textsuperscript{31}}\text{P}\) is also released from other acid-labile intermediates earlier in the carboxylase reaction sequence. Complete conversion of the ribulose-P\(_2\) to products would have resulted in the fixation of only 1110 dpm of \(\text{\textsuperscript{14}}\text{C}\). In the same experiment, it was estimated, from the amount of acid-labile \(\text{\textsuperscript{13}}\text{C}\) released, that the reaction intermediates (I, II, III, and IV if present), Scheme 1) accumulated to a collective total of 12.3\% of the ribulose-P\(_2\) supplied. Even in the unlikely circumstance that all of this \(\text{\textsuperscript{13}}\text{C}\) release was attributable to the aci-carbanion, it would have produced only 137 dpm of radioactivity in the form of pyruvate upon acidification, making its detection (as lactate after a chromatographic procedure) rather difficult. Lesser (and more reasonable) amounts of the aci-carbanion would have escaped detection. We may conclude that the aci-carbanion is not an abundant intermediate in the carboxylase reaction, but more sensitive rapid-quench experiments would be required to establish its level precisely.

Another possible unproductive reaction to which the aci-carbanion might be susceptible would be its sterechemically incorrect protonation to form L-P-glycerate. Indeed, there is an unconfirmed report that 10\% of the P-glycerate produced by ribulose-P\(_2\) carboxylase is of the L isomeric form (Branden et al., 1980). However, since complete carboxylation with \(\text{\textsuperscript{14}}\text{CO}_2\) of a limiting amount of ribulose-P\(_2\) yields amounts of D-P-glycerate (measured by the enzymatic assay described in the legend of Table I which presumably recognizes only the D isomer) that are consistent with the amount of \(\text{\textsuperscript{14}}\text{C}\) fixed (data not shown), it seems unlikely that the extent of L-P-glycerate production can be this large. Whether or not smaller traces of the L isomer are formed remains a question for future study.

Conversion of the gemdiol form of 3-keto-carboxyarabinitol-P\(_2\) (III) (Scheme 2) to two molecules of D-P-glycerate requires that the stereochemical configuration about C-2 of the intermediate be inverted to form the "upper" molecule of P-glycerate. This requires that C-1 and its attached phosphate moiety must move a considerable distance with respect to the carboxyl and hydroxyl groups attached to C-2, first to form the planar aci-carbanion (IV), and then the transition state (V) for proton transfer to P-glycerate. The structure of the catalytic site, as revealed by crystallographic studies of the spinach ribulose-P\(_2\) carboxylase with bound carboxyarabinitol-P\(_2\) (Andersson et al., 1989), shows that the C-1 phosphate group and the carboxyl and hydroxyl groups attached to C-2 are all firmly tethered. The phosphate interacts with mainchain N atoms in loop 8, the carboxyl interacts with lysine 334 in loop 6, and the carboxyl and hydroxyl groups are both coordinated to the active site metal ion which is, in turn, coordinated to carbamylated lysine 201 and other acidic groups in loop 2. (These loops are the sequences which connect the carboxyl termini of \(\beta\) strands to the amino termini of \(\alpha\) helices in the \(\beta/\alpha\) barrel domain of the large subunit (Andersson et al., 1989).) Therefore, in order for the essential inversion of configuration to be accomplished, either some of these interactions must be broken (and presumably new interactions formed) or there must be considerable movement of these active site loops with respect to each other during this final stage of the catalytic cycle. Such complicated movements might be slow and could contribute to ribulose-P\(_2\) carboxylase's slow rate of catalytic turnover. Comparison of the structure of the carbamylated active site with P-glycerate bound with the already determined structure with carboxyarabinitol-P\(_2\) bound would shed light on this question.

Stereochemical considerations also indicate that movement within the active site during catalysis must be necessary. The aci-carbanion will be most resistant to \(\beta\) elimination (to form enolpyruvate) when the bridge oxygen of the phosphate is in the same plane as the double bond, as is represented in Scheme 2. This is because there will be minimal orbital overlap between the C-O bond to the phosphate and the \(\pi\) system of the double bond in this condition (Rose, 1981). However, 3-keto-carboxyarabinitol-P\(_2\) is bound in an extended configuration and the bridge oxygen is positioned very far away from the plane of the bond joining the carboxyl carbon to C-2 (Andersson et al., 1989), which will become the double bond of the aci-carbanion. Furthermore, a similar requirement for coplanarity also affects the stability of the five-carbon enediol intermediate earlier in the reaction sequence and the bridge oxygen cannot adopt a single position which would be planar with the double bonds of both intermediates. Therefore, considerable movement must be necessary to stabilize the aci-carbanion and, at any stage during that movement when the bridge oxygen is out of the plane of the double bond, the phosphate group will be particularly vulnerable to \(\beta\) elimination (Rose, 1981). This would lead to enolpyruvate formation at the catalytic site.

On the other hand, if the aci-carbanion intermediate were to escape from the active site, it would \(\beta\) eliminate very readily in solution. This is because alkaline hydrolysis of 3-keto-carboxyarabinitol-P\(_2\) yields predominantly pyruvate from the "upper" three carbons (Lorimer et al., 1986). Perhaps the active site cannot afford to bind the aci-carbanion very tightly because of its resemblance to the product, P-glycerate. Since P-glycerate must be released rapidly before the next cycle of catalysis can commence, an active site conformation which bound the aci-carbanion tight enough to prevent any significant release might be susceptible to severe product inhibition by P-glycerate.

The constancy of the fraction of product partitioned to pyruvate between pH 6.4 and 9.1 (Table I) could be interpreted to mean that the acidic group (BH in Scheme 2) which donates the proton which becomes attached to C-2 of P-glycerate does not change its ionization state significantly in this pH range. This would probably eliminate carboxyl groups and the hydroxyl of the aci-carbanion's phosphate group from consideration, as well as the \(\alpha\)-amino groups of the catalytically essential lysines, 354 and 175. Lysine 354 of carbamylated spinach ribulose-P\(_2\) carboxylase has a \(pK_c\) of 9.0 and the \(pK_c\) of lysine 166 of carbamylated \textit{Rhodospirillum rubrum} ribulose-P\(_2\) carboxylase (analogous to lysine 175 of the spinach enzyme) is 7.9 (Hartman et al., 1985). However, there is a caveat that must be attached to this reasoning. These conclusions would be invalid if a proton-abstracting basic group with a \(pK_c\) similar to that of the proton donor was instrumental in producing the aci-carbanion (e.g. by abstracting a proton from one of the hydroxyl groups attached to C-3.
of the gemdiol form of 3-keto-carboxyarabinitol-P₂ to initiate cleavage of the C-2/C-3 bond). In this circumstance, both the production of the aci-carbanion and its protonation would be affected similarly by pH variation with little effect on the steady-state level of the aci-carbanion.

It is particularly noteworthy that the ratio between pyruvate and P-glycerate production (in H₂O at 25 °C) is also invariant in the face of varying CO₂ concentration, with different divalent metal ions at the active site, and with ribulose-P₂ carboxylases from different sources with widely differing kinetic properties and turnover rates (Table I). All of these variables, in addition to pH, will affect the rate of catalytic throughput and, therefore, the rate of aci-carbanion formation. So it is clear that partitioning between pyruvate and P-glycerate as products is unaffected by the rate of aci-carbanion formation. One suspects that there may be some intrinsic mechanistic reason for the singular constancy in the relative rates of β elimination and protonation of the aci-carbanion. β elimination is a unimolecular reaction not requiring addition of other atoms from the enzyme or solvent, and this may confer on this reaction some degree of indifference to conditions within the active site. But the protonation reaction must be similarly indifferent to changes in the active site which might be expected when pH is varied or when different metals are present or between the active sites of different ribulose-P₂ carboxylases. In particular, the reactivity of the proton donor seems to be remarkably constant. This might be easier to understand if the proton was donated by a water molecule, rather than by an acidic group on the enzyme.

It is possible to alter the partitioning ratio towards pyruvate, however, and we discovered two ways of doing this. First, the amount of pyruvate formed by this abortive elimination is a unimolecular reaction not requiring processes in the chloroplast, such as fatty acid and amino acid synthesis (Schulze-Siebert et al., 1984). Moreover, pyruvate production by ribulose-P₂ carboxylase provides a direct route to pyruvate within the chloroplast which bypasses the reaction catalyzed by phosphoglycerate mutase, whose presence in the chloroplast has been questioned (Stitt and ap Rees, 1979). Direct incorporation of ¹²C₀₂ into pyruvate also provides a ready explanation for the rapidity with which pyruvate and its transamination product alanine become carboxyl-labeled from ¹⁴C₀₂ in the leaves of some species (Kennedy and Laetsch, 1974). Since pyruvate is not inhibitory and is readily and usefuly metabolized, there might be little selective advantage for ribulose-P₂ carboxylase to evolve a more potent mechanism for protonating its aci-carbanion intermediate in order to suppress pyruvate formation totally.

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