Role of Asparagine-111 at the Active Site of Ribulose-1,5-bisphosphate Carboxylase/Oxygenase from *Rhodospirillum rubrum* as Explored by Site-directed Mutagenesis*

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Crystallographic studies of ribulose-1,5-bisphosphate carboxylase/oxygenase from *Rhodospirillum rubrum* suggest that active-site Asn^{111} interacts with Mg$^{2+}$ and/or substrate (Lundqvist, T., and Schneider, G. (1991) *J. Biol. Chem.* 266, 12604-12611). To examine possible catalytic roles of Asn^{111}, we have used site-directed mutagenesis to replace it with a glutaminyl, aspartyl, seryl, or lysyl residue. Although the mutant proteins are devoid of detectable carboxylase activity, their ability to form an octameric complex comprised of CO$_2$, Mg$^{2+}$, and a reaction-intermediate analogue is indicative of competence in activation and substrate binding. The mutant proteins retain enolization activity, as measured by exchange of the C3 proton of ribulose bisphosphate with solvent, thereby demonstrating a preferential role of Asn^{111} in some later step of overall catalysis.

The active sites of this homodimeric enzyme are formed by interactive domains from adjacent subunits (Larimer, F. W., Lee, E. H., Mural, R. J., Soper, T. S., and Hartman, F. C. (1987) *J. Biol. Chem.* 262, 15327-15329). Crystallography assigns Asn^{111} to the amino-terminal domain of the active site (Knight, S., Andersson, I., and Brändén, C.-I. (1990) *J. Mol. Biol.* 215, 113-160). The observed formation of enzymatically active heterodimers by the *in vivo* hybridization of an inactive position-111 mutant with inactive carboxyl-terminal domain mutants is consistent with this assignment.

The smallest functional unit of ribulose-bisphosphate carboxylase/oxygenase (EC 4.1.1.39) is a dimer, because the active sites are comprised of interactive domains from adjacent subunits. Interfacial active sites were first demonstrated with the homodimeric enzyme from *Rhodospirillum rubrum* by formation *in vivo* of enzymatically active heterodimers from two distinct site-directed mutant proteins (K166G and E48Q) that were devoid of significant carboxylase activity (1). This conclusion was confirmed by x-ray crystallography of the spinach enzyme, in which K175 (analogous to K166 of the *R. rubrum* enzyme) was located within the $\beta/\alpha$ barrel of the carboxyl-terminal domain and E60 (analogous to E48 of the *R. rubrum* enzyme) was located within the amino-terminal domain of the adjacent subunit (2). Hence, interfacial active sites also prevail in this more complex hexadecameric form of the enzyme (Lo$\delta$S) in which the octameric core of large catalytic subunits can be viewed as a tetramer of the dimeric *R. rubrum* carboxylase. The small subunits are remote from the active sites and thus not involved directly in catalysis (3).

Most active site residues of the carboxylase are located within the $\beta/\alpha$ barrel of the carboxyl-terminal domain (2-4). However, at least 2 residues, E48 and N111, are sufficiently close to bound substrate or analogues thereof, as visualized in the crystallographic structure (Fig. 1), to intimate catalytic roles. In fact, E48 was implicated in catalysis prior to elucidation of the three-dimensional structure. Site-directed mutagenesis demonstrated a very high catalytic stringency for E48, which was not required for subunit assembly, normal activation (carbamylation), substrate binding, nor enolization of ribulose-P$_2$ (the initial step in overall catalysis) (5, 6). Furthermore, the inactive E48C mutant protein became catalytically competent upon carboxymethylation, which, in effect, completes the replacement of the original glutamyl residue with a carboxymethylcysteinyl residue. This novel engineered carboxylase displayed a 5-fold-enhanced oxygenase/carboxylase activity ratio, indicating that segments of the amino-terminal domain might serve as determinants of the specificity factor for gaseous substrates (7). Such a postulate is consistent with the crystallographic discovery that in the activated (carbamylated) form of the enzyme E48 and K329 (a catalytic residue in flexible loop 6 of the $\beta/\alpha$ barrel) are engaged in intersubunit, electrostatic interaction (3). In concert, loop 6 and a segment of the amino-terminal domain, which encompasses E48, partially cover the "mouth" of the $\beta/\alpha$ barrel, thereby rendering the active site less accessible to solvent.

Less is known about the function (if any) of N111, the other contact residue provided by the amino-terminal domain of the active site. Based on the crystal structure of the activated form of the *R. rubrum* carboxylase, the amide side chain of N111 serves as one of the ligands for Mg$^{2+}$ (8). However, when the activated enzyme is complexed with ribulose-P$_2$, the amide of N111 is too far from Mg$^{2+}$ to participate in inner sphere coordination but is less than 5 Å from C3 of bound substrate, prompting the chemically unusual notion that N111 could be the base that facilitates the initial.

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1. 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.

2. The abbreviations used are: ribulose-P$_2$, d-ribulose-1,5-bisphosphate; ribulose-P$_2$ carboxylase, d-ribulose-1,5-bisphosphatase/carboxylase/oxygenase; carbobyrainitol-P$_2$, 2-carboxy-D-arabinitol-1,5-bisphosphate; Bicine, $N,N$-bis(2-hydroxyethyl)glycine.
enolization (4). The corresponding residue (N129) of the spinach carboxylase is located within hydrogen bonding distance of the C2 carboxylate of the bound reaction-intermediate analogue carboxyarabinitol-P_2 (3). Hence, three-dimen-
sional structures of different complexes of ribulose-P_2 carbox-
ylase are compatible with various roles of N111. To clarify this situ-
ation, we have turned our attention to characterization of site-directed mutants of R. rubrum ribulose-P_2 carbox-
ylase, which contain substitutions for N111.

EXPERIMENTAL PROCEDURES

Materials—Commonly used chemicals and reagents were of the highest purity readily available. Other materials and vendors were as follows: reagents for oligonucleotide synthesis, American Bio-
tech; restriction endonucleases, Bethesda Research Laboratories or New England Biolabs; sodium [14C]bicarbonate (60 mCi/mmol), Amersham Corp.; tritiated water (5 Ci/ml), ICN. Ribulose-P_2, car-
boxyarabinitol-P_2, and [14C]carboxyarabinitol-P_2 were synthesized by literature procedures (9, 10). [3-3H]Ribulose-P_2 (specific activity, 11,000 cpm/nmol) was prepared by incubation of ribulose-P_2 in tritiated water in the presence of the K329C mutant carboxylase, which catalyzes exchange between the C3 proton and solvent protons, and resorption of ribulose-P_2 (6). Mutant carboxylases were purified by ion-exchange chromatography on MonoQ columns in association with a fast-protein liquid chromatography unit (Pharmacia LKB Biotechnology Inc.) as described previously (11). Wild-type carboxylase was isolated from R. rubrum (12).

Expression and Mutagenesis—rbc genes, cloned into pFL200 (13), were expressed in Escherichia coli strain MV1190 Aflac-proAB) thi supEA(srl-recA)306::TnlO(tet)

The corresponding residue (N123) of the
rubrum enzyme; B after E48 and N111 denotes their location
in the adjacent subunit. (Adapted from the schematic of the spinach enzyme illustrated in Ref. 2.)

Formation of Hybrid Enzymes—The system for generating hybrid enzymes has been described (1, 13). Heterodimers were formed by coexpression of two mutant rbc genes carried by plasmids of different incompatibility groups. In our system, one vector (bearing the muta-
tion for replacement of N111) includes the ColEl origin of replication for isoelectric focusing, polyacrylamide gel electrophoresis was per-
ned at 24 °C in the presence of 6 M urea. Gel composition and buffers
were as described earlier (21) except for the omission of the detergent and increasing the acrylamide concentration in the gel to 7%. The gels contained 0.5% of amphotolyte (pH 3.5-10) and 2% amphotolyte (pH 5-8). Focusing was continued for 16 h at 150 V, followed by 15 min each at 500 and 1000 V. Coomassie Brilliant Blue (0.1%) in methanol-acetic acid-water (5:1:5) was used to visualize the proteins; destaining was achieved in the same solvent lacking dye. Other than for isoelectric focusing, polyacrylamide gel electrophoresis was performed with a Phast System (Pharmacia). Under nondenaturing conditions, separations were achieved on 20% gels, under denaturing conditions in the presence of sodium dodecyl sulfate, 8-25% gradients of acrylamide were used.

RESULTS

Gross Characteristics of Position-111 Mutants—The levels of mutant carboxylases in cell-free extracts of E. coli transformed by pFL200-derived constructs were 5-10% of total soluble protein, as estimated by polyacrylamide gel electro-

FIG. 1. Active-site residues of ribulose-P_2 carboxylase in the immediate vicinity of bound carboxyarabinitol-P_2 as determined by x-ray crystallography (2-4). Residue numbers refer to the R. rubrum enzyme; B after E48 and N111 denotes their location in the adjacent subunit. (Adapted from the schematic of the spinach enzyme illustrated in Ref. 2.)

FIG. 2. Synthetic oligonucleotide primers were used to mutate the codon for N111 (boldface) of R. rubrum ribulose-P_2 carboxylase. A segment of the rbc gene encompassing the codon for N111 is included, and the base substitutions necessary for the desired amino acid replacements are underlined.

For replacement of N111 includes the ColEl origin of replication and confers resistance to ampicillin, and the other vector (bearing the mutation for replacement of E48, K166, K191, or K329) includes the p15A origin of replication and confers chloramphenicol resistance. These plasmids can be introduced into the same cell and selected for resistance to both ampicillin and chloramphenicol. The host used in these experiments is Rec- to eliminate any likelihood of formation of a wild-type rbc gene by recombination.

Protein and Enzyme Assays—Protein concentrations were deter-
mained by a dye binding assay (17); pure ribulose-P_2 carboxylase isolated from R. rubrum served as the standard. Carboxylase activity was monitored by the [14CO_2] fixation assay (18) as recently modified (19).

Exchange of Substrate and Solvent Protons—The enolization partial reaction was assayed by monitoring the enzyme-catalyzed detri-
tiation of [3-3H]ribulose-P_2 (6, 20). The assay mixture (100 μl) at pH 8.0 contained 0.4 mM [3-3H]ribulose-P_2, 50 mM Bicine, 10 mM MgC\textsubscript{2}, 66 mM NaHC\textsubscript{3}, 1 mM EDTA, and carboxylase (wild-type or mutant), which was added last. Periodically, 10-μl aliquots were mixed with 100 μl of 50 mM NaBH\textsubscript{4} to reduce the residual ribulose-P_2 to acid-
stable pentitol-P_2. Excess NaBH\textsubscript{4} was decomposed 10 min later by the addition of 0.5 ml of 1 N acetic acid. The quenched aliquots were taken to dryness in a convection oven at 110 °C and were redissolved in 1 ml of H\textsubscript{2}O prior to scintillation counting.

Binding of Carboxyarabinitol-P_2 to Mutant Proteins—To a solution (0.5 ml) of the carboxylase (1 mg/ml) in a pH 8.0 buffer (50 mM Bicine, 10 mM MgCl\textsubscript{2}, 66 mM NaHC\textsubscript{3}, 1 mM EDTA) was added a 1.2-fold molar excess (relative to subunit) of [14C]carboxyarabinitol-P_2 (1.2 x 10\textsuperscript{6} cpm/nmol). 30 min later, unlabeled carboxyarabinitol-P_2 (20 times the amount in the first addition) was then added to the incubation mixture. A 100-μl aliquot was subjected immediately to gel filtration on a 0.9 x 30-cm column of Sephadex G-50 (fine). Fractions were monitored for protein and for radioactivity so that the stoichiometry of carboxyarabinitol-P_2 binding could be calculated. This value established maximal binding at time "zero." The rate of exchange of labeled for unlabeled ligand was determined by periodically subjecting additional 100-μl aliquots to gel filtration under precisely the same conditions.

Electrophoretic Separations—A vertical microslab apparatus (Idea Scientific, Corvallis, OR), which accommodates gels that are 100 mm high x 90 mm wide x 0.5 mm high x 0.5 mm thick, was used for isoelectric focusing at 24 °C in the presence of 6 M urea. Gel composition and buffers were as described earlier (21) except for the omission of the detergent and increasing the acrylamide concentration in the gel to 7%. The gels contained 0.5% of amphotolyte (pH 3.5-10) and 2% amphotolyte (pH 5-8). Focusing was continued for 16 h at 150 V, followed by 15 min each at 500 and 1000 V. Coomassie Brilliant Blue (0.1%) in methanol-acetic acid-water (5:1:5) was used to visualize the proteins; destaining was achieved in the same solvent lacking dye. Other than for isoelectric focusing, polyacrylamide gel electrophoresis was performed with a Phast System (Pharmacia). Under nondenaturing conditions, separations were achieved on 20% gels, under denaturing conditions in the presence of sodium dodecyl sulfate, 8-25% gradients of acrylamide were used.
phoresis. These levels are similar to that of the wild-type carboxylase encoded by pFL200 (13). As in the cases of other mutant carboxylases studied previously, the position-111 mutants are readily purified by fast-protein liquid chromatography on MonoQ columns. Based on their electrophoretic mobilities, which are very similar to that of wild-type enzyme under non-denaturing conditions, the mutant proteins are also dimeric (Fig. 3). Hence, even the nonconservative replacement of N111 by K does not preclude subunit-subunit association.

Comigration of wild-type and mutant subunits on polyacrylamide gels in the presence of sodium dodecyl sulfate confirms that the mutant genes are expressed as full-length translation products and that even limited proteolysis has not occurred during isolation (data not shown).

None of the mutant proteins retain readily measurable carboxylase activity. Under our standard assay conditions for measuring $^{14}C\text{-CO}_2$-fixation with wild-type enzyme at 20 μg/ml, we observe the fixation of ~30,000 cpm per 50-μl aliquot of reaction mixture per min. With the mutant proteins at 200 μg/ml, <1000 cpm per 50-μl aliquots of reaction mixture are fixed per h, corresponding to <0.01% of wild-type activity. These observed deficiencies appear to be in $k_{cat}$, because increasing the concentration of ribulose-5P from 0.4 to 3 mM in the assays did not stimulate carboxylase activity; the $K_m$ for wild-type enzyme is only 10 μM.

Exchange of the C-3 Proton of Ribulose-5P with Solvent—The enzyme-catalyzed conversion of ribulose-5P and CO$_2$ to 3-phospho-D-glycerate entails a series of discrete partial reactions (22–24). The initial step in the overall transformation is abstraction of the C-3 proton of ribulose-5P to form the enediol(ate) intermediate. This partial reaction is readily monitored based on deprotonation of [3-$^3$H]ribulose-5P. Obviously, the actual rate of deprotonation of ribulose-5P must be at least as great as that of the overall $k_{cat}$ of carboxylation. However, when measured with trace-labeled [3-$^3$H]ribulose-5P, deprotonation appears ~2-fold slower than $k_{cat}$, signifying enolization as partially rate limiting in overall catalysis (20, 25). Thus, if the deficiency of a mutant carboxylase reflects substitution of the proton acceptor required for the initial catalytic event, this step should become even more rate-limiting, resulting in an increased kinetic isotope effect and a decrease in the relative rates of enolization and carboxylation, compared to wild-type enzyme.

The possible involvement of N111 in enolization was examined by ascertaining the ability of the mutant proteins to catalyze deprotonation of [3-$^3$H]ribulose-5P. As illustrated in Fig. 4, each of the position-111 mutant proteins does display significant deprotonation activity. Rates relative to wild-type enzyme are as follows: N111Q, 30–40%; N111D, 4–8%; N111S, 3–6%; N111K, 2–5%. The $K_m$ (ribulose-5P) for the N111Q mutant is very close to the value for wild-type enzyme (15 μM versus 10 μM). Although the $K_m$ values have not been determined for the other mutant proteins, the enolization rates were not significantly increased by the use of 3 mM rather than 0.4 mM ribulose-5P in the assays.

Formation and Stability of Complexes with Reaction-Intermediate Analogues—Carboxyarabinitol-5P, an analogue of the six-carbon reaction intermediate in the carboxylation reaction pathway, is a slow, tight-binding inhibitor of the carboxylase (10). The activated (carbamylated) form of the enzyme binds carboxyarabinitol-5P with such tenacity that a quaternary complex (protein–CO$_2$–Mg$^{2+}$–inhibitor) may be isolated by gel filtration (28). In contrast, the binary complex of nonactivated enzyme and inhibitor is insufficiently stable to permit its isolation in this fashion. Hence, a convenient diagnostic is provided for ascertaining the ability of catalytically deficient mutant proteins to undergo normal activation and to bind phosphorylated ligands.

By analogy with wild-type enzyme, position-111 mutant proteins (in the presence of Mg$^{2+}$ and HCO$_3^{-}$) bind [2-$^{13}$C]carboxyarabinitol-5P stoichiometrically. However, the stabilities of these complexes are compromised relative to that of wild-type enzyme, as demonstrated by the rate of ligand exchange induced by a 20-fold excess of unlabeled inhibitor (Fig. 5). The half-times of exchange are 21, 8, 1.5, and 1 h for wild type, N111Q, N111D, and N111S, respectively. Complex formation is not observed with N111K.

Heterodimer Formation—In vivo generation of catalytically active heterodimers from two inactive mutant proteins, each with a single amino acid substitution, demonstrated that the active sites of the carboxylase are created by interacting domains from adjacent subunits (1). This approach provides a facile, direct identification of the domain location of any active-site residue. Based on the crystallographic assignment of N111 to the amino-terminal domain, position-111 mutants should be complemented by mutants that contain amino acid substitutions within the carboxyl-terminal domain (Fig. 6). Indeed, hybrids of N111S with K166G, K191C, or K329G are all active (Table I). (K166, K191, and K329 are located within

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**Fig. 3.** Polyacrylamide gel electrophoresis under non-denaturing conditions. Lanes 1 and 6, wild-type enzyme; lane 2, N111Q; lane 3, N111D; lane 4, N111S; lane 5, N111K.

**Fig. 4.** Rates of deprotonation of [3-$^3$H]ribulose-5P as catalyzed by wild-type carboxylase (50 μg/ml) ( ), N111Q (110 μg/ml) ( ), N111D (165 μg/ml) ( ), N111S (165 μg/ml) ( ), and N111K (95 μg/ml) ( ). See “Experimental Procedures” for further details.
FIG. 5. Exchange of bound [2-14C]carboxyarabinitol-P2 from quaternary complexes of wild-type and mutant carboxylases with free unlabeled analogue. Wild type (O); N111Q (■); N111D (△); N111S (▲). See “Experimental Procedures” for further details.

FIG. 6. Schematic diagram of the different species that can be formed in a cell coexpressing the rbc genes for the K166G and the N111S mutant subunits. The carboxyl-terminal domain of the active site is illustrated by the rectangular indentations, and the amino-terminal domain of the active site is illustrated by the triangular indentations. Indentations outlined in black represent nonfunctional domains due to specific amino acid substitutions. Heterodimer formation from the two mutant subunits generates a species with one wild-type active site per dimeric molecule. Statistical distribution predicts a 1:2:1 ratio of K166G homodimer; K166G/N111S heterodimer; N111S homodimer and hence a specific enzyme activity for purified preparations of 25% the wild-type value.

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The levels of activity (measured in crude extracts or purified preparations) are similar to those observed with hybrids of K166G/E48Q or K166D/E48Q, indicative of a statistical distribution of inactive homodimers and active heterodimers with one functional active site per molecule. In contrast, coexpression of the genes for N111S and E48Q (both substitutions in the amino-terminal domain) does not result in the formation of an enzymically active species. The homo- and heterodimers in purified preparations are not resolved by electrophoreses under nondenaturing conditions and comigrate with wild-type enzyme (Fig. 7A). However, the predicted subunit heterogeneity of these preparations is clearly demonstrated by isoelectric focusing under denaturing conditions (Fig. 7B).

DISCUSSION

High resolution x-ray crystallographic analyses of ribulose-P2 carboxylase identified N111 as an active-site residue (2-4). As the precise location of its side chain depends on the ligand-binding state of the enzyme, extrapolation of structure to function becomes unusually challenging. Thus, we were motivated to ascertain the consequences of replacement of N111 by other amino acid residues. These included serine to retain the polar character of asparagine without introducing additional bulk, glutamine to retain the amide functionality, aspartate to introduce a negatively charged side chain sterically similar to that of asparagine, and lysine to introduce a positive charge.

Even the more conservative substitutions resulted in complete loss of measurable carboxylase activity, thereby demonstrating the high stringency for an asparaginyl residue at position 111. Several observations appear to exclude major conformational changes as the basis of catalytic deficiencies of the mutant proteins. Subunit folding is not perturbed in that position-111 mutant subunits form stable homodimers and can also associate with carboxyl-terminal mutant subunits giving rise to enzymatically active heterodimers. Excepting N111K, the other mutants in the series are able to form an isolable complex, in the presence of Mg2+ and HCO3−, with carboxyarabinitol-P2. By analogy with wild-type enzyme, tight-binding of the inhibitor by the mutant proteins is indicative of prior carbamylation and coordination of Mg2+. Further evidence for an active-site topology very similar to that of wild-type enzyme is provided by the catalytic competence of the mutant proteins in the enolization partial reaction. About 2% of the wild-type rate in catalyzing detritiation of [3-3H]ribulose-P2 is retained even upon the nonconservative replacement of N111 by K. The N111Q protein displays >30% of the wild-type activity in enolization with a Ka for ribulose-P2 which is increased less than 2-fold. Thus, the structural and functional features of ribulose-P2 carboxylase necessary for subunit-subunit association, activation, binding of ribulose-P2, and the initial step in overall catalysis remain intact, at least qualitatively, in the position-111 mutant proteins.

Their inability to catalyze the overall conversion of ribulose-P2 to 3-d-phosphoglycerate presumably reflects a preferential impact at some step beyond the initial proton abstraction.

Barring disruptions of subunit folding and association, the crystallographic assignment of N111 to the amino-terminal domain of the active site prompts the prediction that heterodimers composed of one position-111 mutant subunit and one carboxyl-terminal domain subunit will be enzymatically active in contrast to the two parental homodimers (Fig. 6). Beyond confirmation of the domain location of N111, the demonstrated formation of active heterodimers emphasizes the tolerance of proper subunit association even to substitutions of active-site residues of the amino-terminal domain that are in direct contact with residues of the adjacent subunit. Despite the intersubunit interaction between N111 and E194 and the proximity of several lysyl residues, replacement of N111 by D or K is not disruptive of subunit-subunit association. In contrast, replacement of K166 or K168 (located within
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Ribulose-1,5-bisphosphate carboxylase activity from E. coli cells expressing various alleles of the rbc gene from R. rubrum

<table>
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<td>N111S/K329G</td>
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* Previous investigations (6, 19, 33) have shown that E48Q, K166G, K191C, and K329G mutant proteins are virtually devoid of carboxylase activity, and hence their alleles are not included in this table.

The failure to detect complexation of N111K with carboxyarabinitol-P_2 by gel filtration is probably due to weakened ligand-protein interactions rather than absence of binding per se. This interpretation is predicated on the assumption that a mutant protein capable of binding ribulose-P_2 will also bind carboxyarabinitol-P_2; binding of ribulose-P_2 is proven by the enolization activity retained by N111K. Stable complexation of the other position-111 mutant proteins with carboxyarabinitol-P_2 occurs only in the presence of Mg^{2+} and HCO_3^-, consistent with carboxylation as a prerequisite for tight binding of the reaction-intermediate analogue. The exchange rates between bound and free carboxyarabinitol-P_2 from quaternary complexes of the mutant proteins are considerably greater than with the wild-type counterpart, signifying weaker interactions. Among the mutant proteins examined, the complex of N111Q is the most stable, but even in this case the ligand-exchange rate is about 3-fold greater than that of wild type. Hence, a plausible role of N111 is to stabilize the transition state mimicked by carboxyarabinitol-P_2, consistent with proximity of the amide side chain to substituents at C2, C3, and C4 of the bound inhibitor or bound substrate as identified by x-ray crystallography.

One mechanistic suggestion based on considerations of the three-dimensional structure of the carbamylated R. rubrum carboxylase complexed with ribulose-P_2 is that the amide side chain of N111 might serve as the base that abstracts the C3 proton from ribulose-P_2 and thereby initiates the overall reaction pathway (4). The retention of substantial levels of enolization activity, despite the absence of detectable carboxylase activity, by mutant proteins with nonconservative substitutions (i.e. N111K) casts serious doubt on any postulate that invokes a critical role of N111 in enolization. Directly or indirectly, N111 may modestly facilitate enolization, because N111Q (with retention of an amide side chain) is considerably more efficient than either N111D, N111S, or N111K in promoting this reaction. However, the 1-A displacement of the N111 side chain by residue substitution with Q reduces carboxylase activity by >10^4-fold despite only a ~3-fold impact on enolization rate. Thus, the more crucial demand for the asparaginyl side chain must be fulfilled beyond the formation of enediol(ate) in the reaction coordinate.

Residual enolization activity inherent to various site-directed mutants of ribulose-P_2 carboxylase has also been used as an argument (6, 28) against some of the other candidate bases offered by structural studies, which include K329, S368, and H321 (2-4). A counter assertion could be made that removal (substitution) of the initial proton acceptor would only reduce the enolization rate by 10- to 100-fold due to a basal, noncatalyzed ionization of the C3 proton of ribulose-P_2. If this were the case, mutagenesis studies reported thus far could not be used to eliminate from consideration any of the side chains put forward by crystallography. However, substitutions for known bases of other enzymes that abstract protons from carbon atoms generally result in kcat values that are reduced by >10^4-fold (29–32). Therefore, we conclude that neither K329, S368, H321, nor N111 is the base that initiates catalysis. Additional candidates for this catalytic group based on evaluations of the three-dimensional structure include H287, the carbamate nitrogen of K191, and the C1 phosphate group of substrate (2–4). Functional analyses demonstrate some catalytic role of the two former groups but do not permit conclusions as to their potential involvement in enolization (33, 34).

K166 is an active-site residue endowed with properties anticipated of the key base responsible for C3 proton abstraction. The ε-amino group of this residue is unusually nucleophilic yet highly acidic with a pK_a of ~7.9 (35), similar to an ionization observed in the pH dependence of the deuterium isotope effect with [3-^2H]ribulose-P_2 as substrate (25). Removal of the side chain functionality (i.e. K166G) reduces kcat for carboxylation by 10^4-fold (36); furthermore, the mutant protein is devoid of detectable enolization activity, but is competent in catalyzing the conversion of an isolated reaction intermediate into product (37). Despite these diverse indicators of a direct role for K166 as the initial proton acceptor, the three-dimensional structural analyses (3, 4) place its ε-amino group too remote from C3 of ribulose-P_2 to function in the proposed fashion. Further investigations will be required to reconcile some mechanistic interpretations derived from structural and functional analyses, certainly with respect to the identity of the proton abstractor. Crystallographic anal-
yses of some of the more relevant mutants is one rather obvious need.

The present study provides the first demonstration that N111 of ribulose-P2 carboxylase is intimately associated with catalysis. Although the data do not pinpoint the precise role of N111, they appear incompatible with the asparaginyl side chain fulfilling a requirement for enolization. Transformation of the initial enediol(ate) intermediate to final products is absolutely dependent on the occupancy of position 111 by an asparaginyl residue. As its side chain is nonionizable, a contribution to stabilization of some transition state that occurs subsequent to enediol(ate) formation would seem more credible than suggesting a direct role in any proton transfer step. Indirect facilitation of such a step cannot be discounted.

Acknowledgments—We express sincere gratitude to Professors C.-I. Brändén and G. Schneider of the Swedish University of Agricultural Sciences, Uppsala, Sweden, for providing crystallographic coordinates for the spinach carboxylase quaternary complex and the R. rubrum carboxylase binary complex with 3-phospho-D-glycerate.

Note Added in Proof—The N111Q mutant is also grossly deficient in oxygenase activity, thereby precluding precise measurements of catalytic rates. However, based on the product ratio of labeled [3-phosphoglycerate]/[2-phosphoglycolate] derived from [1-14C]ribulose-P2 as substrate, the specificity factor for the mutant protein is only ~1 in contrast to a value of ~10 for the wild-type enzyme.

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