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Site-directed mutagenesis to determine essential residues of ribulose-bisphosphate carboxylase of *Rhodospirillum rubrum*

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Abstract. Both Lys-166 and His-291 of ribulosebisphosphate carboxylase/oxygenase from *Rhodospirillum rubrum* have been implicated as the active-site residue that initiates catalysis. To decide between these two candidates, we resorted to site-directed mutagenesis to replace Lys-166 and His-291 with several amino acids. All 7 of the position-166 mutants tested are severely deficient in carboxylase activity, whereas the alanine and serine mutants at position 291 are ~40% and ~18% as active as the native carboxylase, essentially ruling out His-291 in the *Rhodospirillum rubrum* carboxylase (and by inference His-298 in the spinach enzyme) as a catalytically essential residue. The ability of some of the mutant proteins to undergo carbamate formation or to bind either ribulosebisphosphate or a transition-state analogue remains largely unimpaired. This implies that Lys-166 is not required for substrate binding; rather, the results corroborate the earlier postulate that Lys-166 functions as an acid-base group in catalysis or in stabilizing a transition state in the reaction pathway.

Keywords. Ribulose-P_2 carboxylase; site-directed mutagenesis; essentiality of Lys-166; non-essentiality of His-291.

Introduction

In recent years, site-directed mutagenesis of proteins, or protein engineering, has developed into a powerful tool for studies of structure-function relationships of proteins. Among the various techniques of site-directed mutagenesis (Smith, 1985), the use of oligonucleotide-directed mutagenesis has provided a general method for producing desired mutations, such as base substitutions, deletions and insertions, at specific locations of the gene thereby leading to specific amino acid changes at desired sites of the protein under study (Smith and Gillam, 1981). Of the oligonucleotide-directed mutagenesis protocols, the most versatile is based on the use of a circular single-stranded (ss) DNA vector (usually M13 DNA) containing the target gene sequence and a synthetic DNA oligonucleotide containing the desired mutation to prime the synthesis *in vitro* of the complementary strand (Hutchison *et al.,* 1978; Razin *et al.,* 1978; Gillam and Smith, 1979a,b; Zoller and Smith, 1982, 1983).

Following transformation of competent cells with the resulting heteroduplex (usually enriched by band sedimentation in alkaline sucrose) containing the incorporated oligonucleotide, semi-conservative replication *in vivo* leads to the formation of homoduplexes derived from mutant and parental strands. Preliminary

Abbreviations used: ss, Single-stranded; ribulose-P_2 carboxylase, D-ribulose 1,5-bisphosphate carboxylase/oxygenase; *rbc* gene, gene for ribulose-P2 carboxylase; Td, temperature of dissociation; carboxyarabinitol-P_2, 2-carboxyarabinitol 1,5-bisphosphate; RF, replication form.

screening for mutants is usually done by differential annealing with radioactively labelled mutagenic oligonucleotide, based on the substantial difference in the melting temperature of a perfectly matched duplex and that of a mismatched one (Wallace *et al.,* 1979). A convenient secondary screening often takes advantage of either the introduction of a new restriction endonuclease site or removal of an existing one. Final confirmation is obtained by direct sequence determination of the region encompassing the desired mutation.

D-ribulose 1,5-bisphosphate carboxylase/oxygenase (Ribulose-P_2 carboxylase) (EC, 4·1·1·39), which catalyzes the carboxylation of ribulose-P_2 to yield two molar equivalents of D-3-phosphoglycerate, is ubiquitous to photosynthetic organisms and provides the only significant route by which atmospheric CO_2 is converted to carbohydrate and therefore is absolutely essential to all higher forms of life. The enzyme also catalyzes an energy-wasteful, physiologically nonessential oxygenation reaction which competes with CO_2 utilization (see Lorimer, 1981; Miziorko and Lorimer, 1983, for reviews). Hence, an important long-term goal of our programme is to attempt alteration of the enzyme's substrate specificity to favor carboxylase activity, thereby increasing agricultural yields.

Active-site characterization is a key step in the elucidation of any enzyme mechanism. For ribulose-P_2 carboxylase, the complex yet well-established reaction pathway (figure 1) entails (a) enolization of ribulose-P_2 *via* abstraction of its C3 proton, (b) stereospecific carboxylation of the enediol intermediate, (c) hydration and carbon-carbon scission of the resultant 2-carboxy-3-keto intermediate, and (d) inversion and protonation of the aci-acid of 3-phosphoglycerate to complete product formation (Miziorko and Lorimer, 1983; Lorimer *et al.,* 1984; Jaworowski and Rose, 1985; Pierce *et al.*, 1986). The essential base initiating catalysis in step (a) has a p*K*a of 7·5 as established by the pH dependence of V_{max} and the pH dependence of the deuterium isotope effects with [3-^{2}H]ribulose-P_2 as substrate (Van Dyk and Schloss, 1986).

The use of affinity labels has proved invaluable in mapping and characterizing the active sites of ribulose-P_2 carboxylase (see Hartman *et al.,* 1984, for a review). The structural complexity of ribulose-P_2 carboxylase and lack of absolute specificity of the affinity labels used prompted the reliance on comparative amino acid sequence analysis to reveal whether residues implicated at the active-site by affinity labeling are indeed species invariant and thus likely to be essential to function (Hartman *et al.,* 1984; Nargang *et al.,* 1984). Because of their evolutionary diversity and structural dissimilarities, the carboxylases from spinach and *Rhodospirillum rubrum* (a purple non-sulphur photosynthetic bacterium) provide a stringent test of structural conservation. The quaternary structure of ribulose-P_2 carboxylase from spinach is typical among all higher plant and most bacterial carboxylases in being a hexadecamer with 8 large (53,000-Da) and 8 small (14,000-Da) subunits (Rutner, 1970; Martin, 1979; Zurawski *et al.,* 1981), whereas the functionally analogous enzyme from *R. rubrum* is a homodimer of 53,000-Da subunits (Tabita and McFadden, 1974; Schloss *et al.,* 1982; Hartman *et al,* 1984). Moreover, compared to the > 80% sequence homology among most ribulose-P_2 carboxylases (Miziorko and Lorimer, 1983), the homology between the *R. rubrum* carboxylase and the large subunit of the spinach enzyme is only 31 % (Hartman *et al.,* 1984; Nargang *et al.*, 1984) (see figure 2 for complete primary structures and alignments for both carboxylases).

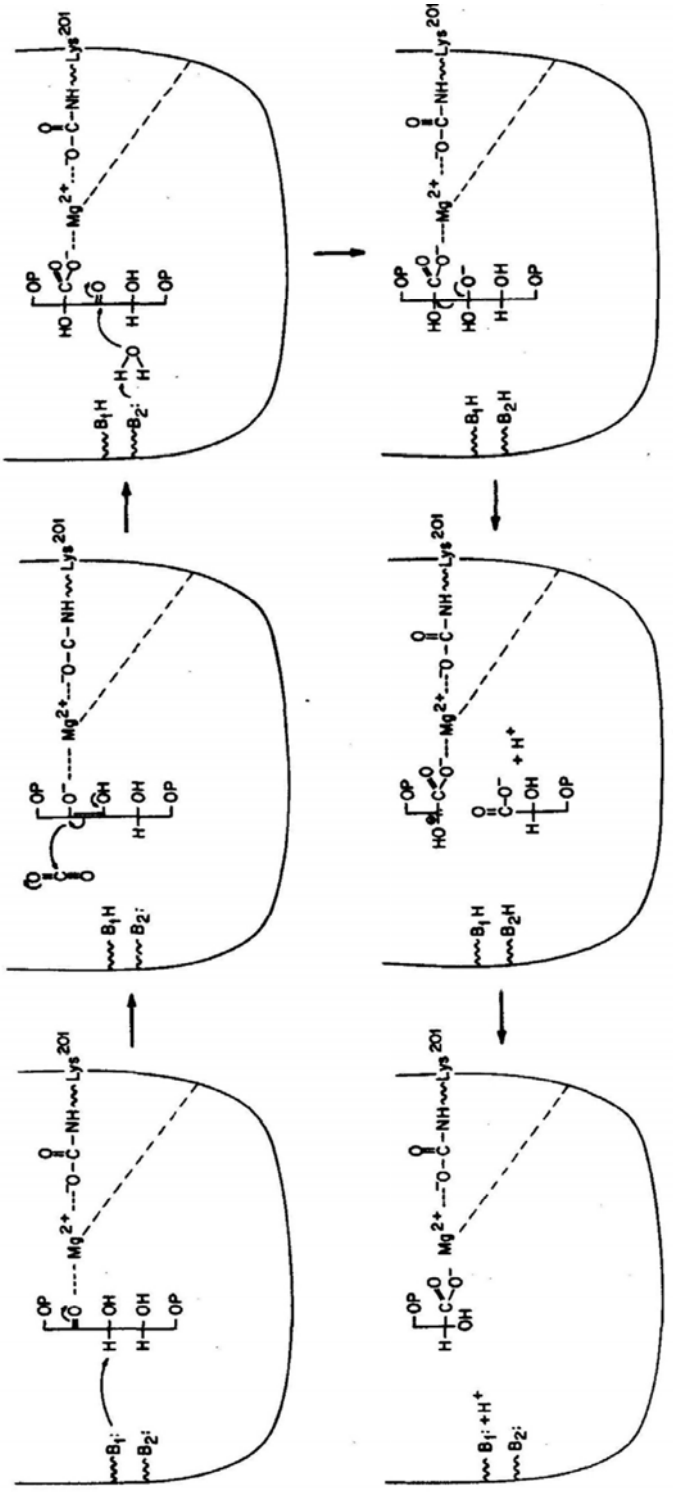

Figure 1. Reaction pathway for carboxylation of ribulose-P_2 as catalyzed by ribulose-P_2 carboxylase (Miziorko and Lorimer, 1983; Jaworowski and Rose, 1985). Reprinted from Hartman *et al.* (1986), with permission of the publisher.

Figure 2. Amino acid sequences of ribulose-P_2 carboxylase from spinach (upper) (Zurawski *et al.,* 1981) and *R. rubrum* (lower) (Hartman *et al.,* 1984; Nargang *et al.,* 1984). Alignments were made by visual inspection. Residues identical in both enzymes are enclosed in boxes. Gaps, attributed to deletions or insertions, appear as blank spaces. Peptide notations are explained in Hartman *et al.* (1984). Cysteinyl residues and residues identified at the active site or activator site by selective chemical labelling are illustrated with larger type.
(Abbreviations: Asp, D; Asn, N; Glu, E; Gln, Q; Thr, T; Ser, S; Pro, P; Gly, G; Ala, A; Cys, C; Val, V; Met, M; Ile, I; Leu, L; Tyr, Y; Phe, F; His, H; Lys, K; Trp, W; Arg, R). Reprinted from Hartman *et al.* (1984), with permission of the publisher.

Based on studies mentioned above, both Lys-175 and Lys-334 in the spinach enzyme, which correspond to Lys-166 and Lys-329 in the *R. rubrum* enzyme, appear essential for enzyme activity. The extreme nucleophilicities and acidities of the ε-amino group of Lys-166 (p*K*a ~7·9) of the *R. rubrum* enzyme and Lys-334 (p*K*a of 9·0) of the spinach enzyme, as revealed by the pH dependencies of inactivations of the two enzymes by the lysine-selective reagent trinitrobenzene sulphonate, further support the postulate that both are important to catalysis rather than to substrate binding (Hartman *et al.,* 1985). Lys-166 may correspond to the essential base that enolizes ribulose-P_2 to initiate catalysis and that displays a p*K*a of 7·5 in the pH curve for V_{max}/K_m. Aurthermore, cross-linking experiments with 4,4'-diisothiocyano-

2,2'-disulphonate stilbene showed that the two active-site lysines of the *R. rubrum* enzyme are within 12 Å (Lee *et al.,* 1987).

His-298 of the spinach enzyme was initially implicated as an essential active-site constituent based on inactivation of the enzyme by diethyl pyrocarbonate (Saluja and McFadden, 1982) and the demonstration that the loss of activity correlated with modification of this residue (Igarashi *et al.,* 1985). A study of the pH dependence of enzyme inactivation by diethyl pyrocarbonate led to the suggestion that His-298 reflected an essential base (p*K*a ~6.8) observed in the pH profile for V_{max} (Paech, 1985).

It appeared unlikely that both a lysyl and an histidyl residue serve to abstract the C3 proton from ribulose-P_2. Both, however, could be essential acid/base groups, since discrete steps in the overall reaction pathway (figure 1) entail proton transfers. In order to clarify the suggested roles of Lys-166 and His-291 in enzyme catalysis, we resorted to site-directed mutagenesis of the *R. rubrum* gene encoding ribulose-P_2 carboxylase at these two sites.

Experimental Procedures

Materials

Chemicals and Enzymes: These have been described earlier (Niyogi *et al.,* 1986).

Strains, plasmids, and phage: Escherichia coli JM107 [(Δ(*lac pro*), *end A*1, *gyr*A96, *thi*-1, *hsdR* 17, *supE*44, *rel*A1 (F' *traD*36, *proAB*$^+$, *lacI*^{q}Z ΔM15)] (Yanisch-Perron *et al.,* 1985) was used as the host for M13 vectors and for expression of the *rbc* gene. *E. coli* BNN45 (*met*B, *thi*$^-$, *hsdR, lacY, supE*44, *supF*), derived from ED8654 (Murray *et al.,* 1977), was used as a cloning host.

The construction of plasmid pFL34 containing the *rbc* gene which expresses wild-type promoter has been described (Larimer *et al.,* 1986). The 1546-base pair *Bam*HI fragment containing the *rbc* gene was transferred into M13 mpl9 vector (pAL19) in the orientation such that the ss phage DNA contains the strand complementary to the mRNA of the *rbc* gene. The phage DNA was purified from CsCl-banded phage as described earlier (Niyogi and Mitra, 1978).

Methods

Synthesis of Oligonucleotides: Oligonucleotides (figure 3) used to produce mutations at the 166 and 291 sites in the *rbc* gene were synthesized by the phosphoramidite method (Sinha *et al.,* 1984) using a Systec model 1450 automated DNA synthesizer and were purified by electrophoresis on 20% Polyacrylamide gels containing 8M urea (Atkinson and Smith, 1984).

Oligonucleotide-directed mutagenesis of the rbc gene: This was done as described before (Niyogi *et al.,* 1986) with some modifications. For annealing (in the single primer extension method—Smith, 1985; Nisbet and Beilharz, 1985; Niyogi *et al*., 1986) the phosphorylated oligonucleotide (20 pmol) was heated with pFL19 DNA (1 pmol) for 10 min at a temperature 5-10°C higher than the empirically calculated temperature of dissociation (Td) (Meinkoth and Wahl, 1984), then at 10°C below the

A.

G T I I K P K L G L R
5'-GGCACGATCATCAAGCCGAAGCTCGGCCTGCGT-3'
3'-CCGTGCTAGTAGTTCGGCTTCGAGCCGGACGCA-5'

Mutant	Number	Oligonucleotide sequence	Strand	Monitored Restriction Site
Ala-166	1	5'-GATCATCGCGCCGAAGCTTGGCCTGC-3'	+	HindIII (+)
Arg-166	2	5'-TCATCAGGCCGAAGCTTGGCCT-3'	+	HindIII (+)
Cys-166	3	5'-CACGATCATCTGCCCGAAGCTCGGCCTGCGT-3'	+	HindIII (-)
Cys-166	4	3'-CATGGTAGTAGACGGGCTTCGA-5'	-	
Gln-166	5	3'-CATGCTAGTAGGTCGGCTTCGA-5'	-	KpnI (-)
Gly-166	6	3'-CATGGTAGTAGCCGGGCTTCGA-5'	-	
His-166	7	5'-GATCATCCACCCGAAGCTTGGCCT-3'	+	HindIII (+)
Ser-166	8	5'-ATCATCAGCCCGAAGC-3'	+	

B.

R A G H G A V
5'-CGGGCTGGCCACGGCGCCGTC-3'
3'-GCCCGACCGGTGCCGCGGCAG-5'

Mutant	Number	Oligonucleotide sequence	Strand	Monitored Restriction Site
Ala-291	I	5'-CGGGCTGGCGCCGGCGCCGTC-3'	+	BalI (-)
Ser-291	II	5'-CGGGCTGGCAGCGGCGCCGTC-3'	+	BalI (-)
Leu-291	III	5'-CGGGCTGGCCTCGGCGCCGTC-3'	+	BalI (-)
Lys-291	IV	5'-CGGGCTGGCAAGGGCGCCGTC-3'	+	BalI (-)
Arg-291	V	5'-CGGGCTGGCCGCGGCGCCGTC-3'	+	BalI (-)

Figure 3. Nucleotide sequences and encoded protein sequences of the *rbc* gene in the vicinity of the codon for (A) Lys-166 and (B) His-291, and relevant features of oligonucleotides used to introduce amino acid substitutions at these positions. The position-166 and position-291 codons or anticodons are underlined and the base substitutions which give rise to the planned mutations are denoted by asterisks. Overlining indicates a restriction site.

Td for 15 min, followed by cooling at room temperature for 1 h. The yields of RF I DNA ranged between 5-50% of the input ssDNA template, depending on the choice of replication protocol and the (G + C) content of the oligonucleotide site (discussed later). Aliquots of RF I DNA were used to transfect *E. coli* JM107 according to the procedure of Hanahan (1985).

Screening and characterization of mutants: Plaque hybridization (Norrander *et al.*, 1983) with appropriate [^{32}P]-labelled oligonucleotides as probes was performed for 1 h at a temperature 20°C below the empirically calculated Td (Meinkoth and Wahl, 1984), then washing at an appropriate temperature to discriminate between wild type and mutant phages, followed by autoradiography (Niyogi *et al.,* 1986). The candidate mutant phages from individual plaques were then grown, and RF DNA was isolated by the alkali lysis procedure (Maniatis *et al.,* 1982). Further screening was performed by appropriate restriction endonuclease treatments (see 'results' section). Mutations were finally confirmed by direct sequencing of ss phage DNA by the dideoxynucleotide chain termination protocol (Sanger *et al.,* 1977) using appropriate primers upstream of the target sequences.

Expression and induction of mutant carboxylases: Fragments harboring mutations generated by single primer extension were obtained by appropriate restriction endonuclease treatment of the corresponding RF I DNAs and subcloned into expression vectors. Mutants were also generated by 'bandaid mutagenesis'* directly into the expression vectors. Such mutations were screened by restriction enzyme analysis and confirmed by sequencing of double-stranded plasmid DNA (Chen and Seeburg, 1985; Zagursky *et al.,* 1985). The induction by isopropylthiogalactoside and expression of normal or mutant carboxylase have been described (Niyogi *et al.,* 1986). Purification to near homogeneity, by immunoaffinity chromatography, of normal and mutant carboxylases from *E. coli* cell extracts has been described earlier (Niyogi *et al.*, 1986).

Protein and enzyme assays: Protein concentrations were determined according to Bradford (1976). Carboxylase and oxygenase activities were determined according to Lorimer *et al.,* (1977). Quantitation of carboxylase protein by dot immunobinding was performed as described earlier (Niyogi *et al.,* 1986).

Results

Use of restriction enzyme digestion for additional screening

As evident from figure 3, three of the mutants used in these studies, namely, Cys-166, Gin-166, and Gly-166, were introduced by bandaid mutagenesis. Silent mutations

*Bandaid mutagenesis (Mural and Foote, 1986), independently reported as notch cloning (Childs *et al.*, 1985), uses a ss oligodeoxynucleotide, which codes for the desired mutation, to span a gap in the target gene which is created by digestion of the plasmid with two restriction endonucleases, one of which leaves a 3' protruding end while the other leaves a 5' protruding end. Since this technique involves a gap in the target sequence, it is similar to cassette mutagenesis (Wells *et al.,* 1985).

were introduced to create a *Kpn* I site and a *Hind* III site in the region flanking the lysine codon at position 166, thereby creating a vector which can be used for bandaid mutagenesis. The other mutants were generated by single primer extension. Silent mutations were introduced to create a *Hind* III restriction site, originally absent in the wild-type, in Ala-166, Arg-166, and His-166. For the His-291 site, a mutation leading to an amino acid substitution at that site also causes loss of the *Bal* I restriction site normally present in that region. These alterations provided convenient additional screening, besides differential hybridization, of potential mutants. Final confirmations of mutants was always obtained by direct sequence analysis of the mutant DNA.

As seen in figure 3, the Cys-166 mutation was generated by both the bandaid protocol and by the single primer method. No differences were found between these two independently derived mutant proteins.

Purity and quaternary structure of the mutant proteins

The mutant proteins, isolated by immunoaffinity chromatography, were >90% pure. The almost identical electrophoretic mobilities of the native and mutant proteins on nondenaturing gels suggest that the mutants are also dimeric like the native enzyme (Niyogi *et al.,* 1986; Hartman *et al,* 1987). Gel filtration on Sephadex G-150 further confirmed this conclusion (data not shown). Gel electrophoresis in the presence of sodium dodecyl sulphate showed that the mutant proteins are physically indistinguishable from wild-type enzyme (Niyogi *et al.,* 1986; Hartman *et al.,* 1987).

Enzymatic properties of mutant carboxylases

Based on enzyme assays with purified mutant proteins, it is clear that the position-166 mutants either lack or display very low levels of carboxylase activity (table 1). None of the mutant proteins show detectable oxygenase activity (results not shown). It was possible to determine the K_m and k_{cat} of the serine mutant: these values are 35μM and 0·0072 s^{-1} (Hartman *et al.,* 1987) compared to 6 μM and 3·6s^{-1} for wild-type enzyme (Niyogi *et al.,* 1986). The catalytic efficiency (k_{cat} /K_m) of the serine mutant is therefore only 0·03% of that of the wild-type enzyme.

Among the position-291 mutants, Ala-291 and Ser-291 display considerable levels of carboxylase activity (table 1). The oxygenase activities of these mutants were correspondingly reduced in comparison to the native enzyme (results not shown). The lowered specific activity of the alanine mutant reflected both a decreased k_{cat} and an increased K_m, with ribulose-P_2 as the substrate: these values are 1·5 s^{-1} and 90 μM compared to 3·6 s^{-1} and 6 μM for the native enzyme (table 1; Niyogi *et al.*, 1986). Hence, the catalytic efficiency of the alanine mutant is about 27% of that of the native enzyme.

Carbamylation and complexation of mutant carboxylases with carboxyarabinitol-P_2

The transition-state analogue 2-carboxyarabinitol 1,5-bisphosphate (carboxyarabinitol-P_2) is a tight-binding inhibitor of CO_2/Mg^{2+} -activated (*i.e.*, carbamylated), native carboxylase and forms a stable quaternary complex that is isolable by gel

Table 1. Enzymatic properties of mutant proteins.

Amino acid substitution	Specific activity (% wild-type)	K_m (ribulose-P_2)	Carbamate formation (mol/mol subunit)	Carboxy-arabinitol-P_2 binding (mol/mol subunit)
A. Mutations at Lys-166 Site				
Lys (wild-type)	100	6 μM	1·0	1·0
Ser	0·2	35 μM	0·6	0·8
Ala	0·1		0·4	0·6
Arg	0·02		ND	ND
Gln	⩽0·01		ND	ND
His	⩽0·01		ND	ND
Cys	⩽0·01		ND	ND
Gly	⩽0·001		0·5	0·5
B. Mutations at His-291 Site				
His (wild-type)	100	6 μM	1·0	1·0
Ala-291	40	90 μM	0·9	0·9
Ser-291	18			0·8
Leu-291	0·9		0·8	0·6
Lys-291	0·05		0·9	0·8
Arg-291	⩽0·01			

ND indicates 'not detectable' under the assay conditions. A blank space indicates that no determination was made.

filtration (Miziorko and Sealy, 1980). Separate samples of each mutant protein were incubated in the presence of Mg^{2+} with either $H^{14}CO^{-}_3$ and unlabelled carboxy-arabinitol-P_2 or unlabelled HCO3 and [^{14}C]-carboxyarabinitol-P_2; these samples were then individually subjected to gel filtration.

With the Arg, Gln, His, and Cys mutants at position-166, binding of neither $^{14}CO_2$ nor [^{14}C]-carboxyarabinitol-P_2 is detected· However, with the Ser, Ala and Arg mutants at position-166 and the Ala, Ser, Leu and Lys mutants at position-291, quaternary complexes are formed, as determined with either $^{14}CO_2$ or [^{14}C]-carboxyarabinitol-P_2 (Hartman *et al.,* 1987; Niyogi *et al.,* 1986). The observed stoichiometrics of binding CO_2 or carboxyarabinitol-P_2 are listed in table 1. When the complexes of the position-166 mutant proteins with [^{14}C]-carboxyarabinitol-P_2 are challenged with a 10-fold molar excess of unlabelled carboxyarabinitol-P_2 prior to gel filtration, the levels of protein-bound radioactivity were reduced 10-fold. Similar results were obtained with the Ala-291 mutant. These results suggest complete ligand exchange. Under identical conditions, no exchange of ligands takes place with the quaternary complex prepared from wild-type enzyme (Hartman *et al.,* 1985).

Discussion

Because of our long-term goal of applying site-directed mutagenesis to different regions of the carboxylase gene, the majority of the mutants at position-166 and all

the mutants at position-291 were generated by the generally applicable approach of single-primer extension in the M13 system (Zoller and Smith, 1983). However, satisfactory yields of replication form (RF) I DNA, a crucial requirement for the subsequent transfection step, were obtained only upon modification of published reaction protocols (Nisbet and Beilharz, 1985; Zoller and Smith, 1983). For example, the pre-melting step (an addition to the usual protocol) and the annealing reaction were performed in the absence of $MgCl_2$ and dithithreitol—usual reaction constituents—and at temperatures that reflected the Td of the particular oligonucleotide-M13 ssDNA complex. These and other modifications and the choice of the temperature and enzyme concentrations during the replication/ligation reaction (Niyogi, S. K. and Yette, M. L., unpublished results) were designed to overcome the formation of hairpin structures in ssDNA due to self-pairing (Niyogi and Mitra, 1978). The high (G + C) contents, 65% for the entire *rbc* gene, 55-65% for the 16-31-nucleotide region surrounding the Lys-166 codon, and 90% for the 21-nucleotide region surrounding the His-291 region, are particularly conducive to the formation of hairpin structures. These could presumably interfere with the annealing of the mutagenic oligonucleotide and also inhibit the elongation process during the replication reaction. The potential advantages of using bandaid mutagenesis in circumventing problems encountered during *in vitro* replication and in the facile introduction of multiple mutations at a given site will be detailed in a future publication.

Site-directed mutagenesis was employed to determine which of the two postulated amino acid residues, Lys-166 or His-291, may serve to abstract the C3 proton from ribulose-P2. The stringency of the requirement was evaluated by substitution with a number of different amino acids at each position. The rationale for choosing a particular amino acid is as follows: For Lys-166, (a) glycine, total removal of the lysyl side-chain; (b) alanine, substantial removal of the lysyl side-chain but less likely to introduce conformational changes in the polypeptide backbone; (c) serine and glutamine, to more closely approximate (in contrast to glycine and alanine) the hydrophilicity of lysine without increasing its steric bulk; (d) arginine, retention of a cationic side-chain; (e) cysteine and histidine, retention of acid/base groups for partial fulfillment of the function of the lysyl side-chain, inspite of possible perturbation caused by the heterocyclic ring of histidine. Similarly, a list of rationales can be generated for the substitutions for His-291.

The substantial activities of the Ala-291 and Ser-291 mutants (table 1) definitely exclude His-291 in the *R. rubrum* carboxylase (and by inference His-298 in the spinach carboxylase) as a catalytically essential residue. However, the decreased k_{cat} and increased K_m (for ribulose-P_2) of the Ala-291 mutant, in comparison to the corresponding parameters of the native enzyme, suggest that His-291 could be a residue in the active site region. Alternatively, the changes in the kinetic parameters could signify conformational changes brought about by substitution for a residue somewhat remote from the active site. Because of the extensive sequence homology of the regions encompassing His-291 in the *R. rubrum* enzyme and His-298 in the spinach enzyme (figure 2), the acknowledged universality of the carboxylase mechanism and the species invariance of residues assigned to the catalytic and activator sites by chemical modification data (Hartman *et al.,* 1984), one can also reasonably exclude His-298 in the spinach enzyme as a catalytically essential residue.

What about Lys-166 in the *R. rubrum* carboxylase (and by inference Lys-175 in the

spinach enzyme)? Since all 7 of the mutant proteins display very low levels or are devoid of carboxylase activity, Lys-166 appears to be essential for catalytic activity. However, the role in catalytic activity can hardly be due to substrate binding, since the K_m of the serine mutant (the most active among the mutants) for ribulose-P_2 is only 6 times greater than that observed with authentic *R. rubrum* carboxylase. The very low activity of the arginine mutant (a rather conservative replacement for lysine) also argues against a direct role of Lys-166 in substrate binding.

The ability of the Ala-166, Ser-166 and Gly-166 mutants to undergo carbamylation and subsequently bind the substrate analogue carboxyarabinitol-P_2 (table 1) clearly argues for the case that Lys-166 is not required for the activation process or for substrate binding. This argument becomes particularly convincing with values obtained with the glycine mutant (table 1) which is devoid of enzyme activity. Because of the proximity of the carbamate to the active site (Pierce and Reddy, 1986), one would have expected major perturbations of carbamate formation if Lys-166 was indeed intimately involved in the activation process.

Since Lys-166 is not essential for carbamate formation or substrate binding and given the observations that the position-166 mutants are similar to the native enzyme in terms of their dimeric structure and gross conformation, we are left with the conclusion that the ε-amino group of Lys-166 serves as a general base (perhaps the one that enolizes ribulose-P_2) or somehow stabilizes a transition state in the reaction pathway.

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