Functional Analysis of the Putative Catalytic Bases His-321 and Ser-368 of *Rhodospirillum rubrum* Ribulose Bisphosphate Carboxylase/Oxygenase by Site-directed Mutagenesis*

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Numerous candidates have been suggested according to chemical and structural criteria for the active site base of ribulose bisphosphate carboxylase/oxygenase that catalyzes substrate enolization. We evaluate the functional significance of two such candidates, His-321 and Ser-368 of the *Rhodospirillum rubrum* enzyme, by site-directed mutagenesis. Position 321 mutants retain 3–12% of wild-type rates of both overall carboxylation and the initial enolization, with little effect on *Kₐ* for CO₂ or ribulose bisphosphate. Position 368 mutants exhibit ~1% of wild-type carboxylation but 4–9% of enolization, also accompanied by little effect on *Kₐ* values. The modest catalytic facilitations elicited by these residues are incompatible with either acting as the crucial base. The enhanced efficiency of the position 368 mutants in enolization versus carboxylation clearly indicates that Ser-368 effects catalysis preferentially beyond the point of proton abstraction. Both sets of mutants bind the reaction intermediate analogue, 2-carboxy-D-arabinitol bisphosphate, stoichiometrically. Ligand exchange from complexes with position 321 mutants is increased relative to wild type, whereas complexes with position 368 mutants are more exchange-inert. Therefore, His-321 may assist stabilization of the transition state mimicked by the analogue.

Ribulose bisphosphate carboxylase/oxygenase (EC 4.1.1.39) catalyzes the carboxylation of ribulose-P₄ by atmospheric CO₂ to yield two molecules of PGA. This process is comprised of several distinct partial reactions (1–3): formation of the 2,3-enediol(ate) of ribulose-P₄, by C3 proton abstraction, reaction of the activated enediol(ate) intermediate with CO₂ at C2 and with H₂O at C3 (either successively or in a concerted fashion (4)), C2–C3 bond cleavage to liberate one molecule of PGA (derived from C3, C4, and C5 of ribulose-P₄), and inversion of configuration and protonation of the remaining terminal aci-acid to form the second molecule of PGA (derived from CO₂ and C1 and C2 of ribulose-P₄). The activated enediol(ate) intermediate may also react with molecular oxygen, which leads to oxygenolytic cleavage of substrate and diversion of cellular carbon into energetically wasteful photorespiration (5, 8). In contrast to the limited understanding of protein determinants that distinguish between the two gaseous substrates (7, 8), the involvement of several acid/base groups at the active site has been invoked on sound chemical grounds to fulfill the minimal requirements of the overall reaction pathway (3). Diverse approaches have identified such groups and have provided insights into some of their precise functions (for reviews, see Refs. 9 and 10 and the citations therein). However, the identity of the base responsible for abstracting the C3 proton of the substrate to form the enediol(ate) intermediate common to both the carboxylation and oxygenation pathways remains an unresolved issue.

Despite a wealth of chemical, mutagenic, and kinetic evidence supporting Lys-166" of the *Rhodospirillum rubrum* ribulose-P₄ carboxylase (and, by analogy, Lys-175 of the spinach enzyme) as the base that mediates the initial enolization step (11–13), crystallographic studies of both the *R. rubrum* and spinach enzymes place this conserved residue too far from the C3 proton of substrate for such a role (14, 15). Although no other amino acid side chain has, in fact, been found that fulfills this structural criterion in any of the crystallographic structures of the carboxylase yet determined (a limitation due, in part, to the ill-defined orientation of bound ligands (14, 15)), a number of other candidates have been offered (14–16). These include His-321 and Ser-368, which are evolutionarily conserved amino acids between the simpler dimeric (Ld) form of bacterial carboxylases and the more complex hexadecameric (LsSs) form of plant, algal, and cyanobacterial carboxylases. In the x-ray structure of the spinach ribulose-P₄ carboxylase complexed with the reaction intermediate analogue carboxyarabinitol-P₃, His-327 (His-321 of the *Rubus ulmarius* enzyme) is a ligand for one of the phosphate groups, and Ser-379 (Ser-368 of the *R. rubrum enzyme*) forms a hydrogen bond with the C3 hydroxyl group of the bound analogue (Fig. 1) (14). The closest approach of an imidazole nitrogen of His-327 to C3 of carboxyarabinitol-P₃ is ~5.1 Å, whereas the hydroxyl group of Ser-379 at ~3.7 Å is the closest of any side chain with the potential to mediate proton transfer. These two residues also engage each other as part of an extensive hydrogen-bonding network conserved in LsSs forms of the carboxylase that ultimately leads to a buried glutamyl residue. However, the absence of several members of this hydrogen-bonding chain in the *R. rubrum* enzyme indicates that the entire network is not crucial to carboxylase activity. Recently,
designations for the various mutant proteins (in parentheses) and mutagenic primers are as follows.3

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pFL243 (H321S): 5' - pAGCGCATCAGACCGACC
pFL248 (H321N): 5' - pAGCGCATCAGACCGACC
pFL252 (H321A): 5' - pAGCGCATCAGACCGACC
pFL253 (H321Q): 5' - pAGCGCATCAGACCGACC
pFL321 (H321R): 5' - pCCGATCTGGCCATG
pFL322 (S368A): 5' - pCCGATCTGGCCATG
pFL332 (S368C): 5' - pCCGATCTGGCCATG
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The underlined bases correspond to the normal codon for His-321 (CAC) or Ser-368 (AGC). Mutations were verified by dyelex sequencing (24) of single-stranded template across the mutation site using oligonucleotides (5'-GCCCTGTCCATTG for position 321, and 5'-TTCTACCCTGAATCC for position 368) that prime 39 and 36 bases upstream from the sites of mutation, respectively.

**Protein and Activity Assays—** Carboxylase activity was assayed by a modified radiometric filter disk assay (25). Assays were routinely performed at ambient temperature in a buffer (pH 8.0) containing 50 mM Bicine, 10 mM MgCl₂, 1 mM EDTA, 25 mM NaHCO₃ (~8 mM), and either 400 or 1000 µM ribulose-1-P. Enzymes were preincubated at room temperature (15-30 min) in the same buffer lacking ribulose-1-P to ensure full activation (spontaneous carbamylation of Lys-191 and coordination of active site Mg²⁺ (26)). The reactions were initiated by addition of ribulose-1-P. Aliquots were periodically applied to trifluoracetic acid-soaked filter disks, which were then dried and counted by liquid scintillation (Eco-Lite liquid scintillant, ICN). The Kₚ values for ribulose-1-P were determined by varying its concentration from 18 to 320 µM at a fixed bicarbonate concentration of 25 mM. Bicarbonate concentration was varied from 10 to 100 mM (~5 mM) for the determination of its Kₚ values while holding the ribulose-P₂ concentration constant at 1 mM. In these latter experiments, bovine erythrocyte carbonic anhydrase (Sigma) was added at 0.1 mM just prior to initiation of reaction; the ionic strength was maintained at a constant level by suitable additions of NaCl.

The enolization partial reaction was assayed by monitoring the enzyme-catalyzed detrinitiation of [3-³H]ribulose-P₂ under conditions used above for carboxylase assays (18, 27). Despite a lack of rate dependence upon bicarbonate concentration by the mutant enzymes studied here (data not shown), 66 mM NaHCO₃ (unlabeled) was used to ensure full activation (i.e. carbamylation) throughout the duration of the assays. Reactions were initiated by the addition of [3-¹H] ribulose-P₂ (2 mM, 0.2 mCi/mmol); periodically, 15-µl aliquots were quenched by dilution with 100 µl of freshly prepared 100 mM NaBH₄. Quenched samples were evaporated to dryness and subjected to scintillation counting.

Stabilities of the quaternary complexes of the enzymes with carboxyarabinitol-P₂ were determined by a gel filtration procedure (11, 28). The complexes were formed by incubation of enzyme (10-15 µM) with ¹C-labelled inhibitor (100 µM) for 1 h. Exchange of bound ligand was carried out by adding unlabeled ligand (1 mM) to these incubations and periodically subjecting portions to gel filtration.

Protein concentrations were determined by the use of Bradford's (29) reagent obtained from Bio-Rad. Pure ribulose-P₂ carboxylase isolated from _R. rubrum_ was used as the standard.

**Enzyme Purifications—all buffers used during purifications contained 10% glycerol. Cell-free extracts were prepared by passing cell suspensions (1 g of cell paste/2 ml of pH 8.0 activation buffer (50 mM Bicine, 10 mM MgCl₂, 1 mM EDTA, and 66 mM NaHCO₃) that contained 1 µM leupeptin and 1 mM phenylmethylsulfonyl fluoride) three times through a French pressure cell (Aminco) at 1000 psi. The mutant enzymes were purified by a modification of a fast protein liquid chromatography protocol that utilizes two consecutive MonoQ (Pharmacia LKB Biotechnology Inc.) anion exchange chromatographic steps (30). Carboxylase-containing fractions were located by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Pharmacia PhastSystem) or by assays for catalytic activity when practical. To process a larger amount of cell paste (5-10 g) than

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3 The single-letter code is used to describe mutant proteins. The first letter denotes the amino acid present in the wild-type enzyme at the numbered position. The final letter denotes the amino acid present at the corresponding position in the mutant protein.
previously (1-3 g), an isocratic ion exchange separation (1 ml of DEAE Fast Flow Sepharose (Pharmacia) per 1 ml of cell-free extract) was carried out prior to chromatography on MonoQ. The extract was diluted 1:1 with additional extraction buffer and applied to the DEAE-Sepharose column, which was washed with two bed volumes of activation buffer followed by two bed volumes of activation buffer containing 20 mM NaCl. The carboxylase was then eluted with the same buffer containing 150 mM NaCl and concentrated to <5 ml by ultrafiltration (Amicon Centriprep-30). Prior to application to the first MonoQ column, the concentrate was diluted to 10 ml with a low ionic strength, pH 8.0 buffer (5 mM Bicine, 5 mM MgCl₂, 0.5 mM EDTA, and 55 mM NaHCO₃) and filtered (Millex-HA 0.45 μm filter, Millipore). The first MonoQ column (HR 10/10) was eluted with a NaCl gradient and the second (HR 10/10) with a Tris-citrate gradient, as described previously (30). The pooled fractions of purified carboxylase obtained from the second MonoQ column were concentrated (Amicon Centricron-30) and dialyzed against activation buffer containing 20% glycerol. The dialyzed enzyme solutions were frozen in liquid N₂ and stored at ~80 °C. Wild-type carboxylase was isolated from *R. rubrum* (31).

**RESULTS**

**Expression, Purification, and Structural Integrity of Mutant Carboxylases**—The levels of mutant carboxylases in cell-free extracts of *E. coli* transformed by pFL200- or pFL245-derived constructs were observed to be 5-10% of the total soluble protein. These levels are comparable to those of wild-type carboxylase produced from these vectors (20). The mutant enzymes were purified to 80-90% homogeneity as determined by both non-denaturing and denaturing electrophoresis (Fig. 2; data not shown for position 368 mutants). All of the mutant enzymes comigrate with wild-type enzyme during electrophoresis under denaturing conditions, indicating that full-length gene products are produced. Mobilities identical to that of wild-type carboxylase during non-denaturing electrophoresis (Fig. 2B; data not shown for position 368 mutants) indicate that all of the mutant carboxylases form stable dimers like the wild-type enzyme.

**Kinetic Parameters (Table I)**—With the exception of the Lys and Arg substitutions, the position 321 mutants exhibit substantial carboxylase activities (3-12% of wild-type *kcat*). The apparent *Km* values for ribulose-P₂ are only moderately perturbed (<10-fold increased), indicating that the side chain at position 321 has only minor effect upon the productive binding of substrate. The comparative *kcat/Km* values illustrate that a 50-100-fold reduction of catalytic efficiency in the carboxylation reaction accompanies substitution of His-321. Among this series of mutant proteins, the rather conservative Asn and Gln substitutions exhibit the higher levels of activity.

The extremely low activities of the mutant proteins with Arg and Lys at position 321 (~0.005%) approach the lower limits for quantification of carboxylase activity. The rates of ribulose-P₂-dependent ¹⁴CO₂ fixation catalyzed by these mutants are inhibited by low concentrations (1.5-fold excess over active site) of carboxyarabinitol-P₂ (data not shown), demonstrating that the incorporation of radioactivity reflects true carboxylase activity. Precise *Km* values of H321K and H321R for ribulose-P₂ could not be determined because of the high concentration of these proteins (>10-20 μM active site) required to reliably measure carboxylation rates. However, a *Km* ~50 μM was estimated in each case. The inherently low activities of the H321K and H321R mutant enzymes apparently reflect conformational disruption (see below) in addition to the direct consequences of altering a key active site side chain.

The mutant enzymes with substitutions for Ser-368 exhibit lower, yet significant, levels of carboxylase activity than the enzymes with substitutions for His-321. The *Km* for ribulose-P₂ with S368A is essentially unchanged from the wild-type value, so removal of the seryl hydroxyl group has no apparent consequence on the binding of ribulose-P₂. Replacement of the hydroxyl with a sulphydryl increases the observed *Km* for ribulose-P₂ by 10-fold, perhaps reflecting the larger van der Waals radius. The comparable catalytic deficiencies of the S368C and S368A mutant enzymes do not support a nucleophilic role for Ser-368 in catalysis.

The *Km* for bicarbonate is unchanged between wild-type and both the H321N and S368A mutants, demonstrating that neither residue is necessary for interactions with substrate CO₂.

Under the routine conditions of the ¹⁴C fixation assay (25 mM NaHCO₃), no significant decrease in the endpoint of ¹⁴C fixation (reflective of ribulose-P₂ exhaustion) relative to that with wild-type enzyme is observed in any of the mutant carboxylases (data not shown), implying that the carboxylase:oxygenase activity ratios are not drastically reduced. However, these assays would not have revealed elevated carboxylase:oxygenase ratios.

**Catalysis of Enediol Formation**—The putative involvement of His-321 and Ser-368 in the enolization partial reaction was directly examined by assaying the efficiencies of the mutant carboxylases in catalyzing detritiation of [3-²H]ribulose-P₂. Like any partial step of an enzyme-catalyzed reaction, the rate of deprotonation of ribulose-P₂ must be at least as great as that of the overall *kcat*. However, deprotonation is partially rate limiting in the overall reaction, appearing ~2-fold slower than *kcat* when measured with trace-labeled [3-²H]ribulose-P₂ (27, 32). Therefore, if the deficiency of a mutant carboxylase is not due to altering the proton acceptor, the apparent rate of enolization could be enhanced relative to overall carboxylation, whereas replacement of the proton acceptor with a functionally inert side chain could result in the initial proton abstraction step becoming solely rate limiting with a consequential increase in kinetic isotope effect and reduction in the ratio of enolization and carboxylation rates.

The relative enolization rates for the His-321 and Ser-368 mutant carboxylases at saturating levels of [3-²H]ribulose-P₂ are shown in Table II. The position 321 mutant carboxylases

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**Fig. 2.** Polyacrylamide gel electrophoresis of the wild-type and position 321 mutant carboxylases in the presence (A) and absence (B) of sodium dodecyl sulfate. Approximately 0.1 μg of protein was applied in each lane. Standards (Bio-Rad) are phosphor- 

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**Active Site Residues of Ribulose-P₂ Carboxylase**
Kinetic characteristics of ribulose-P₂ carboxylases in substrate carboxylation and analogue binding

| Enzyme     | Vₘₐₓ * | Kₐ (ribulose-P₂) | Kₐ(HCO₃) | kₐ/Kₐ (ribulose-P₂) | kₐ/Kₐ (ribulose-P₂) | Quaternary Complex Exchange Rate, tₑ |
|------------|--------|-----------------|----------|---------------------|---------------------|-------------------------------------|
| Wild type  | 100    | 12              | 27       | 2.1 x 10^3          | 0.08                | 0.9                                 |
| H321N      | 11.8   | 48              | 30       | 6.2 x 10^2          | 1.7                 | 3.0                                 |
| H321Q      | 7.8    | 80              | ND       | 2.5 x 10^3          | 1.9                 | 1.8                                 |
| H321S      | 3.2    | 45              | ND       | 1.7 x 10^3          | 0.8                 | 0.6                                 |
| H321N      | <0.01  | ~50             | ND       | <3                  | <0.002              | ~                                    |
| H321R      | <0.01  | ~50             | ND       | <3                  | <0.002              | ~                                    |
| S368A      | 1.7    | 11              | 20       | 3.9 x 10^3          | 1.9                 | 130                                 |
| S368C      | 0.8    | 125             | ND       | 1.6 x 10^2          | 0.08                | 48                                  |

* Determined as described under "Experimental Procedures" at saturating ribulose-P₂ concentration and 25 mM NaHCO₃. Under these conditions, the wild-type enzyme exhibits a specific activity of 3 µmol of CO₂ fixed min⁻¹ mg⁻¹.

To simplify comparisons among laboratories, values are reported as concentrations of bicarbonate rather than CO₂, the true substrate. Assays were performed aerobically (~250 µM O₂) so that the Kₐ values given are "apparent," where Kₐ = Kₐ (1 + [O₂]/[O₂]).

Not determined.

Approximations due to low rates of carboxylation activity (see text).

Complex not formed (see text).

Relative rates of detritiation of [3⁻¹⁴C]ribulose-P₂ and net carboxylation catalyzed by ribulose-P₂ carboxylases

| Enzyme     | Detritiation | Carboxylation |
|------------|--------------|---------------|
| Wild type  | 100          | 100           |
| H321N      | 8.8          | 11.8          |
| H321Q      | 7.0          | 7.8           |
| H321S      | 3.1          | 3.8           |
| H321A      | 2.5          | 3.2           |
| H321K      | <0.1         | <0.01         |
| H321R      | <0.1         | <0.01         |
| S368A      | 8.8          | 1.7           |
| S368C      | 3.6          | 0.8           |

Formation and Stability of Reaction-Intermediate Analogue Complexes—The catalytic competence of ribulose-P₂ carboxylase requires prior derivitization of an active site lysyl residue (Lys-191) by nonsubstrate CO₂ to form a carbamate that provides one ligand for the essential Mg²⁺ (26). Because only the activated form of the enzyme binds the reaction-intermediate analogue carboxyarabinitol-P₂ with great tenacity (19, 28), formation of this quaternary complex (E-CO₂-Mg²⁺-carboxyarabinitol-P₂) provides a convenient diagnostic for the ability of mutant carboxylases to undergo proper activation and to properly bind phosphorylated ligands. Position 321 substituted carboxylases (except for H321K and H321R) bind [2⁻¹⁴C]carboxyarabinitol-P₂ stoichiometrically, in analogy with wild-type enzyme. However, the rates of ligand exchange following challenge with a 10-fold excess of unlabeled inhibitor are increased significantly relative to the exchange rate for wild-type carboxylase (Fig. 3, Table I). Even H321N, which is the least deficient mutant catalytically, exhibits an 8-fold decrease in the complex exchange half-time. Therefore, interactions necessary for optimal binding of the reaction intermediate analogue are compromised by replacement of His-321.

Virtually no binding of carboxyarabinitol-P₂ to H321R or H321K could be detected, indicating that complexes are either not formed or are insufficiently stable to permit isolation during gel filtration (~1 h). The fact that these mutants catalyze the carboxylation of ribulose-P₂, albeit at extremely low levels (see above), proves that they do bind ribulose-P₂, and by inference, carboxyarabinitol-P₂. Additionally, because small molar excesses of carboxyarabinitol-P₂ inhibit the ac-
tivities of these mutants, carboxyarabinitol-P₉ appears to bind in competition with substrate. Nonetheless, the inability to isolate a quaternary complex with these mutants argues that Lys or Arg at position 321 structurally perturbs the active site to a much greater extent than do the other replacements. The interaction of carboxyarabinitol-P₉ with ribulose-P₉ carboxylase has been characterized as a two-step process involving initial rapid, reversible binding that induces a slow conformational change to form the final exchange-resistant complex (19, 33). Conceivably, the introduction and/or improper alignment of positively charged side chains into the polycvalent, polar region of the active site (14) may preclude or alter the conformational change which normally severely restricts solvent accessibility to this site. Such a disruption could contribute to the catalytic deficiencies of these mutants.¹

In contrast to the His-321 replaced enzymes, the position 368 mutant carboxylases form complexes with carboxyarabinitol-P₉ (Figure 3, inset; Table I) that exhibit similar (t₁/₂≈2 days for S368C) or greater (t₁/₂≈5.5 days for S368A) stabilities than wild type.

DISCUSSION

This study represents a continuation of our efforts to define the roles of active site residues of ribulose-P₉ carboxylase and to uncover the identity of the elusive base that initiates the catalytic pathway by abstraction of the C₃ proton of ribulose-P₉. Previous chemical and mutagenesis studies revealed Lys-166 as a residue that embodies salient features anticipated for the base involved in proton abstraction. These include enhanced nucleophilicity and an unusually low pKᵦ of 7.9 (34), which matches an inflection observed in the pH dependence of the deuteron isotope effect with [3-²H]ribulose-P₉ as substrate (32). Furthermore, the inflection was insensitive to the solvent dielectric constant, compatible with an amine serving as the base. Even more compelling, K166G lacks detectable carboxylase and enolization activities (12) (which defines the contribution of Lys-166 to rate enhancement as >10⁶), yet is able to catalyze the turnover of the 6-carbon carboxyketone reaction intermediate to PGA (13). A direct catalytic role of the lysyl e-amino group was verified by the partial restoration of activity effected by selective aminoethylcysteinyl side chain relative to that of lysyl group.

The inhibition of S368C is provided by the key base of A5-3-ketosteroid isomerase (Asp-38), which enhances the rate of substrate enolization by 10⁶ (42). This value, too, may be low because of evidence for competition of an alternate, inefficient base such as H₂O (43). Thus, a rate enhancement of at least 10⁶ provided by a general base which abstracts a proton from a carbon atom appears commonplace among diverse enzymes. Although our results demonstrate stringency for both His-321 and Ser-368 for maximal catalytic efficiency of ribulose-P₉ carboxylase, the rather modest catalytic facilitation (10–100-fold) provided by these residues, in contrast to the literature examples cited, appears incompatible with either side chain directly mediating the initial proton abstraction step.

¹ The inability of positively charged residues to functionally replace His-321 could indicate that its side chain is neutral in the native enzyme. While this may indeed be the case, such an interpretation must be considered speculative in the absence of direct measurements. The charged functionalities of the substitutions (Arg or Lys) may merely extend further into the active-site cavity to globally disrupt the active site, perhaps by approaching Arg-288, the primary residue involved in neutralizing the negative charge of the bound phosphate group.
based on comparisons presented in the preceding paragraph. One consequence of the preponderance of interacting charged and polar residues at the active site may be that the potential for functionally conservative replacements simply does not exist among the nineteen choices available. A seemingly modest alteration (e.g. S368C) could disrupt the delicately balanced microscopic charge and polarity at the active site with concomitant catalytic consequences. Both His-321 and Ser-368 interact directly with the reaction intermediate analogue and with other proximal side chains. The localized structural perturbation wrought by any amino acid substitution might then be propagated so as to impair catalysis indirectly, even though the gross conformational integrity of these mutants does not appear compromised in that they are dimeric, undergo carbamylation (activation), interact with phosphorylated ligands, and retain some catalytic activity.

If neither His-321 nor Ser-368 serves the role of initial proton acceptor, what are their possible functions? The similar impairments in both enolization and carboxylation activities that accompany substitution of His-321 appear to exclude a preferential influence of this residue on any single step of catalysis and are compatible with conformational perturbations as indirect causes of catalytic deficiencies. Alternatively, the decreased stabilities of the complexes of position 321 mutant proteins and carboxyarabinitol-P₂ could be viewed as evidence that His-321 assists in stabilizing a transition state mimicked by the reaction-intermediate analogue. Such an interpretation is consistent with the three-dimensional structure that shows proximity of the histidyl side chain and the C5 phosphate of the bound analogue (see Fig. 1). Credence to this interpretation is furthered by the observation that the Kₐ for ribulose-P₂ is insignificantly altered by replacement of His-321, thereby prompting the view that the histidyl residue contributes to the preferential binding of a transition state relative to substrate.

The 5-fold enhanced enolization activities relative to the residual carboxylation rates of position 368 mutants clearly eliminate Ser-368 as a viable candidate for the primary base. Furthermore, if Ser-368 were to mediate enolization, removal of the β-hydroxyl (S368A) should dramatically reduce the rate of enolization, whereas the introduction of a better nucleophile (e.g. the sulfhydryl of S368C) might be less detrimental. Instead, the S368A mutant is even more efficient than S368C in promoting enolization. The increased stability of the carboxyarabinitol-P₂ complex of both position 368 mutants appears to rule out stabilization of the mimicked transition state by Ser-368; however, absence of the seryl hydroxyl could allow an alternate binding conformation that leads to a dead-end complex. In the crystallographic structure of the spinach carboxylase with bound carboxyarabinitol-P₂, the relative orientation of the hydroxyls at C2 and C3 (and therefore the exact placement of the C3 hydroxyl group) cannot be defined from the electron density map (14). The denoted interaction of Ser-368 with carboxyarabinitol-P₂, which resembles the gem-diol intermediate (a hydrated ketone) but lacks one of the hydroxyls at C3, can only occur if the analogue binds in the conformation with trans-hydroxyls (Fig. 1). Depending on which gem-diol hydroxy group of the true reaction intermediate interacts with Ser-368, a potential role in assisting addition of H₂O to enediolate should be considered.

If Lys-166 is eliminated by crystallographic studies (which may be premature) and if His-321 and Ser-368 are eliminated by the observations described herein, the only structurally plausible amino acid side chains that remain as proxies for the base that enolizes ribulose-P₂ are His-287 and the carbamate of Lys-191. Position 191 mutants totally lack detectable carboxylation and enolization activities. Restoration of partial catalytic competence to inactive K₁₉₁C was achieved by interaction with aminothanesulfonate, thereby invoking a catalytic requirement for the carbamate nitrogen but not defining that role (44). The high acidity of carbamate nitrogens does not appear suitable for a role in proton transfer, but rigorous exclusion is not yet warranted by direct experimental observations. In preliminary experiments (45–47), confirmation or refutation of substrate-assisted carboxylase activity will require further investigation.

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