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Mutations in the Small Subunit of Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase Increase the Formation of the Misfire Product Xylulose-1,5-Bisphosphate[1]

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The small subunit (S) increases the catalytic efficiency of ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39) by stabilizing the active sites generated by four large subunit (L) dimers. This stabilization appears to be due to an influence of S on the reaction intermediate 2,3-enediol, which is formed after the abstraction of a proton from the substrate ribulose-1,5-bisphosphate. We tested the functional significance of residues that are conserved among most species in the carboxy-terminal part of S and analyzed their influence on the kinetic parameters of *Synechococcus* holoenzymes. The replacements in S (F92S, Q99G, and P108L) resulted in catalytic activities ranging from 95 to 43% of wild type. The specificity factors for the three mutant enzymes were little affected (90–96% of wild type), but $K_m(CO_2)$ values increased 0.5- to 2-fold. Mutant enzymes with replacements Q99G and P108L showed increased mis-protonation, relative to carboxylation, of the 2,3-enediol intermediate, forming 2 to 3 times more xylulose-1,5-bisphosphate per ribulose-1,5-bisphosphate utilized than wild-type or F92S enzymes. The results suggest that specific alterations of the L/S interfaces and of the hydrophobic core of S are transmitted to the active site by long-range interactions. S interactions with L may restrict the flexibility of active-site residues in L.

Photosynthetic carbon assimilation is initiated by the carboxylase activity of Rubisco. The competing oxygenase activity, catalyzing the first reaction of the photorespiratory pathway, is often considered deleterious, because photorespiration decreases photosynthetic yield by up to 20% (Ogren, 1984). The first step in Rubisco catalysis is the abstraction of the C-3 proton of RuBP to form a carbanion, which is stabilized on the enzyme as a 2,3-enediol intermediate (Pierce et al., 1986). The second step involves competition for the enediol by three substrates: CO_2, O_2, or a proton. The reaction with CO_2 and O_2 leads to either two molecules of P-glycerate or phosphoglycolate and P-glycerate, respectively (for review, see Hartman and Harpel [1994]). Competition of the two gases is unavoidable because the enediol-RuBP attacked by CO_2 in carboxylation is also susceptible to O_2 in oxygenation (Lorimer and Andrews, 1973). Re-protonation of the enediol, instead of the forward reactions with either CO_2 or O_2, an result in reversion back to RuBP, whereas mis-protonation of the enediol can form either an epimer or an isomer of RuBP, XuBP, or 3-ketoarabinitol-1,5-bisphosphate (Edmondson et al., 1990; Zhu and Jensen, 1991b).

We were interested in the function of S, which is found in many Rubisco enzymes as part of the holoenzyme complex L_8S_8. The functional unit in the complex is L_2, with two Ls sharing two active sites and four L_2 units assembled with eight S. S is not directly involved in catalysis, but its presence increases V_{max} by more than 2 orders of magnitude (Andrews, 1988). However, no residue of S is part of the active sites in any Rubisco enzyme (Knight et al., 1990; Newman and Gutteridge, 1993). How S exerts its effect on L_8 or L_2 is not clear, but several mutagenesis approaches have provided information about residues in S that are required for assembly into the hexadecameric enzyme (Wasmann et al., 1989; Fitchen et al., 1990; Flachmann and Bohnert, 1992) and for catalysis (Voordouw et al., 1987). The addition of S to isolated cyanobacterial L_8 core complexes causes a significant, albeit small, difference in pK of Lys-198, reflecting the influence of S on the carbamylation of L_8S_8 (Smrcka et al., 1991). The long-range interaction of S on the active site was also shown in competition studies between 6-phosphogluconate and RuBP. Carboxylase activity of *Synechococcus* L_8 was more sensitive to the same concentration of 6-phosphogluconate than carboxylation in L_8S_8 (Lee et al., 1991a). Generally, it has been concluded that S may influence the binding of effectors. However, the mechanisms of L and S interaction promoting catalytic competence are unclear.

We based our strategy for mutagenesis on one of the three regions in S that show very high amino acid-sequence invariance. These regions include residues 10 to 21, 54 to 63, and 88 to 104 in Rubisco of *Synechococcus* when aligned with S from other cyanobacteria and higher plants. To explore the structural and functional significance of such

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Abbreviations: CABP, 2-carboxy-D-arabinitol-1,5-bisphosphate; L and S, the large and small subunit proteins of Rubisco, respectively; P-glycerate, glycerate-3-phosphate; RuBP, ribulose-1,5-bisphosphate; τ, CO_2/O_2 specificity factor; XuBP, xylulose-1,5-bisphosphate.

residues, we focused on the third conserved region. Three side chains in *Synechococcus* S were altered by site-directed mutagenesis: Phe-92 to Ser (Phe-92 in cyanobacteria is equivalent to Phe-104 in higher plants), Gln-99 to Gly (higher plants: Gln-111) and Pro-108 to Leu (higher plants: Pro-120). We report the effects of these substitutions on kinetic parameters relative to the wild-type enzyme. Mutants Q99G and P108L produced 2 and 3 times more of the "misfire" product, XuBP, respectively, during catalysis than did the mutant F92S or wild-type Rubisco, but the discrimination between carboxylase and oxygenase activities was only slightly changed in mutants in a comparison with the wild type.

MATERIALS AND METHODS

Site-Directed Mutagenesis of S

To introduce mutations in *Synechococcus* PCC6301 *rbcS* (Shinozaki et al., 1983), a *Bam*HI/*Hin*cII DNA fragment was subcloned into M13mp18 (Yanish-Perron et al., 1985). Single-stranded DNA templates were isolated as described previously (Maniatis et al., 1982), and site-directed mutagenesis was conducted following the procedure described by Kunkel (1985). Three replacements were introduced: F92S (primer: 5′-GTCGCTGGCTCCG-ACAACATCAAG-3′), Q99G (primer: 5′-AAGCAGTGCGG-AACCGTGAGCTTC-3′), and P108L (primer: 5′-GTTCAT-CGTCTCGGCCGCTACTAA-3′). Appropriate regions were sequenced using the dideoxy chain-termination method (Sanger et al., 1977) to confirm the mutations.

Expression and Purification of Proteins

To express *Synechococcus* wild-type and mutant Rubisco, the *rbcS* gene (encoding S) (Smrcka et al., 1991) was cloned 3′ of the *rbcL* gene (encoding L) as a *Bam*HI/*Hin*cII fragment in pLANL (Smrcka et al., 1991). Expression was driven by the heat-inducible bacteriophage λ-promoter, P_L, to allow for simultaneous expression of both GroEL and GroES, which are known to participate in Rubisco folding (Goloubinoff et al., 1988). *Escherichia coli* BR2 cells were grown to an optical density of 0.5 at 600 nm in a 14-L fermenter (New Brunswick Scientific Co., Inc., Edison, NJ) at 28°C prior to heat induction at 42°C. Purification of the enzymes was as described previously (Smrcka et al., 1991). Purified enzymes were made 100 mM in KCl, 10% glycerol and stored at −70°C.

Rubisco Assays

Holoenzyme activity was determined at 30°C in a 500-μL assay solution containing 100 mM Bicine-NaOH, pH 8.0, 40 mM $KH^{14}CO_3$ (1 Ci/mol), 10 to 20 mM $MgCl_2$, and 3 mM DTT. Reactions were initiated by the addition of RuBP to 1 mM and stopped after 30 s by the addition of 1 N HCl. Samples were dried, washed with an excess of 1 N HCl, and dried again to remove unfixed $^{14}CO_2$. Acid-stable radioactivity was assayed by liquid-scintillation counting.

CABP Binding

[^{14}C]CABP was synthesized and purified from other isomers according to the method of Pierce et al. (1980). Samples containing purified enzymes were incubated in 1 to 5 μM [^{14}C]CABP (10–20 μCi/μmol) in 25 mM Tris-Cl, pH 8.0, 10 mM $KHCO_3$, and 10 mM $MgCl_2$ followed by a 1- to 5-h incubation with unlabeled, purified CABP (50–250 μM). Protein-bound CABP was separated from unbound CABP by gel filtration on an Econo-Pac 10 DG column (Bio-Gel P-6, Bio-Rad). Since one molecule of CABP binds tightly to one active site, protomer amounts were calculated from the amount of [^{14}C]CABP bound.

Specificity Factor Measurements

The specificity factor τ was determined as previously described (Uemura et al., 1996).

Titration of Rubisco Activity with XuBP

XuBP was synthesized by aldolase-catalyzed condensation of glycolaldehyde phosphate (Serianni et al., 1979) and dihydroxyacetone phosphate and purified as previously described (Smrcka et al., 1991). Fully activated Rubisco (10 μg) was incubated with various concentrations of XuBP for 15 min in the Rubisco assay medium, and then 0.6 mM RuBP was added and the reaction stopped after 30 s.

Measurement of XuBP

To analyze the XuBP formed during the reaction, 50 to 80 μg of Rubisco (depending on specific activity) was used. The reactions were conducted in 50 mM Hepes, pH 8.0, 10 mM $MgCl_2$, 5 mM $KHCO_3$, and 1.8 mM RuBP in 1 mL at 25°C. Upon consumption of RuBP, the reaction was stopped by addition of 5 drops of cation-exchange resin (AG 50W-X4, Bio-Rad) in the H^+ form, which lowered the pH to less than 2.0 and denatured the protein. After the resin was removed, the supernatant was concentrated to dryness in a SpeedVac centrifuge (Savant Instruments, Farmington, NY) at room temperature. Residues were dissolved in 500 μL of H_2O, and 200 μL was applied to HPLC for separation of the reaction products.

Chromatographic Analysis of Sugar Phosphates

Sugar phosphate products from Rubisco reaction mixtures were separated by anion-exchange chromatography with an HPLC system (Dionex, Sunnyvale, CA). IonPac AG11 guard and analytical columns (Dionex) were used. Sugar phosphates were eluted with 6 mM $NaHCO_3$ and 30 mM Na_2CO_3 at a flow rate of 1 mL/min. A suppressor anion micromembrane (model AMMS-II, Dionex) was used and regenerated with 25 mM H_2SO_4 at a flow rate of 7 to 10 mL/min. Peaks were detected with a conductivity detector (Uemura et al., 1996).

RESULTS

Mutations in S

Three residues in the third conserved region in S (Fig. 1) were altered. They are highly conserved in Rubisco from

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          80.       90.       100.      110.
Osa       KAYPDAFVRIIGFDNVRQVQLISFIAYKPPGCEESGGN-----
Psa       AAYPQAFVRIIGFDNVRQVQCISFIAHTPESY-----------
Sol       KAPPDAFVRFIGFNDKREVQCISFIAYKPAGY-----------
Pma       KAYPQSFIRIIGFDNVRQVQCISFIAYKPPGV-----------
Les       KAYPQAWVRIIGFDNVRQVQCISFIAYKPEGY-----------
Pvi       KEYPAAFIRVLGFDAKRQVQVAGFLVQKPSIA-----------
Fpr       KEYPQAWIRIIGFDNVRQVQCISFIASKPGGF-----------
Ath       KEYPNAFIRIIGFDNTRQVQCISFVAYKPPSFTG---------
Han       KEYPQAWIRIIGFDNVRQVQCIMFIASRPDGY-----------
Cre       KAFPDAYVRLVAFDNQKQVQIMGFLVQRPKTARDFQPANKRSV
Syn       SEYGDCYIRVAGFDHIKQCQTVSFIVHRPGRY-----------
                    *         *         *
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Figure 1. Mutagenesis of conserved residues in S. Three highly conserved residues (marked by asterisks) in the carboxy-terminal domain of S were changed by site-directed mutagenesis in S of *Synechococcus*. Phe-92 was replaced by Ser, Gln-99 was replaced by Gly, and Pro-108 was changed to Leu. These residues are located at subunit interfaces (F92 and Q99), whereas P108 is part of the hydrophobic core of S. The residue numbering was aligned according to the *Synechococcus* sequence. Osa, Rice (*Oryza sativa*); Psa, pea (*Pisum sativum*); Sol, spinach (*Spinacea oleracea*); Pma, apple (*Pyrus malus*); Les, tomato (*Lycopersicon esculentum*); Pvi, fern (*Pteris vittata*); Fpr, *Flaveria pringlei*; Ath, *Arabidopsis thaliana*; Han, sunflower (*Helianthus annuus*); Cre, *Chlamydomonas reinhardtii;* and Syn, *Synechococcus* PCC6301. Absolutely identical residues and residues that are highly conserved in most species are shown in boldface.

diverse plant orders and families, as well as in the enzymes from *Synechococcus* and other cyanobacteria. Phe-92 was changed to Ser (F92S), Gln-99 to Gly (Q99G), and Pro-108 to Leu (P108L) with numbering according to the *Synechococcus* amino acid sequence (Shinozaki et al., 1983). These replacements were introduced, based on an analysis of the structures of spinach and *Synechococcus* L_8S_8 (Knight et al., 1990; Newman and Gutteridge, 1993; Newman et al., 1993), to selectively alter the L/S interface in the case of F92, to test a putative "loss of function" by removing the long side chain of Q99, and to explore the significance of the internal hydrophobic core of S with P108 participating in its formation.

Active-Site Integrity of Mutant Enzymes

All mutant and wild-type enzymes, when expressed in *E. coli*, behaved similarly throughout the purification process with ion-exchange chromatography. They were isolated with a purity higher than 95%, as judged by SDS-PAGE (Fig. 2). Active-site integrity was judged by [^{14}C]CABP binding to carbamylated sites and was also examined by entrapment of activator $^{14}CO_2$ by CABP. After incubation of the wild-type and mutant enzymes with [^{14}C]CABP in the presence of CO_2 and Mg^{2+}, a stable, quaternary complex indistinguishable from wild type was formed by all mutant enzymes (data not shown). In the assay in which $KH^{14}CO_3$ and excess [^{12}C]CABP were used, both wild-type and mutant enzymes trapped activator $^{14}CO_2$ in an identical way (data not shown), suggesting that the active site was not significantly disturbed.

Activity of Mutant Rubisco Enzymes

The observed rates of $^{14}CO_2$ fixation were proportional to the protein concentration during a 30-s interval. Incubations for more than 30 min without RuBP did not increase the amount of acid-stable radioactivity (data not shown). The carboxylase V_{max} values of mutant enzymes relative to the wild-type enzyme (100%) were: F92S, 94%; Q99G, 60%; and P108L, 43% (Table I).

Determination of Kinetic Parameters

The F92S enzyme did not exhibit significant alterations in K_m(RuBP) but showed a small change in τ and a significant reduction (40%) in its $K_m(CO_2)$ (Table I). The mutant Q99G enzyme remained unaltered in both K_m(RuBP) and τ, but $K_m(CO_2)$ was increased 1.5-fold. The substitution of P108L increased both the K_m(RuBP) and $K_m(CO_2)$ nearly 3- and 2-fold, respectively, in comparison with wild type. However, the change of τ was minor (Table I).

Formation of XuBP during Catalysis

XuBP is a readily detected misfire product (Edmondson et al., 1990; Zhu and Jensen, 1991b). If the enzyme cannot effectively convert enediol-RuBP to products, reprotonation of the enediol may increase. Mis-protonation of C-3 of RuBP in the enediol forms XuBP (Edmondson et al., 1990; Zhu and Jensen, 1991b). As determined by HPLC detection of the amount of XuBP formed during catalysis, the three mutant enzymes behaved differently. The substitution P108L caused a 3-fold increase in the formation of XuBP per RuBP utilized. In the mutant Q99G enzyme, XuBP increased 2-fold; however, the mutant F92S enzyme generated no significant increase in XuBP formation (Table I; Fig. 3).

Titration of Rubisco Activity with XuBP

XuBP is a substrate analog that binds tightly to decarbamylated Rubisco sites and inhibits enzyme activity even

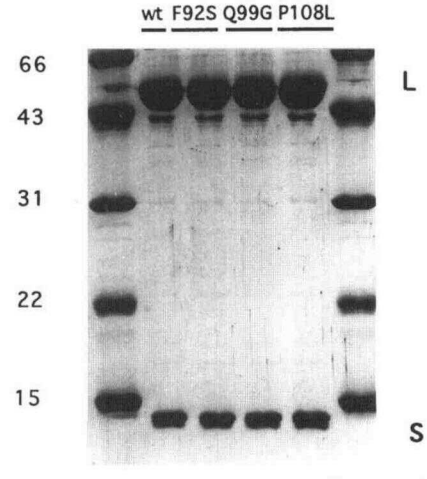

Figure 2. SDS-PAGE of purified recombinant Rubisco. Recombinant Rubisco enzymes were expressed in *E. coli* by heat induction and purified by ion-exchange chromatography using Mono-Q columns. All mutant S assembled into holoenzymes. Preparations were at least 95% pure, as judged by protein staining with Coomassie blue after SDS-PAGE separation. Numbers at the left indicate molecular weights in thousands. wt, Wild type.

Table I. *Kinetic properties of purified Synechococcus wild-type and mutant Rubisco enzymes*

Enzyme	K_m(RuBP)	$K_m(CO_2)$	V_{max}	XuBP[a]	τ[b]
	μM	μM	μmol mg^{-1} min^{-1}	nmol/μmol RuBP	
Wild type	13.5 ± 0.4	112 ± 5	6.1 ± 0.2	1.4	51 ± 2.1
F92S	15.8 ± 2.5	66 ± 4	5.8 ± 0.4	1.6	46 ± 1.7
Q99G	13.1 ± 1.7	171 ± 7	3.7 ± 0.1	2.4	49 ± 3.0
P108L	37.7 ± 1.3	233 ± 9	2.6 ± 0.1	4.0	47 ± 2.5

[a] nmol XuBP produced per μmol RuBP utilized. XuBP was analyzed from three separate reactions at different CO_2 and O_2 conditions. The values of XuBP produced per RuBP consumed were different at different CO_2 and O_2 conditions, but the numerical order remained unchanged. These values came from a reaction with 5 mM $KHCO_3$ at pH 8.0 and under N_2. [b] CO_2/O_2 specificity equals V_cK_o/V_oK_c. V_c and V_o are maximal velocities for carboxylation and oxygenation, respectively; K_o and K_c are the Michaelis constants for CO_2 and O_2, respectively.

in the presence of CO_2 and metal ions (Zhu and Jensen, 1991a; Newman and Gutteridge, 1994). To probe for possible changes in the ability to bind XuBP at the active site resulting from substitutions in S, the inhibitory effect of XuBP was measured. After wild-type and mutant enzymes were incubated with various concentrations of XuBP for 15 min, activity was measured. As shown in Figure 4, the F92S enzyme displayed no difference compared with the wild type, but the P108L and Q99G enzymes exhibited significant differences, suggesting that the substitutions P108L and Q99G have altered the binding affinity for XuBP.

DISCUSSION

Although we understand Rubisco active-site geometry reasonably well from structural studies, with the strongly binding, transition-state analog, CABP, and the weaker inhibitor, XuBP, it is clear that the kinetic behavior of the enzyme is also influenced by a number of long-range interactions and structural movements (Newman and Gutteridge, 1993; Newman et al., 1993). Best known is the impact of the movable loop 6 in L that covers, depending on substrate binding, the active site (Hartman and Harpel, 1994). In contrast, the influence of S on enzyme kinetics is much less understood.

Most mutations introduced previously into *Synechococcus* S allowed assembly into the catalytically active holoenzyme. These mutations clustered at the N terminus, residues 10 to 21 (McFadden and Small, 1988; Kettleborough et al., 1991; Paul et al., 1993). Alternatively, some mutations were introduced in the second conserved domain, residues 54 to 63 (Lee et al., 1991b). Our alterations concentrated on the third conserved domain, residues 88 to 104. All altered enzymes exhibited kinetic properties that made them less active than the wild-type holoenzyme. Of interest, a hybrid enzyme of *Synechococcus* L and *Cylindrotheca* S increased partitioning between carboxylation and oxygenation by nearly 60% relative to *Synechococcus* Rubisco (Read and Tabita, 1992a). Also, distinct S subunits produced under different environmental conditions can significantly affect catalytic properties of the enzyme. Rubisco of blue-light-grown fern gametophytes, for example, contained immu-

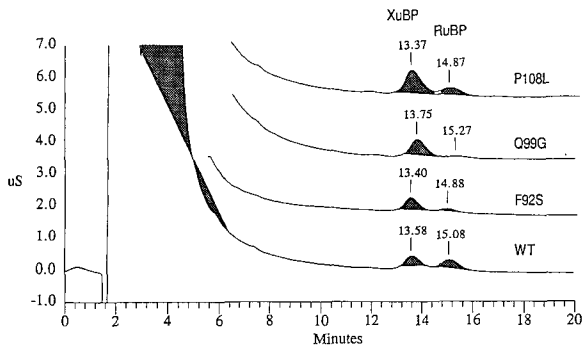

Figure 3. XuBP formation by mis-protonation during catalysis. Portions of representative traces of high-performance anion-exchange separation and detection by conductivity of XuBP and RuBP after carboxylation with wild-type and mutant enzymes are shown. RuBP and XuBP are identified by retention times, indicated above each peak, relative to standards and by spiking the reaction products with standards. The XuBP amount from misfire was quantitated by peak area using XuBP that was standardized by analysis of Pi released. CO_2 fixation with Rubisco used 5 mM $KHCO_3$ in the absence of O_2 for 30 min. The XuBP formed upon consumption of 1.68 μmol of RuBP was 6.66 nmol (P108L), 4.05 nmol (Q99G), 2.70 nmol (F92S), and 2.27 nmol (wild type). Reactions were terminated before RuBP was used up completely, as determined initially in pilot experiments, to avoid the slow, Rubisco-catalyzed XuBP-to-RuBP epimerization reaction.

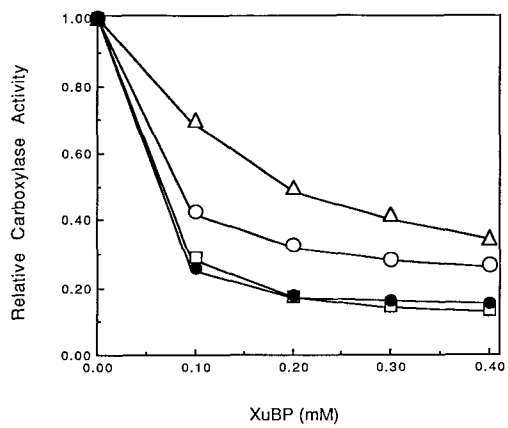

Figure 4. Titration of wild-type and mutant Rubisco activity with XuBP. Relative carboxylation activity of wild-type and mutant enzymes after incubation of the enzymes with XuBP indicates that P108L and Q99G have an altered binding affinity for the inhibitor XuBP. Ten micrograms of activated Rubisco enzymes was first incubated with different concentrations of XuBP for 15 min in the presence of 50 mM Hepes, pH 8.0, 10 mM $MgCl_2$, and 10 mM $KHCO_3$ at 25°C. Enzyme activity after preincubation with XuBP was assayed by the addition of 0.6 mM RuBP for a reaction that lasted 30 s. △, P108L; ○, Q99G; □, F92S; and ●, wild type.

nologically different S and exhibited higher specific activity when compared with the enzyme present after a red-light treatment (Eilenberg et al., 1991).

Structures of *Synechococcus* and higher plant S are similar enough to assemble catalytically active hybrids between *Synechococcus* L and plant S subunits (Smrcka et al., 1991). Therefore, the biochemical analysis of cyanobacterial S is likely to provide an accurate gauge for kinetic analogies that exist with higher plant S, particularly when regions are altered that are highly conserved in all S. All residues changed in this region (residues 88–104) in *Synechococcus* S likely have similar functions in higher plant Rubisco.

Mutant F92S

The cyanobacterial F92, corresponding to the spinach F104, is located at the interface between S and L and becomes buried after assembly of S into L_8S_8. This strictly conserved Phe is part of a hydrophobic cluster (Knight et al., 1990; Newman and Gutteridge, 1993). The mutation of F92S was chosen to alter the hydrophobicity and geometry at the interface by insertion of a polar residue with a maximum in steric change. In contrast to the F92L substitution of Read and Tabita (1992b), The F92S change had no significant effect on the catalytic turnover for carboxylation and K_m(RuBP). There was no significant difference between F92S and the wild-type enzymes in the formation of XuBP during catalysis. Obviously, however, long-range interactions exist. Both F92L and F92S substitutions at the S/L interface significantly increased the affinity for CO_2 (Table I; Read and Tabita, 1992b).

Mutant Q99G

By completely removing the long side chain of Q99 in *Synechococcus* S, a putative loss of function was tested. The change Q to G had no significant effect on τ or K_m(RuBP). The increase of the $K_m(CO_2)$ and a lower $V_{max}(CO_2)$ relative to the wild type demonstrates, however, the importance of this residue for the structure of the active site. It is interesting to note that in *Anabaena*, a Trp-53 that is close to Q99 interfered with the assembly of the holoenzyme (Fitchen et al., 1990). In that example, the exchange W53R disturbed either a Gln-Arg salt bridge or the two adjacent Arg residues inhibited assembly (Fitchen et al., 1990). In the Q99G mutant replacement of Gln with Gly generated a cavity, perhaps interrupting hydrogen bonds between residues in S and L. This Gln is involved in the S-to-L interactions (Knight et al., 1990) and could be responsible for the observed reduced catalytic efficiency and increased formation of XuBP.

Mutant P108L

Most likely, the structural change from Pro, which seems to be part of an S internal hydrophobic core (Fitchen et al., 1990; Knight et al., 1990), to Leu caused a change in the packing of S. This resulted in a change in K_m(RuBP) relative to the wild type, which can only be rationalized by the transmission of this structural change in S to the active site. A 2-fold increase in $K_m(CO_2)$ and a 3-fold increase in misfire production of XuBP (Table I) indicate greater flexibility of the active site, which is probably the result of weakened S-to-L long-range interactions.

Formation of XuBP

Catalytic turnover of RuBP involves predominantly the formation of either two molecules of P-glycerate by carboxylation or the formation of one phosphoglycolate and one P-glycerate by oxygenation. However, the formation of two products, XuBP and 3-ketoarabinitol-1,5-bisphosphate, by mis-protonation of either C-3 or C-2 of the 2,3-enediol intermediate is also significant (Edmondson et al., 1990; Zhu and Jensen, 1991b). Residue substitutions at the active site in L showed that enolization of RuBP does not require all residues that are essential for carboxylation. For example, loop-6 deletion mutant enzymes maintained their ability to enolize RuBP, but their carboxylation activity was totally destroyed (Larson et al., 1995). The substitution E48Q in *Rhodospirillum rubrum* L formed as much XuBP as P-glycerate, and enolization and subsequent carboxylation of the initial enediol were imbalanced, a consequence of the perturbation of active-site geometry accompanying the replacement (Lee et al., 1993).

We report here that, in addition to substitutions in L, alterations in S can also influence the catalytic properties of the active site. Reaction steps subsequent to enediol formation are affected, which result in increased misfiring and an altered affinity for CO_2. Among the three substitutions, P108L caused the largest decrease in V_{max} and the most drastic increase in K_m(RuBP) and $K_m(CO_2)$, suggesting that the active-site geometry may have been significantly altered. However, this change did not affect the competition of CO_2 and O_2 for the enediol intermediate, as deduced from the negligible change in τ. Reduced catalytic activities in the P108L enzyme and, to a lesser degree, the Q99G enzyme are accompanied by an increase in the formation of XuBP, with a decline in CO_2 affinity. The most plausible explanation for increased XuBP formation in these mutant enzymes that we can suggest is that the amino acid changes are the cause for the low efficiency of the recombinant enzymes to stabilize the forward reaction with enediol-RuBP and CO_2.

Although XuBP binds tightly only to decarbamylated Rubisco sites (Zhu and Jensen, 1991a; Newman and Gutteridge, 1994), crystallographic analysis indicated that tight binding of XuBP still requires closure of loop-6 over the active site, with a structure that is similar to the Rubisco-CABP quaternary complex (Newman and Gutteridge, 1994). The juxtaposition of these two results suggests that a disorder, rather than a disruption, of the interaction between L and S resulted from the mutations that we introduced. The subtle changes likely increased the flexibility of active-site residues in L. The changes reported here, other site-directed changes reported by others, and analysis of the kinetic consequences of such changes have provided detailed information about the structure/function relationships of the enzyme (Hartman and Harpel, 1994). More studies, and especially studies that approach site-directed mutagenesis in a more global way, will be necessary. Such studies, in concert with improved crystallographic resolution of mutant

enzymes, will provide the necessary data to finally understand the complex, structural basis of reactions that lead to the competing activities in carboxylation and oxygenation.

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