Examination of the Function of Active Site Lysine 329 of Ribulose-bisphosphate Carboxylase/Oxygenase as Revealed by the Proton Exchange Reaction*

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Diverse approaches that include site-directed mutagenesis have indicated a catalytic role of Lys-329 of ribulosebisphosphate carboxylase/oxygenase from Rhodospirillum rubrum. To determine whether Lys-329 is required for the initial enolization of ribulose bisphosphate or for some subsequent step in the overall reaction pathway, the competence of position 329 mutant proteins (devoid of carboxylase activity) in catalyzing exchange of solvent protons with the C-3 proton of substrate has now been examined. Irrespective of the amino acid substitution for Lys-329, the mutant protein retains 2–6% of the wild-type activity in the proton exchange reaction. The complete stability of ribulose bisphosphate during the enolization catalyzed by mutant protein suggests that the major effect of Lys-329 is to facilitate the addition of gaseous substrates (CO2 or O2) to the enediol intermediate. The exchange reaction requires Mg2+, is CO2-dependent, and is inhibited by the transition-state analogue 2-carboxyarabinitol 1,5-bisphosphate. A mutant protein in which Lys-191, the site for carbamylation by CO2 in an obligatory activation step, is replaced by a cysteinyl residue totally lacks proton exchange activity. Barely detectable exchange activity (~0.2% of wild-type) is displayed by the Lys-166 → Cys mutant protein, consistent with the previously implicated role of Lys-166 in the deprotonation of ribulose bisphosphate. Retention of exchange activity by the Glu-48 → Gin mutant protein, which is slightly active in overall carboxylation, demonstrates that active site Glu-48, like Lys-329, exerts its major effect at some step subsequent to the initial enolization.

Ribulosebisphosphate carboxylase/oxygenase (EC 4.1.1.39) exhibits bifunctionality in catalyzing the competing reactions of ribulose-P3 with atmospheric CO2 or O2. These reactions are the first steps in photosynthetic carbon fixation and energy-wasteful photorespiration, respectively, and reflect the partitioning of a common reaction intermediate, 2,3-enediol of ribulose-P2 (1, 2). In recent years, the structure, mechanism, and regulation of the carboxylase have been under intense study in numerous laboratories, because of its role in plant yields and because modulation of its substrate specificity may provide a means for enhancing plant yields (for a particularly insightful review, see Ref. 3).

Both activities are dependent on a unique activation process in which the ε-amino group of Lys-191 condenses with CO2 yielding a carbamate that is stabilized by catalytically essential Mg2+ (4, 5). The carbamate may be detected and quantified as a component of an exchange-inert quaternary complex formed when the transition-state analogue carboxyarabinitol-P2 binds to the activated enzyme (6).

Subsequent to carbamylation of the enzyme, the initial catalytic step is abstraction of the C-3 proton of ribulose-P2 to generate the corresponding 2,3-enediol. Carboxylase or oxygenase activity then reflects whether CO2 or O2 reacts with the enediol (7). In the carboxylation pathway, reaction of CO2 with the 2,3-enediol gives rise to 2-carboxy-3-keto-D-arabinitol 1,5-bisphosphate (7). This six-carbon reaction intermediate undergoes hydration and carbon-carbon scission to liberate one molecule of PGA derived from C-3, C-4, and C-5 of ribulose-P2. The other product of the scission reaction, a carbanion derived from CO2 and C-1 and C-2 of ribulose-P2, must then undergo inversion and protonation to form the second molecule of PGA (for a more detailed description of the overall reaction pathway, see Ref. 3). Among these partial reactions, the enolization of ribulose-P2 and the formation of PGA from the six-carbon intermediate may be assayed independently of the overall carboxylation reaction (7, 8). Enolization is monitored by the exchange of solvent protons with the C-3 proton of ribulose-P2; with six-carbon intermediate, labeled with 3H in the carbonyl group, its conversion to PGA is followed as an increase in acid-stable radioactivity.

The dissection of partial reactions, if catalyzed by site-directed mutant proteins devoid of overall carboxylation activity, provides an avenue for ascribing the involvement of active-site residues to discrete steps. For example, K166G (despite lacking detectable carboxylase or oxygenase activity) undergoes carbamylation and catalyzes the hydrolysis of the six-carbon intermediate; it does not, however, catalyze the enolization reaction (9). These observations provided direct support for the earlier suggestion (10) that the ε-amino group of active-site Lys-166 serves as a base required for the enolization of ribulose-P2.

2 Residue numbers refer to the R. rubrum carboxylase unless noted otherwise.

3 Mutant proteins are designated with one-letter abbreviations of amino acids. The first residue name is that found in the wild-type enzyme; the number identifies its location in the polypeptide chain.
Lys-329 is another active site residue of ribulose-P₇ carboxylase that has also been probed by site-directed mutagenesis (11). Irrespective of the nature of the amino acid replacement, each of the mutant proteins lacked enzymic activity. The ability of these mutant proteins to bind phosphorylated ligands suggested a role of Lys-329 in catalysis rather than in substrate binding. Conversion of Lys-329 to aminoethylcysteine (mutant K329C) yielded an active enzyme displaying a 4-fold reduced kₘₐₜₜₛ but normal Kₐ values relative to those of wild-type enzyme (12). Thus, the consequences of introducing amino acid substitutions for Lys-329 did not appear to reflect indirect conformational effects but rather the removal of a group involved in catalysis.

If the assignment of Lys-166 to the initial enolization is correct, the participation of Lys-329 likely occurs at some subsequent step in the reaction pathway. Position 329 mutant proteins might then retain the ability to catalyze the enolization of ribulose-P₂. Confirmation of this postulate is provided by the present study, in which characteristics and requirements for enolization are also described.

**EXPERIMENTAL PROCEDURES**

**Materials**—Commonly used chemicals and reagents were purchased at the highest level of purity readily available. Other commercial materials and vendors were as follows: Bicine, PEP, PEP carboxylase (maize), and malate dehydrogenase (bovine heart) from Sigma; tritiated water (6 Ci/ml) from ICN Radiochemicals. Ribulose-P₂ and carboxyarabinitol-P₇ were prepared according to published procedures (13, 14). [3-³H]Ribulose-P₂ (specific activity = 11,000 cpm/nmol) was kindly provided by Dr. George H. Lormier of DuPont or prepared by incubation of ribulose-P₂ in tritiated water in the presence of K329C, which catalyzes exchange between the C-3 proton and solvent protons, and resolution of the labeled ribulose-P₂. Details of this procedure will be published elsewhere.

Wild-type ribulose-P₂ carboxylase from Rhodospirillum rubrum and its site-directed mutants cloned in Escherichia coli were purified as described earlier (13, 16) and stored at -70 °C as concentrated stocks (10–70 mg/ml) in a pH 8.0 buffer consisting of 50 mM Bicine, 86 mM NaHCO₃, 10 mM MgCl₂, 1 mM EDTA, and 10–20% (w/v) glycerol. As needed, aliquots of the stocks were dialyzed at room temperature against storage buffer lacking glycerol. Protein concentrations were based on absorbancies at 280 nm and an ε₁⁰₀ of 120. (15). The wild-type enzyme had a specific activity of 4–5 units/mg in the direct fixation assay.

**Exchange of Solvent Tritium with the C-3 Proton of Ribulose-P₂**—The assay procedure was that described by Saver and Knowles (18). Reaction mixtures (final volumes = 200 µl) at pH 8.0 and 23°C contained 20 µCi of tritiated water, 3 mM Bicine, 10 mM MgCl₂, 50 mM Bicine, 10 mM NaHCO₃, 1 mM EDTA, and 5–300 µg of wild-type or mutant carboxylase (the amount depending on the activity level), which was added last. In those cases in which the exchange reaction was to be examined, the assay solution containing the enzyme was depleted of 300–500 µM [³H]ribulose-P₂ (specific activity ~11,000 cpm/nmol). All assays were conducted at the same ionic strength by appropriate adjustments with NaCl. Reactions were initiated with either ribulose-P₂ or enzyme (2–5 µg of wild-type or 20–50 µg of mutant). Periodically, aliquots (20 µl) were quenched by diluting into 100 µl of 75 mM NaBH₄ contained in scintillation vials; the excess borohydride was decomposed 5 min later with 500 µl of 2 M acetic acid. Samples were dried in an oven at 110°C for 20 min; residues were dissolved in 500 µl of H₂O and, after the addition of 10 µl of scintillant (ACS, Amersham Corp.), counted. In those instances in which the exchange reaction was to be examined in the absence of CO₂, the following components were introduced into the assay solution 45 min prior to initiation: PEP carboxylase (200 units), malate dehydrogenase (5 units), NADH (3 mM), and PEP (3 mM). Based on the monitoring of NADH oxidation in a separate control, which was identical to the exchange reaction assay solution including 3 mM ribulose-P₂ but lacking any form of ribulose-P₂ carboxylase, the consumption of endogenous CO₂/H₂O (300–500 µM) was complete within 90 s. Additional 500 µM NaHCO₃ subsequently added to the same solution was also rapidly consumed, demonstrating the efficiency and excess capacity of the CO₂ sink.

**RESULTS**

**General Features of Exchange of Solvent Tritium with the C-3 Proton of Ribulose-P₂ as Catalyzed by K329C**—When ribulose-P₂ is incubated with activated wild-type enzyme in the presence of tritiated water, HCO₃⁻, and Mg⁷⁺, the resolubilated substrate (prior to its complete conversion to PGA) contains tritium at C-3, reflecting protonation of the enediol intermediate (18). This observation is reproduced by the data illustrated in Fig. 1A. Before the addition of enzyme, chromatography on DEAE-cellulose of a solution of ribulose-P₂, subsequent to borohydride reduction for stabilization as the corresponding pentitol-P₂, does not reveal any radioactivity emerging at the positions for phosphate esters. (The labeled water that remains after lyophilization is eluted during the extensive washing prior to initiation of the gradient and is not shown). Ten min after addition of the wild-type enzyme, the processed reaction mixture contains labeled PGA and labeled pentitol-P₂. The carboxylation of ribulose-P₂ is completed within 45 min, and a single peak of labeled PGA is observed. The specific radioactivity of this PGA is about one-sixth that of solvents protons, in agreement with the reported isotope effect for the terminal proton transfer step (18).

As illustrated in Fig. 1B, K329C also catalyzes exchange of solvent protons into ribulose-P₂. Note even a trace of PGA can be detected, consistent with the earlier claim, based on conventional assays with [¹⁴C]CO₂, that position 329 mutant proteins lack carboxylase activity (11). Omission of bicarbonate from the reaction mixture results in only modest reduction of tritium incorporation. The exchange reaction requires Mg⁷⁺, as in the case of wild-type enzyme (18), and is inhibited by the transition-state analogue carboxyarabinitol-P₂. The enhanced labeling of ribulose-P₂ (Fig. 1B) compared to that of PGA at completion of the reaction depicted in Fig. 1A suggests that ribulose-P₂ fully equilibrates with solvent due to repetitive deprotonation/reprotonation.

Even if the exchange reaction as catalyzed by K329C is allowed to proceed for 6 h (the data in Fig. 1B represents 1 h incubation), the ribulose-P₂ concentration, as measured with wild-type carboxylase, remains unchanged.

By analogy with wild-type carboxylase, the incorporation of solvent tritium into ribulose-P₂ during incubation with K329C presumably entails exchange of the C-3 proton. The experiment shown in Fig. 1C confirms this expectation. After incubation of ribulose-P₂ with mutant protein in tritiated water for 3 h (other conditions as described for Fig. 1, A and B), wild-type enzyme is introduced. Subsequent chromatography reveals PGA as the only radioactive product. Hence, all of the radioactivity associated with the pentitol-P₂ peak in
buffered at pH 8.0 and included 66 mM NaHCO₃ and 10 mM MgCl₂, and were quenched at 10 min.

Other two samples contained the wild-type carboxylase at 45 pg/ml unless stated otherwise. Prior to chromatography, the reaction mixtures were treated with borohydride, thereby converting ribulose-P₂ to pentitol-P₂ and lyophilized to remove most of the labeled water. In A, one sample was processed that lacked the carboxylase (C). The other two samples contained the wild-type carboxylase at 45 μg/ml and were quenched at 10 min (●) and 45 min (○), respectively. In B, each sample contained K329C at 200 pg/ml, and the incubation period was for 1 h. Ligand compositions were as follows: no other additions (●), 50 μM carboxyarabinitol-P₂ (○), minus MgCl₂ (□), minus NaHCO₃ (△). The profile shown in C represents a sample in which ribulose-P₂ was first incubated with K329C (200 μg/ml) for 3 h at which time wild-type carboxylase (1.5 mg/ml) was added; the reaction was quenched 3 h later. Additional details are described under "Experimental Procedures."

Fig. 1B must have reflected ribulose-P₂ rather than a derived product or contaminant; otherwise, some radioactivity would have remained at the bisphosphate region in Fig. 1C. Furthermore, the peak area for PGA in Fig. 1C matches that of the end-point sample in Fig. 1A rather than the much larger peak area for pentitol-P₂ in Fig. 1B. This pattern is entirely consistent with the fact that the C-3 hydrogen (or titrnum) is lost to solvent during the catalytic turnover of ribulose-P₂ to PGA. Thus, the degree of labeling of PGA when the carboxylase reaction is carried out in tritiated water must be the same whether [3-3H]ribulose-P₂ or unlabeled ribulose-P₂ is used.

The time course of the exchange reaction catalyzed by K329C, under the same conditions as those for the experiments summarized by Fig. 1, is shown in Fig. 2. The specific radioactivity of ribulose-P₂ reaches a maximum of ~720 cpm/nmol, which is ~53% that of the solvent (~1360 cpm/nmol ³H₂O). Thus, complete equilibration is reached with an indication of a small equilibrium isotope effect.

![Fig. 1. DEAE chromatography of samples of ribulose-P₂ (3 mM) incubated in tritiated water in the presence of wild-type carboxylase (A), K329C (B), or both proteins (C). Samples were buffered at pH 8.0 and included 66 mM NaHCO₃ and 10 mM MgCl₂, unless stated otherwise. Prior to chromatography, the reaction mixtures were treated with borohydride, thereby converting ribulose-P₂ to pentitol-P₂ and lyophilized to remove most of the labeled water. In A, one sample was processed that lacked the carboxylase (C). The other two samples contained the wild-type carboxylase at 45 μg/ml and were quenched at 10 min (●) and 45 min (○), respectively. In B, each sample contained K329C at 200 pg/ml, and the incubation period was for 1 h. Ligand compositions were as follows: no other additions (●), 50 μM carboxyarabinitol-P₂ (○), minus MgCl₂ (□), minus NaHCO₃ (△). The profile shown in C represents a sample in which ribulose-P₂ was first incubated with K329C (200 μg/ml) for 3 h at which time wild-type carboxylase (1.5 mg/ml) was added; the reaction was quenched 3 h later. Additional details are described under "Experimental Procedures."](image1)

![Fig. 2. Time dependence of exchange of solvent tritium with the C-3 proton of ribulose-P₂ as catalyzed by K329C. Ribulose-P₂ was incubated with K329C under conditions described in the legend to Fig. 1B. Periodically, samples were quenched and analyzed by DEAE chromatography as described under "Experimental Procedures."](image2)
FIG. 3. Rates of deprotonation of [3-3H]ribulose-P₂ as catalyzed by mutant (A and B) and wild-type carboxylase (C). In all of these experiments, the final concentration of ribulose-P₂ was 0.4 mM; unless otherwise noted, stock solutions of proteins had been dialyzed against buffer containing 66 mM NaHCO₃ and 10 mM MgCl₂ to ensure complete carbamylation, assay solutions contained these same high levels of NaHCO₃/MgCl₂, and reactions were initiated by the addition of enzyme. Sample descriptions follow: A, K329C (150 μg/ml) (○); K329C (150 μg/ml) which had been dialyzed against buffer lacking NaHCO₃/MgCl₂ and was thus non-carbamylated (△); K166C (1.5 mg/ml) (□); K166C (1.5 mg/ml) (●); control lacking any carboxylase (□). B, K329C (150 μg/ml) (○); K329C (150 μg/ml) without added bicarbonate but with the PEP carboxylase system in the assay solution (△); K329C (150 μg/ml) without added bicarbonate but with the phosphoenolpyruvate carboxylase system in the assay solution (◇); K329C (150 μg/ml) without added bicarbonate but with the PEP carboxylase system in the assay solution (○); K329C (150 μg/ml) without added bicarbonate but with the PEP carboxylase system in the assay solution (○); K329C (150 μg/ml) without added bicarbonate but with the PEP carboxylase system in the assay solution (○). The insets in B and C show the specific activity (units/mg) as a function of final bicarbonate concentrations in the assay solutions. The open symbols at zero bicarbonate represent the assays conducted in the presence of the PEP carboxylase system with reactions initiated with ribulose-P₂. Additional details are described under "Experimental Procedures."

Table 1
Relative overall carboxylase and exchange activities of mutant carboxylases

| Protein       | Carboxylase activity | Exchange activity |
|---------------|----------------------|-------------------|
| Wild-type     | 100                  | 100               |
| K329C        | <0.01                | 4–5               |
| K329G        | <0.01                | 2–3               |
| K329E        | <0.01                | 2–3               |
| K329R        | <0.01                | 2–3               |
| K329Q        | <0.01                | 4–5               |
| K329A        | <0.01                | 5–6               |
| K329S        | <0.01                | 5–6               |
| K191C        | <0.001               | <0.05             |
| K166C        | <0.01                | 0.15–0.25         |
| K166G        | <0.001               | 0.05–0.08         |
| E48Q         | 0.05                 | 1–2               |

The evaluation of position 329 mutants of the carboxylase as catalysts for the enolization of ribulose-P₂ is instructive in several ways. First, the retention of this activity by proteins that are devoid of carboxylase activity demonstrates that enolization can proceed independently of the other partial reactions in the overall carboxylase pathway. Comparisons of the enolization activity possessed by different mutant carboxylases containing single amino acid substitutions for various active site residues should then reveal which side chains are necessary for the initial proton abstraction step. Second, the demonstration that position 329 mutant proteins are indeed enzymes, albeit not carboxylases, which act on ribulose-P₂ provides evidence of an active-site topology similar to that of the wild-type enzyme. Apparently, the catalytic deficiency of these mutant proteins is due to the absence of a catalytic ε-amino group rather than to indirect conformational changes. Third, the absence of dephosphorylation, isomerization, and/or epimerization of ribulose-P₂ during proton exchange indicates that the enediol never dissociates from the enzyme. Future rapid quench experiments should provide the equilibrium constant between enzyme-bound ribulose-P₂ and the corresponding enediol. Fourth, lack of addition of either gaseous substrate to the enediol generated by the position 329 mutant suggest that with wild-type enzyme neither carbox-
ylation nor oxygenation is spontaneous but require direct participation by amino acid side chains.

To elaborate on the last assertion in the preceding paragraph, the carboxylase-catalyzed reaction proceeds by a Theorell-Chance type of kinetic mechanism: ordered addition and enolization of ribulose-P₂ followed by bimolecular reaction of the enediol with gaseous substrate (1, 2). Evidence in favor of this ordered, sequential reaction includes NMR, direct binding, isotope trapping, and kinetic analyses indicating that the gaseous substrates do not bind to free enzyme or to enzyme-ribulose-P₂. Earlier speculation (24) that the universal oxygenase activity of ribulose-P₂ carboxylases reflects an unavoidable consequence of the inherent reactivity of the enediol is certainly compatible with the elucidated kinetic mechanism. Carboxylation or oxygenation of the enediol could then be viewed as nonenzymic. However, the enolization of ribulose-P₂, as catalyzed by K329C, without concomitant carboxylation or oxygenation (oxygen was never excluded from the incubations), argues that the enzyme plays an active role in facilitating the addition of gaseous substrate to enediol. One possibility is that Lys-329 is needed to enhance the reactivity of the enediol through polarization and development of the nucleophilic center at C-2.

Initial characterizations (11) of position 329 mutant carboxylases were consistent with a role of Lys-329 in catalysis. However, the failure of these mutant proteins, in the presence of bicarbonate and Mg²⁺, to form a stable complex with the transition-state analogue carboxyarabinitol-P₂ did not preclude the possibility of an indirect role in the activation process that entails carbamylation of Lys-191. Present observations that the maximal proton exchange activity displayed by K329C requires both CO₂ and Mg²⁺ argue that it does undergo normal activation chemistry and that the absence of carbamylation activity must be reflective of a function of Lys-329 in catalysis. Based on the increased $k_{cat}$ for ribulose-P₂ exhibited by K329C (180 μM) in contrast to the wild-type enzyme (11 μM), the previously observed (11) lack of tight complexation of carboxyarabinitol-P₂ by position 329 mutants is probably due to reduced affinity for phosphorylated ligands in general.

In our preliminary report (25), we asserted that the non-carbamylated forms of both wild-type and position 329 mutant proteins exhibit inherent activity in the proton exchange reaction. In retrospect, this conclusion was unjustifiably dogmatic due to our failure to adequately deplete assay solutions of CO₂. By the use of PEP carboxylase to render assay solutions virtually CO₂-free, the observed rates of proton exchange with both wild-type and mutant enzymes can be reduced to 5% of their respective maximal values achieved at high bicarbonate concentrations. Whether residual exchange activities are inherent to the non-carbamylated enzyme or reflective of the difficulties of eliminating all CO₂ is unclear. However, the complete absence of exchange activity associated with K191C (Lys-191 is the site of carbamylation), even though this protein binds stoichiometric levels of CO₂ and displays high affinity for carboxyarabinitol-P₂, favors an obligatory role of carbamylation in enolization.

Clearly, Lys-329 is not required for the initial deprotonation of ribulose-P₂. Irrespective of the nature of the amino acid substitution at position 329, which include glycyl or alanyl residues, the resulting mutant proteins display apparent exchange activities 2-6% as great as that for the wild-type enzyme. The absolute rate of deprotonation of C-3 of ribulose-P₂ by the wild-type carboxylase as measured by NMR (1) is 2-3-fold greater than $k_{cat}$ for overall carboxylation; compared to this corrected value, the observed exchange activities of the mutant proteins are reduced to 0.7-3% of wild-type. However, these relative rates are uncorrected for the tritium isotope effect, which may approach the intrinsic kinetic tritium isotope effect as the only chemistry entails cleavage of a C-H (C-T) bond. The intrinsic tritium isotope effect for the R. rubrum carboxylase has been estimated as >2.6 (27) and 10-16 (2). If the higher value is correct and can be applied to the exchange reaction catalyzed by the mutant proteins, their true activity in this partial reaction is about 30% of wild-type enzyme.

Lys-329 must participate in catalysis at some step subsequent to the enolization of ribulose-P₂. As noted above, the apparent lack of addition of CO₂ or O₂ to the enediol of ribulose-P₂ generated by the position 329 mutants suggests that Lys-329 in the wild-type enzyme facilitates this step. Failure of ribulose-P₂ to be cleaved to products, despite its enolization, does not, however, provide conclusive proof that CO₂ or O₂ does not react with enediol. The position 329 mutants could be competent catalyzing reversible addition of gaseous substrates to enediol but incompetent in the conversion of the resultant intermediates to products. However, two observations are inconsistent with this possibility. In the overall carboxylase reaction, the six-carbon, carboxylated intermediate partitions almost exclusively in the forward direction to yield PGA, rather than regenerating enediol via decarboxylation (7). Also, if the deficiency of the position 329 mutant proteins were in the processing of six-carbon, carboxylated intermediate, HCO₂(OH) should inhibit the proton exchange reaction. In contrast, bicarbonate actually stimulates the proton exchange reaction. Thus, a more plausible argument can be made for the participation of Lys-329 in the reaction of enediol with CO₂ or O₂ rather than in some later step.

Although we have not thoroughly examined the exchange reaction as catalyzed E48Q, its level of activity is similar to that of the position 329 mutant proteins. Relative to wild-type enzyme, E48Q is only ~0.05% as active in overall carboxylase activity, but >1% (uncorrected for the tritium isotope effect) as active in enolization activity. Thus, as in the case of Lys-329, the major influence of Glu-48 appears to be at some step subsequent to the initial deprotonation of ribulose-P₂.

The recently published (28) 2.3-Å structure of activated spinach ribulose-P₂ carboxylase, complexed with carboxyarabinitol-P₂, reveals an intersubunit ionic bond between the active site residues Glu-60 and Lys-177 (Glu-48 and Lys-168 of the R. rubrum carboxylase). Therefore, disruption of subunit-subunit association upon replacing Lys-166 or Lys-168 of the R. rubrum carboxylase with an aspartyl or glutamyl residue, respectively, is understandable (29, 30). Lys-334 (which corresponds to Lys-329 of the R. rubrum enzyme) is within ionic bonding distance of the C-2 carbonylate of the bound transition-state analogue, a location compatible with the present postulate that the ε-amino group of Lys-329 might enhance the reactivity of the enediol of ribulose-P₂. However, Andersson et al. (28) include Lys-329 as a candidate for the base that enolizes ribulose-P₂; we believe that this possibility is unlikely due to the substantial enolization activity displayed by the position 329 mutant proteins. Furthermore, this lysyl residue has a pKₐ of 9.0 (10), whereas the base (irrespective of its identity) that abstracts the C-3 proton from ribulose-P₂ has a pKₐ of 7.5 (2). This latter pKₐ approximates that of Lys-166 of the R. rubrum carboxylase (corresponding to Lys-175
of the spinach enzyme); this fact, in conjunction with a wealth of chemical and mutagenesis data, which include lack of significant enolization activity by position 166 mutant proteins, provides rather compelling arguments for Lys-166 as a base that accelerates proton abstraction in the first step of the overall reaction. Some doubt is cast on this conclusion by the crystal structure, which shows the lysyl 175 ε-amino group closer to the C-1 oxygen than to the C-3 proton of ribulose-P₂. The authors state, however, that the structure of the enzyme-carboxyribosylphosphate-R₃ complex "simulates a later stage in the reaction sequence" rather than the initial enolization. This qualification should be taken quite seriously because of the major conformational differences between the deactivated (non-carbamylated) protein, the activated (carbamylated) protein, and the activated protein complexed with carboxyribosylphosphate-R₃. For example, in the 2.9Å structure of non-carbamylated R. rubrum enzyme (31), Lys-329 is far removed from Lys-191 (the carbamylation site) and probably would not even be classified as an active site residue (precise distances cannot be stated because side chains were not fitted to electron densities and a short segment of α-carbon backbone including Lys-329 was ill-defined). Also, whereas both the enzyme-carboxyribosylphosphate-R₃ structure (29) and the non-carbamylated tobacco enzyme structure (32) confirm an intersubunit location of the active site comprised of side chains from adjacent subunits, as first discovered by hybridization of site-directed mutant proteins (22), the earlier structure of non-carbamylated R. rubrum enzyme prompted a somewhat different conclusion. The low kₑₐ₄ for the conversion of the six carbon, carboxylated reaction intermediate to PGA in comparison to the overall kₑₐ₄ for carboxylation of ribulose-P₂ has also been interpreted to reflect conformational differences between carbamylated enzyme and carbamylated enzyme complexed with carboxyribosylphosphate-R₃ (7). Given the multiple conformational states of ribulose-P₂ carbosylyase, the translation of structural, chemical, and kinetic observations into plausible mechanistic inferences remains especially challenging.

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