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

Ralf Flachmann*, Genhai Zhu, Richard G. Jensen, and Hans J. Bohnert

Department of Biochemistry, University of Arizona, Tucson, Arizona 85721

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 dimers. This stabilization appears stabilizing the active sites generated by four large subunit (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 photosylation 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: CO₂, O₂, or a proton. The reaction with CO₂ and O₂ leads to either two molecules of P-glycero or phosphoglycolate and P-glycero, respectively (for review, see Hartman and Harpel (1994)). Competition of the two gases is unavoidable because the enediol-RuBP attacked by CO₂ in carboxylation is also susceptible to O₂ in oxygenation (Lorimer and Andrews, 1973). Re-protonation of the enediol, instead of the forward reactions with either CO₂ or O₂, 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 LpS₆. The functional unit in the complex is L₄, with two L₄ sharing two active sites and four L₄ units assembled with eight S. S is not directly involved in catalysis, but its presence increases V₅₅ 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 L₄ or L₂ 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 L₄ core complexes causes a significant, albeit small, difference in pK of Lys-198, reflecting the influence of S on the carboxylation of LpS₆ (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 L₄ was more sensitive to the same concentration of 6-phosphogluconate than carboxylation in LpS₆ (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

Abbreviations: CABP, 2-carboxy-3-arabinitol-1,5-bisphosphate; L and S, the large and small subunit proteins of Rubisco, respectively; P-glycero, glycero-3-phosphate; RuBP, ribulose-1,5-bisphosphate; τ, CO₂/O₂ specificity factor; XuBP, xylulose-1,5-bisphosphate.

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2 Present address: European Molecular Biology Laboratory, Heidelberg, Germany.

* Corresponding author; e-mail bohnert@biosci.arizona.edu; fax 1-520-621-1697.
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 BamHI/HincII 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-CAACATCAAG-3'), Q99G (primer: 5'-AAGCAGTGCGGCTCTCGGCCGCTACTAA-3'), and P108L (primer: 5'-GTTCATCGTCCTGGCCGCTACTA-3'). Appropriate regions were sequenced using the dideoxy chain-termination method with *XhoI* and *NdeI* 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 BamHI/HincII fragment in pLANL (Smrcka et al., 1991). Expression was driven by the heat-inducible bacteriophage λ-promoter, P3, 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. Purified 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 KH14CO3 (1 Ci/mol), 10 to 20 mM MgCl2, 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 14CO2. Acid-stable radioactivity was assayed by liquid-scintillation counting.

CABP Binding

[14C]CABP was synthesized and purified from other iso-

ers according to the method of Pierce et al. (1980). Samples containing purified enzymes were incubated in 1 to 5 μM [14C]CABP (10-20 μCi/μmol) in 25 mM Tris, pH 8.0, 10 mM KHCO3, and 10 mM MgCl2 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 [14C]CABP bound.

**Specificity Factor Measurements**

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

**Titration of Rubisco Activity with XuBP**

XuBP was synthesized by aldolase-catalyzed condensa-

**Chromatographic Analysis of Sugar Phosphates**

Sugar phosphate products from Rubisco reaction mix-

**RESULTS**

Mutations in S

Three residues in the third conserved region in S (Fig. 1) were altered. They are highly conserved in Rubisco from
active-site integrity of mutant enzymes trapped activator 12
CABP was used, both wild-type and mutant enzymes (data not shown). In the assay in which excess [12]CABP were used, both wild-type and mutant enzymes trapped activator 12
CABP. After incubation, CO2 entrapment of activator 12 was nearly 3-fold (Fig. 3), whereas P108L increased both the Km(RuBP) and Km(CO2) 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 effectively convert enediol-RuBP to products, reprotonation of 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...
Table 1. Kinetic properties of purified Synechococcus wild-type and mutant Rubisco enzymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_{m}$(RubP)</th>
<th>$K_{m}$(CO$_2$)</th>
<th>$V_{max}$</th>
<th>XuBP$^{a}$</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>13.5 ± 0.4</td>
<td>112 ± 5</td>
<td>6.1 ± 0.2</td>
<td>1.4</td>
<td>51 ± 2.1</td>
</tr>
<tr>
<td>F92S</td>
<td>15.8 ± 2.5</td>
<td>66 ± 4</td>
<td>5.8 ± 0.4</td>
<td>1.6</td>
<td>46 ± 1.7</td>
</tr>
<tr>
<td>Q99G</td>
<td>13.1 ± 1.7</td>
<td>171 ± 7</td>
<td>3.7 ± 0.1</td>
<td>2.4</td>
<td>49 ± 3.0</td>
</tr>
<tr>
<td>P108L</td>
<td>37.7 ± 1.3</td>
<td>233 ± 9</td>
<td>2.6 ± 0.1</td>
<td>4.0</td>
<td>47 ± 2.5</td>
</tr>
</tbody>
</table>

$^{a}$ 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 $V_{c}/V_{o}/V_{r}$. $V_{c}$ and $V_{o}$ are maximal velocities for carboxylation and oxygenation, respectively; $K_{c}$ and $K_{o}$ are the Michaelis constants for CO$_2$ and O$_2$, respectively.

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 misine was quantitated by peak area using XuBP that was standardized by analysis of $P_i$ 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 micromolars 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.

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-
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,S,. 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 $\tau$ or $K_m$(RuBP). The increase of the $K_m$(CO$_2$) and a lower $V_{\text{max}}$(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 interfering 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-glyceraldehyde, 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 $V_{\text{max}}$ 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 $\tau$. 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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**LITERATURE CITED**


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