RbcS suppressor mutations improve the thermal stability and CO$_2$/O$_2$ specificity of rbcL-mutant ribulose-1,5-bisphosphate carboxylase/oxygenase

Yu-Chan Du  
*University of Nebraska at Lincoln*

Seokjoo Hong  
*The Samuel Roberts Foundation*

Robert J. Spreitzer  
*University of Nebraska - Lincoln, rspreitzer1@unl.edu*

Follow this and additional works at: [http://digitalcommons.unl.edu/biochemfacpub](http://digitalcommons.unl.edu/biochemfacpub)

Part of the Biochemistry, Biophysics, and Structural Biology Commons

Du, Yu-Chan; Hong, Seokjoo; and Spreitzer, Robert J., "RbcS suppressor mutations improve the thermal stability and CO$_2$/O$_2$ specificity of rbcL-mutant ribulose-1,5-bisphosphate carboxylase/oxygenase" (2000). Biochemistry -- Faculty Publications. 80.  
[http://digitalcommons.unl.edu/biochemfacpub/80](http://digitalcommons.unl.edu/biochemfacpub/80)

This Article is brought to you for free and open access by the Biochemistry, Department of at DigitalCommons@University of Nebraska - Lincoln. It has been accepted for inclusion in Biochemistry -- Faculty Publications by an authorized administrator of DigitalCommons@University of Nebraska - Lincoln.
**RbcS suppressor mutations improve the thermal stability and CO₂/O₂ specificity of rbcL-mutant ribulose-1,5-bisphosphate carboxylase/oxygenase**

Yu-Chun Du, Seokjoo Hong*, and Robert J. Spreitzer†

Department of Biochemistry, University of Nebraska, Lincoln, NE 68588

Communicated by William L. Ogren, United States Department of Agriculture, Hilton Head Island, SC, October 23, 2000 (received for review September 9, 2000)

In the green alga *Chlamydomonas reinhardtii*, a Leu²⁹⁰-to-Phe (L290F) substitution in the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), which is coded by the chloroplast *rbcL* gene, was previously found to be suppressed by second-site Ala²²²-to-Thr and Val²⁶²-to-Leu substitutions. These substituents complement the photosynthesis deficiency of the L290F mutant by restoring the decreased thermal stability, catalytic efficiency, and CO₂/O₂ specificity of the mutant enzyme back to wild-type values. Because residues 222, 262, and 290 interact with the loop between β strands A and B of the Rubisco small subunit, which is coded by *RbcS1* and *RbcS2* nuclear genes, it seems possible that substitutions in this loop might also suppress L290F. A mutation in a nuclear gene, *Rbc-1*, was previously found to suppress the biochemical defects of the L290F enzyme at a post-translational step, but the nature of this gene and its product remains unknown. In the present study, three nuclear-gene suppressors were found to be linked to each other but not to the *Rbc-1* locus. DNA sequencing revealed that the *RbcS2* genes of these suppressor strains have mutations that cause either Asn⁵⁴-to-Ser or Ala⁵⁷-to-Val substitutions in the small-subunit βA/βB loop. When present in otherwise wild-type cells, with or without the resident *RbcS1* gene, the mutant small subunits improve the thermal stability of wild-type Rubisco. These results indicate that the βA/βB loop, which is unique to eukaryotic Rubisco, contributes to holoenzyme thermal stability, catalytic efficiency, and CO₂/O₂ specificity. The small subunit may be a fruitful target for engineering improved Rubisco.

The green alga *Chlamydomonas reinhardtii* serves as an excellent model for the study of photosynthesis because photosynthesis-deficient mutants can be maintained with acetate as an alternative source of carbon and energy. Mutations can be assigned to the nuclear or chloroplast genetic compartments by virtue of Mendelian or uniparental inheritance, and both compartments can be transformed with homologous or heterologous DNA (1). These attributes have been particularly useful for examining the structure–function relationships of eukaryotic ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, Enzyme Commission 4.1.1.39) (2). Because land plants cannot be maintained in the complete absence of photosynthesis, and the subunits of eukaryotic Rubisco cannot be assembled in *Escherichia coli* (3), it has been difficult to examine the effects of random or directed mutations on the function of land–plant Rubisco in *vivo* or in *vitro* (4–6). Tobacco is the only land plant in which the chloroplast genome can be transformed (5, 6).

By screening for photosynthesis-deficient *Chlamydomonas* mutants and selecting for photosynthesis-competent revertants, several regions of the Rubisco large subunit have been identified that influence the ratio of ribulose 1,5-bisphosphate (RuBP) carboxylase to oxygenase activities (2, 7). Because CO₂ and O₂ compete at the same large-subunit active site, the CO₂/O₂ specificity of the enzyme is defined by a kinetic constant, Ω, that equals the catalytic efficiency of carboxylation relative to that of oxygenation (Ω = V₅₀,carboxy/V₅₀,oxygen) (V₅₀, max for carboxylation; V₅₀, max for oxygenation; K₅₀ for O₂; K₅₀ for CO₂) (8). The value of Ω determines the amount of productive CO₂ fixation in photosynthesis relative to the loss of CO₂ via the nonessential photore respiratory pathway (8, 9). Thus, *rbcL* mutations and their intragenic suppressors that affect Ω may define potential sites for ultimately engineering an improved Rubisco enzyme (2, 10).

One photosynthesis-competent revertant of a temperature-conditional *rbcL* mutant has been enigmatic (11–13). It results from a mutation in a nuclear gene, named *Rbc-1*, the product of which acts posttranslationally to correct decreases in holoenzyme stability and Ω caused by a Leu²⁹⁰-to-Phe (L290F) large-subunit substitution (13). *Rbc-1* does not encode the Rubisco small subunit, which is coded by the *RbcS1* and *RbcS2* genes in *Chlamydomonas* (14). Because assembly of 8 large (55 kDa) and 8 small (15 kDa) subunits into the Rubisco holoenzyme appears to depend on the action of the chaperonins (15, 16) and perhaps other assembly proteins (17), *Rbc-1* may be one of a number of genes required for this process (18, 19).

In a previous study, an attempt was made to discern the nature of the *Rbc-1* nuclear gene by selecting and analyzing additional photosynthesis-competent revertants of the *rbcL*-L290F mutant (20). Two were found to result from intragenic suppressor mutations that cause Ala²²²-to-Thr (A222T) and Val²⁶²-to-Leu (V262L) substitutions. Like the *Rbc-1* nuclear-gene mutation (11, 12), these second-site *rbcL* mutations increase the amount of mutant Rubisco at the 35°C restrictive temperature and also increase Ω of the mutant enzyme back to the wild-type value (20). In the absence of the L290F mutation, the A222T and V262L substitutions improve the thermal stability of otherwise wild-type Rubisco in *vitro* (7). Based on the x-ray crystal structure of spinach Rubisco (21), residues Leu²⁹⁰, Ala²²², and Val²⁶² are far from the active site. They reside at the bottom of the large-subunit α/β-barrel domain where they surround a loop between β strands A and B of the Rubisco small subunit.

Because large-subunit residues 222 and 262 are not in Van der Waals contact with residue 290, they may complement L290F via structural perturbations of the small-subunit βA/βB loop (7, 20).

Abbreviations: Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP, ribulose 1,5-bisphosphate; Ω, CO₂/O₂ specificity factor.
*Present address: Plant Biology Division, The Samuel Roberts Noble Foundation, Admore, OK 73402.
†To whom reprint requests should be addressed. E-mail: rspreitzer1@unl.edu.
The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Article published online before print: Proc. Natl. Acad. Sci. USA, 10.1073/pnas.260503997. Article and publication date are at www.pnas.org/cgi/doi/10.1073/pnas.260503997
If such is the case, substitutions in the small-subunit loop might complement L290F directly. In the present study, suppressor mutations were found in a second nuclear-genome locus, and this locus does contain the Rubisco RbcS genes.

**Materials and Methods**

**Strains and Culture Conditions.** *Chlamydomonas reinhardtii* 2137 mt− is the wild-type strain (22). Mutant rbcL-L290F mt− lacks photosynthesis and requires acetate for growth at the 35°C restrictive temperature but is indistinguishable from wild type when growth is compared at 25°C (13, 23). Mutant RbcSΔ-T60−3 mt− was used as the host for nuclear transformation. It lacks photosynthesis and requires acetate for growth because of deletion of the 13-kb locus that contains *RbcS1* and *RbcS2* (24). All *Chlamydomonas* strains are maintained at 25°C in darkness with 10 mM acetate medium containing 1.5% Bacto-agar (22).

**Biocatalysis.** Cytosolic redox enzymes to 0.5 ml of assay buffer containing 50 mM Bicine (pH 8.0) (30). Carboxylase activity was assayed at 25°C by adding the incubated enzymes to 0.5 ml of assay buffer containing 50 mM Bicine (pH 8.0) (31). One unit of activity is equivalent to 1 nmol [1-3H]RuBP formed per min. The reactions were terminated by adding 0.5 ml of 3 M formic acid in methanol.

**Results**

**Molecular Genetics of Photosynthesis-Competent Revertants.** In previous studies, photosynthesis-competent revertants of mutant *rbcL*-L290F were selected on minimal medium in the light at the restrictive temperature of 35°C (11, 20). Recent analysis of 11 of these genetically independent revertants revealed that two arose from unparentally inherited intragenic suppressor mutations that cause A222T and V262L substitutions in the large subunit (7, 20). The remaining nine revertants displayed Mendelian inheritance of the photosynthesis-competent phenotype (20), indicating that they arose from intergenic suppressor mutations in one or more nuclear genes (11). Further genetic analysis in the present study revealed that three of these revertants, named R116–1B, R116–9A, and R116–10C, contained suppressor mutations that arose independent of the suppressor mutation in the Rbc-1 nuclear gene (11). Reciprocal crosses between mt+ and mt− isolates of revertants R116–1B, R116–9A, and R116–10C indicated that the suppressor mutations were genetically linked (at a locus distinct from Rbc-1).

Because the *rbcL*-L290F, *rbcL*-A222T, and *rbcL*-V262L mutations affect residues at the interface between the large subunit and small-subunit βα/ββ loop, and because the new nuclear suppressors were not linked to the *Rbc-1* locus, we reasoned that the R116–1B, R116–9A, and R116–10C revertants might result from mutations that affect the small-subunit βα/ββ loop residues (46–73 in *Chlamydomonas*). When the *RbcS* genes were PCR amplified and sequenced, no mutation was found in the *RbcS1* genes. However, each of the revertants was found to contain a mutation in the *RbcS2* gene. In revertant R116–1B, a transition mutation occurred that would change Asn54 (AAC) to Ser (AGC) (N54S). In both of the R116–9A and R116–10C revertants, a transition mutation occurred that would change Ala57 (GCC) to Val (GTC) (A57V). Because this *RbcS2*-A57V mutation eliminates a HaeIII restriction site (GGCC→GGGT), the mutations in revertants R116–9A and R116–10C were further confirmed by restriction-enzyme analysis. Because R116–9A and R116–10C arose from the same mutation, only one of them (R116–10C) was analyzed further.

When revertants R116–1B mt− (*rbcL*-L290F/*RbcS2-N54S) and R116–10C mt− (*rbcL*-L290F/*RbcS2-A57V) were crossed with the mt− pf-2 centromere marker, the N54S and A57V suppressor mutations were found to be 20 map units from the centromere (Table 1). This map distance provides a point of correlation with the physical map of chromosome II in the *Chlamydomonas* electronic database (1, 34). R116–1B mt− and R116–10C mt− progeny were recovered from the first crosses and crossed with wild-type mt+. Because the *rbcL*-L290F chloroplast mutation is not transmitted from the mt− parent, these crosses would allow the phenotypes of the RbcS2-N54S and RbcS2-A57V mutations to be observed in an otherwise wild-type strain. No discernable (deleterious) phenotype could be ascribed to either of the RbcS2 mutations. PCR amplification and DNA sequencing of RbcS2 from complete tetrads of progeny, and restriction-enzyme analysis in the case of RbcS2-A57V (Fig. 1), confirmed that the N54S and A57V mutations were linked to the suppressor phenotype in the forward crosses and enabled the isolation of RbcS2-N54S and RbcS2-A57V strains (in the absence of...
of the original rbcL-L290F mutation) from the reciprocal crosses.

Because a wild-type RbcS1 gene is also present and expressed at an appreciable level (14, 24), it would be difficult to conclude from crosses alone that the RbcS2-N54S or RbcS2-A57V mutation had no adverse effect on Rubisco assembly or function in the absence of the original rbcL-L290F mutation. Therefore, the mutant RbcS2-N54S and RbcS2-A57V genes were transformed into the RbcS2 strain, which lacks both RbcS1 and RbcS2 and requires acetate for growth (24). Photosynthesis-competent colonies were recovered on minimal medium in the light at frequencies (about 3 per 10^3 cells) comparable to those obtained with the wild-type RbcS2 gene (24). Thus, when the N54S and A57V substitutions are present in all of the small subunits of the holoenzyme, they have no apparent effect on otherwise wild-type Rubisco. These transformant strains are designated RbcS1-D/RbcS2-N54S and RbcS1-D/RbcS2-A57V.

**Small-Subunit Substitutions Improve Rubisco Thermal Stability.** Although the rbcL-L290F/RbcS2-N54S and rbcL-L290F/RbcS2-A57V revertants were indistinguishable from wild type when growth was compared on minimal medium at 35°C, both had less than half the wild-type level of Rubisco holoenzyme when extracts of 35°C-grown cells were fractionated in vivo (11–13). However, both revertants had more than twice the level of holoenzyme as the original rbcL-L290F mutant at 35°C (11–13), which would, in part, account for their ability to grow photoautotrophically at 35°C. Extracts of 25°C-grown mutant and revertant cells had nearly equal amounts of holoenzyme, indicating that the N54S and A57V suppressor substitutions may compensate for the altered stability of the L290F enzyme at only the 35°C restrictive temperature (Table 2). In contrast, the rbcL-L290F mutation, the RbcS2-N54S and RbcS2-A57V suppressor strains had wild-type levels of Rubisco holoenzyme, and the RbcS1-D/RbcS2-N54S and RbcS1-D/RbcS2-A57V transformants had levels of holoenzyme that were not significantly different from those of transformants that contain only the wild-type RbcS2 gene (24) (data not shown).

When the thermal stability of Rubisco purified from 25°C-grown cells was assayed in vitro (Fig. 2), the rbcL-L290F/RbcS2-N54S and rbcL-L290F/RbcS2-A57V revertant enzymes were found to be similar to the original rbcL-L290F mutant enzyme. In fact, the N54S substitution may cause a slight decrease in thermal stability relative to that of the rbcL-L290F enzyme (Fig. 2). Thus, increases in the amount of Rubisco in the rbcL-L290F/RbcS2-N54S and rbcL-L290F/RbcS2-A57V revertant enzymes relative to the amount in the original rbcL-L290F mutant strain (Table 2) may not be explained solely by a direct change in thermal stability. It is more likely that the N54S and A57V suppressor substitutions cause slight improvements in structural stability at 35°C that afford enhanced protection from proteinase in vitro (11–13).

In the absence of the L290F large-subunit substitution, the N54S and A57V small-subunit substitutions significantly improve the thermal stability of wild-type Rubisco in vitro (Fig. 2, compare wild type with RbcS2-N54S and RbcS2-A57V). Furthermore, in the absence of the wild-type RbcS1 gene, the Rubisco holoenzymes isolated from the RbcS1-D/RbcS2-N54S and RbcS1-D/RbcS2-A57V transformants are even more thermally stable. For example, after a 20-min incubation at 65°C, these holoenzymes retained 35 and 80% of their initial carboxylase activities, respectively, but wild-type enzyme was com-

![Figure 1](image-url)

**Fig. 1.** Segregation of the RbcS2-A57V suppressor mutation in reciprocal crosses. DNA was purified from wild-type (lane 1) revertant R116–10C (rbcL-L290F/RbcS2-A57V) (lane 2), a tetrad of progeny from the cross R116–10C × wild-type mt^+ (lanes 3–6), and a tetrad of progeny from the cross wild-type mt^- X R116–10C mt^- (lanes 7–10). By using a pair of oligonucleotides specific for the RbcS2 gene, an 818-bp sequence (bases 640-1457 relative to bases 1–1,267 of the coding region of RbcS2) was PCR amplified from each sample, digested with HaeIII, and separated on a 3.5% agarose gel. The RbcS2-A57V mutation eliminates a HaeIII site, increasing the size of a 198-bp fragment to 210 base pairs. Progeny in lanes 3 and 5 had temperature-conditional acetate-requiring phenotypes. Progeny in lanes 4, 6, and 7–10 had wild-type (photosynthesis-competent) phenotypes at 35°C.

**Table 2. Amount of Rubisco holoenzyme per total cell protein in wild-type, mutant rbcL-L290F, revertant rbcL-L290F/RbcS2-N54S, and revertant rbcL-L290F/RbcS2-A57V grown at 25 or 35°C in darkness.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Holoenzyme</th>
<th>(25°C)</th>
<th>(35°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td></td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>rbcL-L290F</td>
<td></td>
<td>44 ± 6</td>
<td>67 ± 1</td>
</tr>
<tr>
<td>rbcL-L290F/N54S</td>
<td></td>
<td>46 ± 8</td>
<td>18 ± 2</td>
</tr>
<tr>
<td>rbcL-L290F/A57V</td>
<td></td>
<td>40 ± 8</td>
<td>16 ± 1</td>
</tr>
</tbody>
</table>

*Values are means ± SD (n – 1) of three or more sucrose-gradient preparations.
holoenzymes containing only increased thermal stability resulted from the suppressor substi-
tom these "wild-type" holoenzymes or RbcS2 wild-type Rubisco was compared with holoenzymes purified from transformant N54S and L290F wild-type enzyme. This decrease in rbcL activities, respectively. For holoenzyme assembly via transport of small subunits into cells that had been transformed with either RbcS1 or RbcS2 (24). No difference was found in the thermal stabilities of three separate enzyme preparations of three separate enzyme preparations.

Fig. 2. Thermal inactivation of purified Rubisco from wild type (○), mutant rbcL-L290F (■), revertant rbcL-L290F/RbcS2-N54S (□), revertant rbcL-L290F/ RbcS2-A57V (□), suppressor RbcS2-N54S (●), suppressor RbcS2-A57V (●), suppressor RbcS1-Δ/ RbcS2-N54S (●), and transformant RbcS1-Δ/ RbcS2-A57V (●). Purified Rubisco (10 μg/ml) was incubated at each temperature for 20 min. The samples were then cooled on ice, and RuBP carboxylase activity was assayed at 25°C. Activities for each enzyme were normalized against the level of activity measured after the 35°C incubation (wild type, 1; L290F, 0.5; 24°C, 0.5; 29°C, 1.0; L290F/A57V, 1.0; N54S, 1.4; A57V, 1.4, Δ/N54S, 1.2, Δ/A57V, 1.0 μmol·min⁻¹·mg⁻¹). Comparing three separate enzyme preparations at 65°C, wild-type Rubisco was completely inactivated, but transformant Δ/N54S and Δ/A57V Rubisco retained 33% ± 2 SD and 76% ± 3 SD of their initial activities, respectively.

Small-Subunit Substitutions Increase Ω of Mutant Rubisco. As shown here (Table 3) and previously (7, 13, 20), the Ω value of rbcL-L290F mutant Rubisco is about 10% lower than that of the wild-type enzyme. This decrease in Ω arises primarily from a decrease in $V_c$. (Table 3). The N54S and A57V small-subunit substitutions increase Ω of the mutant enzyme back to the wild-type value by decreasing $K_o$ and increasing the value of $K_o/K_c$ (Table 3, compare rbcL-L290F with rbcL-L290F/RbcS2-N54S and rbcL-L290F/RbcS2-A57V). The revertant enzymes also have increased $V_c$ and $V_c/K_c$ values relative to those of the mutant enzyme, indicating that they do, in fact, have improved carboxylation efficiency relative to the mutant enzyme (9, 19). However, despite wild-type Ω values, the rbcL-L290F/RbcS2-N54S and rbcL-L290F/RbcS2-A57V revertant enzymes are not as good as the wild-type enzyme (9, 19). They have significant reductions in $V_c$, $V_c/K_c$, and $V_c/K_o$ (Table 3, compare wild type with rbcL-L290F/RbcS2-N54S and rbcL-L290F/RbcS2-A57V).

Rubisco enzymes isolated from the RbcS1-Δ/RbcS2-N54S and RbcS1-Δ/RbcS2-A57V (which have a wild-type rbcL gene, lack RbcS1, and are homogeneous for the N54S and A57V small-subunit substitutions) were found to have wild-type Ω values (Table 3). However, these small-subunit mutant enzymes also have decreases in $V_c$ and $V_c/K_c$ relative to the wild-type enzyme (Table 3, compare wild type with RbcS1-Δ/ RbcS2-N54S and RbcS1-Δ/RbcS2-A57V). This negative effect on catalysis was absent from the RbcS2-N54S and RbcS2-A57V suppressor-strain holoenzymes (Table 4), which are heterogeneous for wild-type (coded by RbcS1) and mutant (coded by RbcS2) small subunits. Thus, the RbcS2-N54S and RbcS2-A57V enzymes have improved thermal stability in vitro (Fig. 2) with no apparent decline in Ω or carboxylation catalytic efficiency (Table 4).

Discussion

Table 4. Kinetic properties of Rubisco purified from wild-type and suppressor strains RbcS2-N54S and RbcS2-A57V

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>$\Omega$ ($V_c/K_c/V_o$)</th>
<th>$V_c$* (μmol·h⁻¹·mg⁻¹)</th>
<th>$K_c$* (μM CO₂)</th>
<th>$K_o$* (μM O₂)</th>
<th>$V_c/K_c$</th>
<th>$V_c/K_o$</th>
<th>$V_c$/$V_o$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>60 ± 1</td>
<td>138 ± 13</td>
<td>32 ± 3</td>
<td>544 ± 38</td>
<td>4.3</td>
<td>17</td>
<td>3.5</td>
</tr>
<tr>
<td>L290F</td>
<td>54 ± 1</td>
<td>47 ± 1</td>
<td>43 ± 2</td>
<td>763 ± 41</td>
<td>1.1</td>
<td>18</td>
<td>3.0</td>
</tr>
<tr>
<td>L290F/N54S</td>
<td>60 ± 3</td>
<td>87 ± 1</td>
<td>33 ± 4</td>
<td>715 ± 37</td>
<td>2.6</td>
<td>22</td>
<td>2.7</td>
</tr>
<tr>
<td>L290F/A57V</td>
<td>58 ± 1</td>
<td>92 ± 3</td>
<td>32 ± 1</td>
<td>674 ± 79</td>
<td>2.9</td>
<td>21</td>
<td>2.8</td>
</tr>
<tr>
<td>Δ/N54S</td>
<td>61 ± 1</td>
<td>99 ± 8</td>
<td>26 ± 2</td>
<td>485 ± 47</td>
<td>3.8</td>
<td>19</td>
<td>3.2</td>
</tr>
<tr>
<td>Δ/A57V</td>
<td>59 ± 1</td>
<td>84 ± 7</td>
<td>26 ± 3</td>
<td>489 ± 87</td>
<td>3.2</td>
<td>19</td>
<td>3.1</td>
</tr>
</tbody>
</table>

The values are the means ± SD ($n$ = 1) of three separate enzyme preparations.

Table 3. Kinetic properties of Rubisco purified from wild-type, mutant rbcL-L290F, revertants rbcL-L290F/RbcS2-N54S and rbcL-L290F/RbcS2-A57V, and transformants RbcS1-Δ/RbcS2-N54S and RbcS1-Δ/RbcS2-A57V

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>$\Omega$ ($V_c/K_c/V_o$)</th>
<th>$V_c$* (μmol·h⁻¹·mg⁻¹)</th>
<th>$K_c$* (μM CO₂)</th>
<th>$K_o$* (μM O₂)</th>
<th>$V_c/K_c$</th>
<th>$V_c/K_o$</th>
<th>$V_c$/$V_o$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>60 ± 1</td>
<td>138 ± 13</td>
<td>32 ± 3</td>
<td>544 ± 38</td>
<td>4.3</td>
<td>17</td>
<td>3.5</td>
</tr>
<tr>
<td>L290F</td>
<td>54 ± 1</td>
<td>47 ± 1</td>
<td>43 ± 2</td>
<td>763 ± 41</td>
<td>1.1</td>
<td>18</td>
<td>3.0</td>
</tr>
<tr>
<td>L290F/N54S</td>
<td>60 ± 3</td>
<td>87 ± 1</td>
<td>33 ± 4</td>
<td>715 ± 37</td>
<td>2.6</td>
<td>22</td>
<td>2.7</td>
</tr>
<tr>
<td>L290F/A57V</td>
<td>58 ± 1</td>
<td>92 ± 3</td>
<td>32 ± 1</td>
<td>674 ± 79</td>
<td>2.9</td>
<td>21</td>
<td>2.8</td>
</tr>
<tr>
<td>Δ/N54S</td>
<td>61 ± 1</td>
<td>99 ± 8</td>
<td>26 ± 2</td>
<td>485 ± 47</td>
<td>3.8</td>
<td>19</td>
<td>3.2</td>
</tr>
<tr>
<td>Δ/A57V</td>
<td>59 ± 1</td>
<td>84 ± 7</td>
<td>26 ± 3</td>
<td>489 ± 87</td>
<td>3.2</td>
<td>19</td>
<td>3.1</td>
</tr>
</tbody>
</table>

*The values are the means ± SD ($n$ = 1) of three separate enzyme preparations.

†Calculated values.
How Does the Small Subunit Influence Catalysis and Thermal Stability?

In previous studies (7, 20), A222T and V262L large-subunit substitutions were also found to complement the original L290F mutant substitution. On the basis of the x-ray crystal structure of spinach Rubisco (21), Ala222, Val262, and Leu290 are in close contact with residues in the small-subunit βA/βB loop (Fig. 3B). Large-subunit Ala222 is in Van der Waals contact with small-subunit Tyr61 (Tyr67 in the *Chlamydomonas* enzyme), and Val262 is in Van der Waals contact with Pro59 (Cys65 in the *Chlamydomonas* enzyme). Large-subunit Leu290 may be close to small-subunit residues Pro39, Gly60, and Tyr62 (Cys64, Leu66, and Tyr68, respectively) in the *Chlamydomonas* enzyme. Thus, although Ala222 and Val262 are not in Van der Waals contact with Leu290, all three residues interact with the same region of the small-subunit βA/βB loop. Because the x-ray crystal structure of *Chlamydomonas* Rubisco has not yet been solved (44), and because the N54S and A57V small-subunit substitutions replace residues that are apparently absent from the spinach Rubisco structure (Fig. 3A), it is difficult to deduce the location of the Asn54 and Ala57 residues within the *Chlamydomonas* enzyme. However, Arg53 of spinach Rubisco (homologous to the Arg29 of *Chlamydomonas* that is adjacent to the extra residues) is in Van der Waals contact with small-subunit residues Pro59 and Gly60, and hydrogen bonds with Tyr61 across the hydrophobic core of the loop (Fig. 3B). Thus, all of the small-subunit (N54S and A57V) and large-subunit (A222T and V262L) suppressor substitutions occur at residues that may be in contact with the small-subunit region closest to Leu290 (Cys65 through Tyr68 in *Chlamydomonas*; Pro59 through Tyr62 in spinach) (Fig. 3).

The L290F large-subunit substitution may affect catalysis by disrupting a series of hydrogen-bonded residues that extends to His327 in the active site (7, 9, 20). It had previously been proposed that the large-subunit A222T and V262L substitutions complemented the original *rbcL*-L290F mutant enzyme via structural interactions in the hydrophobic core of the large-subunit α/β-barrel, or via structural rearrangements of the small-subunit βA/βB loop (7, 20). Now that small-subunit N54S and A57V substitutions have been identified that can complement the large-subunit L290F substitution, it would seem more likely that the large-subunit A222T and V262L substitutions complement L290F by influencing the structure of the small-subunit βA/βB loop (Fig. 3B).

The large- (A222T and V262L) and small-subunit (N54S and A57V) suppressor substitutions improve the *rbcL*-L290F mutant enzyme by increasing Ω and Vc (refs. 7, 20; Table 3). However, in the absence of the original L290F substitution, none of the small- or large-subunit suppressor substitutions increases Ω or Vc of the otherwise wild-type enzyme (ref. 7; Table 3). In contrast, all of these large- and small-subunit suppressor substitutions substantially improve the in vitro thermal stability of wild-type Rubisco (ref. 7; Fig. 2). Perhaps each of the suppressor substitutions alters the structure of the βA/βB loop in such a way as to create a similar kind of cavity at the small-/large-subunit
interface that accommodates the increased bulk of the Phe side chain. The reduction in steric hindrance at Phe290 would then be responsible for restoring catalysis and improving holoenzyme thermal stability. In the absence of the larger Phe side chain, the cavity produced by each of the suppressor substitutions might permit greater flexibility at the large-/small-subunit interface, and this increased conformational freedom may account for increased thermal stability \textit{in vitro} (45). If such is the case, one might expect that the introduction of a variety of smaller residues at the subunit interface might improve thermal stability without necessarily affecting catalysis.

A mutation in the \textit{Rbc-I} nuclear gene, which is not linked to the \textit{RbcS} locus, also suppresses the \textit{rbcL-L290F} mutation in much the same way as the small- (N54S and A57V) and large-subunit (A222T and V262L) suppressor substitutions (11, 12). The \textit{Rbc-I} nuclear suppressor increases \( \Delta V_c \) and the amount of \textit{rbcL-L290F} Rubisco (11) and, in the absence of the L290F substitution, increases the thermal stability of wild-type Rubisco (12). Considering that the \( \beta A/\beta B \) loop is required for the assembly of the eukaryotic Rubisco holoenzyme (42, 43) and that a number of small- (N54S and A57V) and large-subunit (A222T, V262L, and L290F) substitutions in the \( \beta A/\beta B \) loop region can influence thermal stability, it would seem possible that \textit{Rbc-I} encodes a protein that may participate in Rubisco holoenzyme assembly via conformational arrangement of the \( \beta A/\beta B \) loop. Perhaps the product of the \textit{Rbc-I} mutant gene folds the \( \beta A/\beta B \) loop in a way that also creates a similar kind of cavity at the small-/large-subunit interface.

**Additional Regions of the Small Subunit May Control \( \Omega \).** In previous studies, coexpression of large subunits from the cyanobacterium \textit{Synechococcus} (Rubisco \( \Omega = 41 \)) and small subunits from the diatom \textit{Cylindrotheca} (Rubisco \( \Omega = 107 \)) in \textit{E. coli} produced a hybrid holoenzyme that had an intermediate \( \Omega \) value of 65 (46). By exploiting chloroplast transformation of tobacco, large subunits from sunflower (Rubisco \( \Omega = 98 \)) were assembled with the resident small subunits of tobacco (Rubisco \( \Omega = 85 \)) to produce a hybrid holoenzyme with an \( \Omega \) value of 89 (5). Because the resulting hybrid holoenzymes had greater than 80\% decreases in \( V_c \) (5, 46), one cannot accurately judge their net carboxylation efficiencies solely from the values of \( \Omega \) (8, 9, 19). Nonetheless, it is apparent that the \( \Omega \) values of these hybrid holoenzymes were substantially influenced by the contributed small subunits. The size of the small-subunit \( \beta A/\beta B \) loop in the hybrid enzymes does not account for this influence on \( \Omega \). \textit{Synechococcus} and \textit{Cylindrotheca} small subunits lack the larger loop that is characteristic of plants and green algae, and tobacco and sunflower small subunits have larger loops of identical size (Fig. 3A). However, the results of the present study indicate that specific substitutions in the longer \( \beta A/\beta B \) loop characteristic of plants and green algae can influence \( \Omega \) (Table 3). Thus, either the nature of the differences between these loops accounts for differences in relative values of \( \Omega \) (Fig. 3A), or there are additional regions of the small subunit that can also influence \( \Omega \). It will be possible to examine these alternatives via directed mutagenesis and transformation of \textit{Chlamydomonas} (24).

This study was supported by the Department of Energy (Grant DE-FG03-90ER15044) and by the Nebraska Agricultural Research Division (Journal Series Paper 13124).