Multiple catalytic roles of His 287 of *Rhodospirillum rubrum* ribulose 1,5-bisphosphate carboxylase/oxygenase*

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Abstract

Active-site His 287 of *Rhodospirillum rubrum* ribulose 1,5-bisphosphate (RuBP) carboxylase/oxygenase interacts with the C3-hydroxyl of bound substrate or reaction-intermediate analogue (CABP), water molecules, and ligands for the activator metal-ion (Andersson I, 1996, *J Mol Biol* 259:160–174; Taylor TC, Andersson I, 1997, *J Mol Biol* 265:432–444). To test structure-based postulates of catalytic functionality, His 287 was replaced with Asn or Gln. The mutants are not affected adversely in subunit assembly, activation (binding of Mg^{2+} and carbamylation of Lys 191), or recognition of phosphorylated ligands; they bind CABP with even greater tenacity than does wild-type enzyme. H287N and H287Q are severely impaired in catalyzing overall carboxylation ($\sim 10^3$ -fold and $>10^5$ -fold, respectively) and enolization (each mutant below threshold for detection) of RuBP. H287N preferentially catalyzes decarboxylation of carboxylated reaction intermediate instead of forward processing to phosphoglycerate. Analysis of RuBP turnover that occurs at high concentrations of mutants over extended time periods reveal >10 -fold reduced CO_2/O_2 specificities, elevated misprotonation of the enediol intermediate, and misprocessing of the oxygenated intermediate of the oxygenase pathway. These results are consistent with multifaceted roles for His 287 in promoting enediol formation, enediol tautomerization, and forward-processing of carboxylated intermediate.

Keywords: analysis of partial reactions; reaction-intermediate stabilization; Rubisco; site-directed mutagenesis

Rubisco links photosynthetic energy capture to the net fixation of atmospheric CO_2 by catalyzing the carboxylation of RuBP to form two molecules of the 3-carbon carbohydrate precursor, PGA. The overall carboxylation reaction entails a complex scheme of sequential reactions, including enolization of RuBP, addition of CO_2 and H_2O to the 2,3-enediol of RuBP,¹ C–C cleavage, and stereospecific protonation of one cleavage product (reviewed in Andrews & Lorimer, 1987; Schloss, 1990; Hartman & Harpel, 1993, 1994). Several detailed molecular mechanisms for the Rubisco-catalyzed

carboxylation reaction have appeared recently (Newman & Gutteridge, 1993; Andersson, 1996; Taylor & Andersson, 1997a). Although these mechanisms embrace a wealth of accumulated chemical, kinetic, mutagenic, and structural knowledge about this enzyme, several features remain unresolved, including elucidation of CO_2/O_2 specificity determinants and consensus assignment of functionally relevant residues to precise steps of the overall reaction.

Among the strictly conserved active-site residues in direct contact with bound substrate or intermediate analogues (Knight et al., 1990; Lundqvist & Schneider, 1991; Newman & Gutteridge, 1993; Schreuder et al., 1993; Andersson, 1996; Taylor & Andersson, 1997a, 1997b), His 287² has not been subjected to sufficient functional scrutiny to allow its catalytic roles to be defined. Thus, several different mechanistic interpretations of structural data have been offered concerning the function of His 287. This residue approximates the activator site (comprised of a divalent metal-ion coordinated by Asp 193, Glu 194, and the carbamate of Lys 191), contacts bound ligands, and forms hydrogen bonds with active-site

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*This manuscript is dedicated to the memory of Finn Wold, an inspirational role model, gifted mentor, supportive colleague, and dear friend.

Abbreviations: Rubisco, D-ribulose 1,5-bisphosphate carboxylase/oxygenase (E.C. 4.1.1.39); RuBP, D-ribulose 1,5-bisphosphate; PGA, 3-phospho-D-glycerate; CABP, 2-carboxy-D-arabinitol 1,5-bisphosphate; CKABP, 2-carboxy-3-ketoarabinitol 1,5-bisphosphate; bicine, N,N-bis(2-hydroxyethyl)glycine; PGyc, 2-phosphoglycolate; CTBP, 2-carboxytetritol 1,4-bisphosphate; DiBP, 2,3-pentodiulose 1,5-bisphosphate; XuBP, xylulose 1,5-bisphosphate; DiMP, 1-deoxy-D-glycero 2,3-pentodiulose 5-phosphate.

¹The deprotonated form of RuBP varies in ionic state between enediol and enediolate forms throughout the reaction coordinate; we will collectively refer to this intermediate as “enediol.”

²Residue numbers refer to the sequence position in *R. rubrum* Rubisco. In the designations for the mutants, the first letter refers to the amino acid found in the wild-type enzyme and the second letter denotes the amino acid replacement.

water molecules (Fig. 1).³ As one of several residues within 5 Å of C3 of enzyme-bound RuBP or the reaction-intermediate analogue CABP, His 287 was originally proposed as a candidate for the base that catalyzes RuBP enolization (Knight et al., 1990; Lundqvist & Schneider, 1991). On the basis of its interaction with the C3-oxygen of bound ligands, His 287 was invoked as acceptor of the C3-hydroxyl proton in assisting tautomerization of enediol intermediate [which may be necessary to activate substrate for attack by CO₂ (Schloss, 1990)] or in C-C cleavage of the carboxylated reaction intermediate (Taylor & Andersson, 1997a, 1997b). By virtue of the association of His 287 with an active-site bound water molecule within 6 Å of C3 of CABP, a role as general base in activating solvent for addition to the carboxylated reaction intermediate has also been suggested (Newman & Gutteridge, 1993; Andersson, 1996).

Preliminary mutagenesis studies, prior to high-resolution structures of relevant liganded forms of Rubisco, did not fully clarify the roles of His 287 in catalysis. Asparaginyl substitution of His 287 was reported to result in significant retention of carboxylation and enolization activities (~1% of wild type), whereas lysyl and threonyl substitutions were more detrimental (Lorimer et al., 1987; Gutteridge et al., 1988). Independent preliminary studies indicated that asparaginyl replacement of position 287 abolished essentially all activity (Harpel et al., 1993a). In light of these conflicts, additional insights from higher-resolution crystal structures, and recent technical advances in product analysis for Rubisco mutants, we have replaced His 287 with Asn or Gln in order to retain the hydrogen bonding potential but eliminate acid/base functionality. Thorough evaluation of the properties of these mutants, as described herein, demonstrates the contribution of His 287 at various stages of the reaction coordinate.

Results

Purity and structural integrity of mutant Rubiscos

Purified mutant Rubiscos were judged to be >98% homogeneous by denaturing and nondenaturing PAGE. Comigration of purified mutant enzymes with authentic wild-type *Rhodospirillum rubrum* enzyme during electrophoresis under denaturing conditions verified proper synthesis of full-length gene products in the *Escherichia coli* host. Comigration of the bands for mutant and wild-type enzymes during nondenaturing electrophoresis indicated that the mutants form stable dimers, analogous to wild-type Rubisco.

Carboxylation activity

H287N and H287Q are greatly impaired in overall carboxylation activity (~0.05% and <0.001% of wild type, respectively), based on ¹⁴CO₂-fixation assays. Although the observed rates only slightly exceed the threshold for detection of carboxylation by this assay, they do truly represent catalytic turnover, as judged by protein and RuBP dependence, and by inhibition by CABP [a tight-binding

Fig. 1. Active-site interactions in the E·Ca²⁺·CO₂·RuBP complex of spinach Rubisco (Taylor & Andersson, 1997a). Residue numbers correspond to *R. rubrum* Rubisco. Major interactions (≤4 Å) discussed in the text are denoted by dashed lines. Note that the physiological activator ion, Mg²⁺, has been replaced by Ca²⁺ in order to avert RuBP turnover during crystallization and analysis. Distances from the His 287-coordinated and His 321-coordinated water molecules to C3 of RuBP are about 5 Å and 4 Å, respectively.

inhibitor of wild-type Rubisco (Pierce et al., 1980)] (Fig. 2) (data not shown for H287Q). The ¹⁴CO₂-fixation activities of these mutants are too low for accurate determination of *K_m* and *V_{max}* values; however, the H287N-catalyzed carboxylation rate was unchanged between 0.3 mM and 3 mM RuBP [*K_m*(RuBP) = 12 μM for wild-type Rubisco (Harpel et al., 1991)]. Likewise, *K_m*(CO₂) could

Fig. 2. Carboxylation activity of H287N. Assays were performed at pH 8 with 50 mM bicine, 1 mM EDTA, 10 mM MgCl₂, 10% glycerol, and 66 mM [¹⁴C]NaHCO₃, ±1 mg/mL enzyme (20 μM protomer), ±3 mM RuBP, and ±440 μM CABP.

³Numerous Rubisco structures (different species and different ligand forms) have been elucidated. The constellation of active-site residues is the same in all cases, and the relative positioning of active-site side chains is similar among different species provided that identical ligand forms are compared. The Rubisco·Ca²⁺·CO₂·RuBP structure is particularly instructive mechanistically, because of the unique combination of high resolution, activation state, bound RuBP, and localization of active-site water molecules.

not be determined, but the activity response for H287N was proportionately the same as wild-type Rubisco between 35 mM and 60 mM NaHCO_3 [$K_m \approx 30 \text{ mM NaHCO}_3$ at pH 8 (Harpel et al., 1991)]. Increasing the assay concentration of Mg^{2+} ion from 10 mM to 55 mM led to <15% increase in rate for H287N. Therefore, the impairment of this mutant does not result from disrupted interactions with substrates or activator metal ion. Purified mutant enzymes exhibited identical activity levels and characteristics whether expressed from the pFL342 vector or from the vector designed independently by Lorimer et al. (1987).

Catalysis of partial reactions

Rubisco's carboxylation reaction can be dissected into individual partial reactions, several of which can be assayed independently of the overall reaction. Impairment of any partial reaction implicates involvement of the substituted residue, either directly or indirectly, in that particular step of catalysis.

Enolization activity (C3-proton abstraction) is monitored as the enzyme-catalyzed detritiation of $[3\text{-}^3\text{H}]\text{RuBP}$ (Sue & Knowles, 1982; Hartman & Lee, 1989). As demonstrated in Figure 3, H287N and H287Q are greatly impaired in exchange activity relative to wild-type Rubisco. For comparison, the dotted line in Figure 3 represents the experimentally measured rate of detritiation in a control lacking enzyme, and the dashed line is a theoretical line representing 1% of the wild-type activity at the protein concentration of H287N used in this experiment. Even when assayed at protomer concentrations as high as 60 μM (3 mg/mL) or at eight-

Fig. 3. Time courses for detritiation of $[3\text{-}^3\text{H}]\text{RuBP}$ by wild-type (0.04 mg/mL protein, ■), H287N (1.9 mg/mL protein, ●), and H287Q (3.0 mg/mL protein, ▲) Rubisco. Assay solutions (pH 8.0, 23 °C) contained 50 mM bicine, 10 mM MgCl_2 , 1 mM EDTA, 73 mM NaHCO_3 , 2.9 mM $[3\text{-}^3\text{H}]\text{RuBP}$ ($\sim 0.2 \text{ Ci/mol}$), 10% glycerol, and indicated levels of protein. The dotted line depicts data for a reaction not containing protein and the dashed line depicts a theoretical line representing 1% of wild-type activity at the concentration of enzyme used in the H287N reaction.

Fig. 4. Turnover of $[2'\text{-}^{14}\text{C}]\text{CKABP}$ by (A) wild-type and (B) H287N Rubiscos. PGA present at the beginning of the reactions is carried over from the preparation of $[2'\text{-}^{14}\text{C}]\text{CKABP}$. Experimental details are given in Materials and methods.

fold higher specific radioactivities of $[3\text{-}^3\text{H}]\text{RuBP}$, neither H287N nor H287Q exhibited exchange activities significantly greater than the lower limit of detection ($\sim 0.1\%$ of wild-type).

Forward processing of the carboxylated reaction intermediate, inclusive of hydration and C-C cleavage partial reactions, can also be assayed independently of overall carboxylase activity. The isolated intermediate, provided as $[2'\text{-}^{14}\text{C}]\text{CKABP}$, is acid-labile; hence, its normal turnover to PGA (or pyruvate, derived from misprocessing of the terminal intermediate of catalysis, see Harpel & Hartman, 1996) is reflected by an increase in acid-stable radioactivity and a corresponding decrease in acid-labile radioactivity. A decline in CKABP concentration, disproportionate to the increase in PGA concentration, reflects decarboxylation of the former (Pierce et al., 1986a). Whereas wild-type Rubisco catalyzes the productive forward processing of CKABP, H287N predominantly catalyzes the decarboxylation reaction (Fig. 4), consistent with preliminary reports (Lorimer et al., 1987; Gutteridge et al., 1988). In a separate experiment, a 5:1 molar ratio of H287N protomer:CKABP was used to ensure complete binding and rapid consumption of the added $[2'\text{-}^{14}\text{C}]\text{CKABP}$, in order to minimize spontaneous decarboxylation free in solution [$t_{1/2} \sim 1 \text{ h}$ (Lorimer et al., 1986)]. Even under these conditions, the ratio of hydrolysis/decarboxylation was estimated to be ≤ 0.1 (at the limits of discrimination) for H287N versus ≥ 0.9 for wild-type Rubisco.

Product analysis

Despite the severe kinetic impairment of H287N in catalyzing net carboxylation and partial reactions, chromatographic analyses of

reactions with $[1\text{-}^3\text{H}]\text{RuBP}$ reveal that H287N (when examined at very high concentrations) processes RuBP with formation of multiple products, inclusive of PGA and PGyc (Fig. 5A). When $[1\text{-}^3\text{H}]\text{RuBP}$ is provided as substrate, the ratio of labeled PGA and PGyc provides a measure of flux between the competing carboxylation and oxygenation pathways (v_c/v_o). Expressed in terms of a specificity factor denoted " τ " ($\tau = V_c K_o / V_o K_c = (v_c/v_o) \cdot [\text{O}_2] / [\text{CO}_2]$), this flux provides a gauge of the potential efficiency of a Rubisco variant in photosynthetic carbon assimilation (Laing et al., 1974). Based on the PGA:PGyc peak ratios, the specificity factor of H287N ($\tau \sim 0.9$) is about 12-fold lower than that of wild-type enzyme ($\tau \sim 11$).

The preferential impairment of carboxylase activity of H287N is even greater than surmised solely on the basis of lowered τ -value, because CTBP, the other major turnover product present in the product profile (apart from PGA and PGyc), is derived exclusively from oxygenation of the RuBP-enediol. CTBP is formed by rearrangement of DiBP, which arises from elimination of H_2O_2 from the peroxyketone intermediate of the oxygenation pathway (Fig. 6) (Chen & Hartman, 1995; Harpel et al., 1995). Therefore, appearance of either compound in Rubisco product profiles is diagnostic of misdirected oxygenase activity. The intermediacy of DiBP in the formation of CTBP by H287N was verified by chemical-trapping with 50 mM sodium borate (Chen & Hartman, 1995; Harpel et al., 1995) and by the ability of H287N to convert authentic DiBP (isolated from a reaction mixture in which RuBP is processed by the E48Q mutant of Rubisco; Chen & Hartman, 1995) to CTBP (data not shown).

Fig. 5. Radiometric anion-exchange chromatographic profiles of products formed from $[1\text{-}^3\text{H}]\text{RuBP}$ by (A) H287N or (B) H287Q. The H287N reaction (pH 8) contained 1 mg/mL (20 μM protomer) of the protein, 250 μM $[1\text{-}^3\text{H}]\text{RuBP}$ (~ 5.4 Ci/mol), 50 mM bicine, 10 mM MgCl_2 , 1 mM EDTA, 10.3 mM NaHCO_3 , and 10% glycerol. The H287Q reaction contained the same components except 1.25 mg/mL enzyme (25 μM protomer) and 50.1 mM NaHCO_3 . Reactions were quenched after 3 h and 4.5 h, respectively. The initial peak probably reflects slight phosphatase contamination of the enzyme preparations, which becomes significant at the high protein concentrations and extended incubation times required to process RuBP with these low activity mutants. The identity of the peak at ~ 12 min has not been determined. The elution gradient was the same for both samples.

XuBP, another side product of Rubisco reactions (Fig. 6), is also formed by H287N. XuBP results from misprotonation of RuBP-enediol at C3, and is observed during turnover of RuBP by wild-type spinach, but not wild-type *R. rubrum*, Rubisco (Edmondson et al., 1990; Lee et al., 1993). XuBP formation by H287N therefore indicates an inability of the mutant to completely sequester enediol from solvent. Interestingly, DiMP, the other characterized enediol-derived side product that has been observed with Rubisco mutants (Harpel & Hartman, 1994; Larimer et al., 1994; Morell et al., 1994; Larson et al., 1995), is absent in H287N reaction profiles (expected elution time ~ 21 min). This dicarbonyl results from β -elimination of phosphate from the enediol, either in solution due to its dissociation from the active site, or at the active site due to departure of the intermediate from an extended conformation that maintains the bridge oxygen of the C1-phosphate group coplanar with the double bond (Rose, 1981). H287N, although inefficient at protecting the enediol from misprotonation, therefore appears to bind this intermediate with sufficient tenacity and in a proper orientation to prevent loss of phosphate and/or release of intermediate from the active site. Identical product profiles were obtained with the H287N mutant expressed from the construct of Lorimer et al. (1987), confirming its identity to H287N derived from pFL342.

Although its rate of substrate turnover was considerably slower, H287Q also utilizes $[1\text{-}^3\text{H}]\text{RuBP}$ as a substrate, as visualized by chromatographic analysis (Fig. 5B). Identification of products for H287Q was hampered by the low rate of RuBP consumption and the effect of slight phosphatase contamination in the enzyme preparation (manifested in the large initial breakthrough peak in the chromatogram). However, H287Q exhibits a low τ value (estimated to be ~ 0.8) and forms CTBP, like H287N.

Enzyme activation/CABP binding

Reflective of the high affinity of activated Rubisco for CABP (Pierce et al., 1980), formation of a stable, stoichiometric complex between the enzyme and the reaction-intermediate analogue is diagnostic of proper activation [carbamylation of active-site Lys 191 and binding of Mg^{2+} (Lorimer et al., 1976; Lorimer, 1981; Donnelly et al., 1983)]. H287N and H287Q both form Mg^{2+} -dependent complexes containing ~ 1 molecule of CABP per active site, as reported previously for the H287N mutant (Lorimer et al., 1987; Gutteridge et al., 1988). Correlation between CABP complexation and enzyme activation was verified by use of $^{14}\text{CO}_2$, in which the stoichiometries of bound $^{14}\text{CO}_2$ to active-site concentrations for H287N and wild-type Rubisco were both equal to unity, as also demonstrated previously (Lorimer et al., 1987; Gutteridge et al., 1988). Surprisingly, the complexes of activated H287N and H287Q with CABP are considerably more stable to ligand exchange than the wild-type counterpart (Fig. 7).

Discussion

A critical dependence of Rubisco activity on the imidazole side chain of His 287 is intimated by its hydrogen bonding interaction with the C3-hydroxyl of bound RuBP (or CABP) and with metal-coordinating amino acid side chains at the activator site. However, such a dependence has not been established rigorously heretofore with respect to the quantitative rate-enhancement to overall catalysis provided by His 287. Accordingly, we have addressed this issue directly by examination of catalytic properties of H287N and

Fig. 6. Side reactions of RuBP-enediol pertinent to position-287 mutants. β -Elimination of phosphate (formation of DiMP) is not observed with H287N, but may occur with H287Q. $R = C(H,OH)CH_2OPO_3^{2-}$.

H287Q. Two additional issues concerning catalytic role(s) of His 287 were also explored. First, are any of the measurable partial reactions less impacted than overall carboxylase activity, whereby a primary role of His 287 can be ascribed to a particular step of the transformation of RuBP to PGA and PGyc? Second, if position-287 mutants retain any discernable carboxylase activity, are labile reaction intermediates stabilized as effectively as with wild-type enzyme?

The H287Q mutant displays <0.001% of wild-type carboxylase activity, thereby showing that His 287 contributes at least 10^5 -fold

to overall rate enhancement. The distinctly higher activity of H287N (0.05% of wild-type) suggests that the amide nitrogen of the introduced Asn is spatially equivalent to an imidazole nitrogen of the wild-type enzyme and thus partially fulfills a hydrogen bonding role of the latter. Having purified recombinant H287N generated from two different expression vectors, we can only conclude that earlier reports (Lorimer et al., 1987; Gutteridge et al., 1988) attributing 1% of wild-type activity to H287N were in error.

Because subunit structure, activation chemistry, and substrate binding parameters of position-287 mutants are not appreciably perturbed, greatly diminished k_{cat} values must reflect impacts on catalysis, per se, rather than gross conformational changes. Of particular relevance in this regard, activated (Mg^{2+} -stabilized carbamate) H287N and H287Q form complexes with CABP that are even more stable than the wild-type counterparts.

Conversion of RuBP to PGA and PGyc during prolonged incubations with high concentrations of H287N or H287Q proves that mutants retain the capabilities to enolize RuBP and to hydrolytically cleave the carboxylated reaction intermediate. However, the rates of these partial reactions are so slow that they are not detected when examined independently of overall carboxylation. Furthermore, the normal partitioning of the enediol intermediate between the carboxylation and oxygenation pathways is skewed drastically in favor of oxygenation. Thus, rather than participating preferentially at any single step along the reaction coordinate, His 287 facilitates both enolization and forward processing of the carboxylated reaction intermediate and also influences the relative reactivity of enediol intermediate toward gaseous substrates.

Although His 287 accelerates enolization of RuBP by at least 10^3 -fold, this residue is not optimally positioned to serve as the primary base. The C3 proton of enzyme-bound RuBP or CABP is 4–5 Å removed from the closest imidazole nitrogen and also projected away from the histidyl side chain (Newman & Gutteridge, 1993; Andersson, 1996). In contrast, the Lys 191-carbamate oxygen that is not coordinated to activator Mg^{2+} ion is in-line with the

Fig. 7. Ligand exchange profiles for $E \cdot Mg^{2+} \cdot CO_2 \cdot CABP$ complexes of wild-type (■), H287N (●), and H287Q (▲) Rubiscos.

C3 proton and only 3 Å removed. This exquisite positioning, in combination with the unique resonance properties of the carbamate that allow it to simultaneously act as a metal ligand and catalytic base, offer a compelling argument for its participation as the primary base (Newman & Gutteridge, 1993; Andersson, 1996; Taylor & Andersson, 1997a). Why then does elimination of the imidazole side chain at position 287 have such a negative impact on enolization? His 287, within hydrogen bonding distance to the noncoordinated carbamate oxygen, may stabilize the protonated carbamate (subsequent to C3-proton abstraction), in effect increasing its basicity. Another possibility is predicated on the notion that the carbamate is shielded from bulk solvent, perhaps by bound RuBP or the derived enediol, and can only equilibrate with solvent protons through a proton relay network that includes His 287. Disruption of this network would diminish enolization activity, as assayed by wash-out of tritium from [3-³H]RuBP, even if the primary base (i.e., the carbamate) were left intact. In such a situation, the C3 proton of RuBP would merely be repeatedly shuttled to and from the carbamate.

Similarly, active-site Lys 166 is also critical to enolization, despite being too distant from the C3 proton of enzyme-bound RuBP to serve as the primary base (reviewed in Hartman & Harpel, 1993, 1994). However, the juxtaposition of Lys 166 to C2 (Knight et al., 1990; Taylor & Andersson, 1997b) is consistent with the ϵ -amine serving as a general base or Lewis acid in the tautomerization of the enediol intermediate. Abstraction of the C3 proton of RuBP, probably assisted by polarization of the C2-carbonyl by Mg^{2+} and Lys 166, gives rise initially to an enediolate with the negative charge on the C2 oxygen. Tautomerization, invoked as a discrete catalytic event by presteady-state kinetics (Schloss, 1990), to a species with the negative charge residing on the C3 oxygen, would favor nucleophilic attack of water at C3 and electrophilic attack of CO_2 at C2, as required for normal processing of RuBP. The locations of His 287 and Lys 166, relative to C3 and C2 of RuBP, respectively, are compatible with joint participation in tautomerization and the envisioned proton relay system for the exchange of carbamate protons with solvent protons.

The basis of impaired forward processing of isolated [2'-¹⁴C] CKABP by H287N is also subject to more than one interpretation. Hydrolytic cleavage of CKABP to form PGA entails hydration of the ketone group, followed by deprotonation of the resulting gem-diol and carbon-carbon scission. Hence, elimination of the general base that promotes either of these two distinct events would negatively impact normal processing of CKABP. Thus, our finding that H287N catalyzes decarboxylation rather than forward processing of CKABP is consistent with the prior conclusion, based on the structure of an inactive Rubisco· CO_2 · Ca^{2+} ·RuBP complex (Taylor & Andersson, 1997a), that His 287 serves as the base that deprotonates the hydrated CKABP. However, the possibility that His 287 catalyzes the hydration step cannot be excluded. Although His 287 is insufficiently close to the water molecule nearest C3 of RuBP to suggest such a role, we note⁴ that His 287 is within ~4 Å and hydrogen bonded to another water molecule positioned ~5 Å from C3 in this same complex (Fig. 1). Furthermore, His 287 is hydrogen bonded to an active-site water about 6 Å from C3 in the Rubisco· CO_2 · Mg^{2+} ·CABP complex (Andersson, 1996). This dis-

tance could be even less during turnover of the actual reaction intermediate.

Taylor and Andersson (1997a) favor the use of His 327 of spinach Rubisco (equivalent to His 321 of the *R. rubrum* enzyme) as the general base for hydration, based on the proximity of a water molecule to both the imidazole side chain and C3 of bound ligand (about 3 Å and 4 Å, respectively) as observed in the crystallographic structure of enzyme complexed with Ca^{2+} and RuBP. Consequences of substitution of this histidyl residue run counter to such a role. The H321N mutant retains nearly equivalent levels (~10%) of wild-type activity in overall carboxylation and in the enolization partial reaction, demonstrating that the rate of forward processing of CKABP is not reduced by more than 10-fold. Yet, based on comparison of the rates of CKABP hydration in solution to the k_{cat} for RuBP turnover by wild-type enzyme, the hydration step must be enhanced by at least 10³-fold (Lorimer et al., 1986; Pierce et al., 1986a). Taking into account both structural and functional information, we thus believe that a stronger argument can be made for the participation of His 287, rather than His 321, in the hydration step.

Decarboxylation of CKABP, as catalyzed by nonactivated wild-type Rubisco, gives rise to DiMP as a consequence of β -elimination of phosphate from the 2,3-enediol of RuBP (the initial product of decarboxylation) (Jaworowski et al., 1984; Pierce et al., 1986a). However, we do not detect DiMP in product profiles from the turnover of [1-³H]RuBP, even though H287N decarboxylates isolated CKABP. This apparent discrepancy is reconciled if hydration and carboxylation of the enediol are indeed concerted, as suggested by Cleland (1990), and thus form hydrated CKABP directly. Given such a mechanism, the enzyme would never encounter a free ketone intermediate. Because CKABP exists in solution predominantly as the free ketone (Pierce et al., 1986a), a mutant lacking the capability to hydrate this compound efficiently would be prone to decarboxylate it. The extremely slow rate of formation of PGA from [1-³H]RuBP by H287N is compatible with slow, and perhaps spontaneous, hydration in concert with carboxylation of enediol.

Although H287N is severely impaired in the enolization reaction, the enediol intermediate that is formed does not show any tendency to undergo β -elimination of phosphate. We conclude that the mutant protein, analogously to the wild-type enzyme, stabilizes this intermediate by maintaining C1, O1, and P1 atoms coplanar with the C2,C3 double bond. However, the enediol generated by H287N is more prone to misprotonation, perhaps indicative of increased residency time at the active site. The impaired carboxylation of the enediol (denoted by increased amounts of PGyc and CTBP formed in product profiles relative to PGA) may have a more direct basis. Because CO_2 and O_2 react with enediol through bimolecular collision (Pierce et al., 1986b), differential binding affinities of gaseous substrates cannot be the source of discrimination. Instead, partitioning between carboxylation and oxygenation has been attributed to geometric deformation of the planar enediol as a prerequisite to the carboxylation transition state (Tapia et al., 1995) or preferential stabilization of incipient transition states (Chen & Spreitzer, 1991, 1992; Lorimer et al., 1993). In either case, direct interaction of His 287 with the enediol could well influence its reactivity. The enhanced affinities for CABP (which mimics the hydrated-carboxyketone intermediate) and the misprocessing of the peroxyketone intermediate displayed by position-287 mutants may reflect perturbations of normal interactions between His 287 and the carboxylated or oxygenated intermediates (or their respective transition states).

⁴These distances, not provided in the original publication, were measured from a model built from Brookhaven Protein Data Bank file 1RXO (Rubisco· Ca^{2+} · CO_2 ·RuBP) (Taylor & Andersson, 1997a).

In conclusion, our studies clearly demonstrate multifaceted catalytic roles of His 287, high stringency for an imidazole side chain at position 287, and a large contribution of His 287 to overall rate enhancement. Are these properties attributed to direct effects of His 287 or its influence on other primary players with which it interacts? The answer may be "both." The imidazole nitrogen of His 287 that interacts with the carbamate oxygen is also nearly equidistant between the metal-coordinated carboxylate oxygen of Glu 194 and the C3 oxygen of bound ligand in the Rubisco $\cdot \text{Ca}^{2+} \cdot \text{CO}_2 \cdot \text{RuBP}$ structure (Fig. 1). Although His 287 is not needed for enzyme activation, this residue contributes to the electrostatic balance in the vicinity of the metal ion, which has been proposed to modulate aspects of catalysis (Lu et al., 1992; Andersson, 1996). Interestingly, in preliminary studies, position-194 mutants were observed to lack the ability to catalyze carboxylation or enolization, but could decarboxylate CKABP (Lorimer et al., 1987; Gutteridge et al., 1988); inhibition of decarboxylation by high magnesium concentration (without concomitant rescue of forward processing) suggested that the primary impairment of these mutants was in metal binding. The characteristics of position-287 mutants are more complex and show that the contributions of His 287 transcend any subtle impact upon metal-ion binding.

Materials and methods

Materials

Unenriched RuBP, $[1\text{-}^3\text{H}]\text{RuBP}$, $[3\text{-}^3\text{H}]\text{RuBP}$, and $[2'\text{-}^{14}\text{C}]\text{CABP}$ (used as a mixture of the *arabino* and *ribo* epimers) were synthesized and purified as reported (Horecker et al., 1958; Kuehn & Hsu, 1978; Pierce et al., 1980; Hartman & Lee, 1989). RuBP concentrations in stock solutions were determined as total PGA produced by wild-type *R. rubrum* Rubisco (Schloss et al., 1982). $[2'\text{-}^{14}\text{C}]\text{CKABP}$, prepared by acid-quenching of a reaction containing RuBP, $^{14}\text{CO}_2$, and an excess of wild-type *R. rubrum* Rubisco (Pierce et al., 1986a), was a generous gift of Dr. G.H. Lorimer.

Mutant *R. rubrum* *rbc* genes were constructed by site-directed mutagenesis of the wild-type *rbc* gene carried by the expression vector pFL245 and were expressed in *E. coli* strain MV1190. Conditions for mutagenesis and heterologous expression were as described (Lorimer et al., 1990; Harpel et al., 1991). Plasmid designations and the corresponding mutagenic oligonucleotide primers (mutated codons underlined) are pFL342 (encoding H287N), 5'-TTGCATTATAACCGGGCTGGC, and pFL334 (encoding H287Q), 5'-TTGCATTATCAGCGGGCTGGC. Mutations were verified by dideoxy sequencing in the region of mutation (Sanger et al., 1977). The vector utilized for production of H287N in previous studies (Lorimer et al., 1987; Gutteridge et al., 1988) was transferred into *E. coli* MV1190 for expression in the current study. Mutant Rubiscos and authentic wild-type enzyme (isolated from *R. rubrum*) were purified as described (Schloss et al., 1982; Harpel et al., 1991; Harpel & Hartman, 1994).

Protein analysis and activity assays

Protein concentrations were determined from the absorbance of Rubisco at 280 nm (1.2 A.U. for 1 mg/mL; Schloss et al., 1982). Carboxylase activity was determined at pH 8 and 23 °C as acid-stable counts derived from $[^{14}\text{C}]\text{NaHCO}_3$ in the presence of RuBP (Niyogi et al., 1986; Harpel et al., 1991). The enolization partial

reaction was assayed by monitoring the enzyme-catalyzed "wash-out" of tritium from $[3\text{-}^3\text{H}]\text{RuBP}$ (Sue & Knowles, 1982; Hartman & Lee, 1989).

$[2'\text{-}^{14}\text{C}]\text{CKABP}$ turnover by Rubisco was assayed as described (Pierce et al., 1986a). Reaction mixtures (pH 8.0) containing 0.005 mg/mL of wild-type or 0.02 mg/mL of H287N Rubisco, 50 mM bicine, 10 mM MgCl_2 , 1 mM EDTA, 10% glycerol, and 66 mM NaHCO_3 were preincubated for 15 min at 23 °C before addition of 1.6 μM $[2'\text{-}^{14}\text{C}]\text{CKABP}$ ($\sim 4,300$ dpm/nmol). $[^{14}\text{C}]\text{PGA}$ (2.3 μM) and RuBP (10.4 μM) were also introduced as carry-over from preparation of the intermediate. Reaction aliquots were quenched by treatment with 1% SDS, followed immediately by either acid or borohydride, then processed for scintillation counting (Lee et al., 1993).

Product analyses

Product profiles were obtained by anion-exchange chromatography of reactions containing $[1\text{-}^3\text{H}]\text{RuBP}$ and the appropriate Rubisco variant (Harpel et al., 1993b). Reactions were quenched with 1% SDS and ultrafiltered (Amicon Centricon-10) to deproteinate the samples. Samples were then applied to a column of Mono Q anion-exchanger (Pharmacia HR 5/5, 5 mm $\times$ 50 mm) and eluted with a gradient of NH_4Cl (containing 10 mM sodium borate) at pH 8.0. Column eluates were assayed for radioactivity by in-line radiometric detection (IN/US β -RAM). Because both PGA (derived from CO_2 plus C1 and C2 of RuBP during carboxylation) and PGyc (derived from O_2 and C1 and C2 of RuBP during oxygenation) retain tritium from $[1\text{-}^3\text{H}]\text{RuBP}$, the CO_2/O_2 specificity factor (τ) was calculated from the radiometric peak areas for PGA and PGyc, respectively (Laing et al., 1974). The CO_2 concentration was estimated from the Henderson-Hasselbach equation, assuming $pK_{a1} = 6.1$; $[\text{O}_2]$ was assumed to be 255 μM .

Reaction-intermediate analogue binding and enzyme activation

The binding stoichiometries and stabilities of quaternary complexes between activated Rubisco variants [entailing carbamylation of active-site Lys 191 by CO_2 and binding of Mg^{2+} (Lorimer & Mizioro, 1980; Lorimer, 1981; Donnelly et al., 1983)] and CABP were evaluated by gel filtration (Mizioro & Sealy, 1980; Smith et al., 1988). Solutions of the respective Rubisco (10–20 μM active sites) were incubated at 23 °C in a pH 8.0 buffer (50 mM bicine, 10 mM MgCl_2 , 1 mM EDTA, and 10% glycerol) supplemented with either 100 μM $[2'\text{-}^{14}\text{C}]\text{CABP}$ and 66 mM unlabeled NaHCO_3 (for analysis of CABP binding) or 100 μM unlabeled CABP and 66 mM $[^{14}\text{C}]\text{NaHCO}_3$ (for analysis of carbamylation efficiency) for 1 h prior to gel filtration ($t = 0$ h) versus incubation buffer (lacking CABP but containing 66 mM unlabeled NaHCO_3). In each case, radiolabel was introduced at ~ 2 Ci/mol. Samples for the measurement of $^{14}\text{CO}_2$ binding were concentrated approximately twofold by ultrafiltration (Amicon minicon-30 units) and diluted with an equal volume of buffer; concentration and dilution were repeated once prior to gel filtration. Binding stoichiometries were established from the specific radioactivity of the ligand and the protein concentrations in peak fractions. Stabilities of the complexes prepared with radioactive CABP were determined by the rate of ligand exchange following addition of 1 mM unlabeled CABP to the incubations and subsequent periodic gel filtration of aliquots therefrom.

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