The temperature-conditional photosynthesis-deficient mutant 68-4PP of *Chlamydomonas reinhardtii* results from a Leu-290 to Phe substitution in the chloroplast-encoded large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39). Although this substitution occurs relatively far from the active site, the mutant enzyme has a reduced ratio of carboxylation to oxygenation in addition to reduced thermal stability in vivo and in vitro. In an attempt to understand the role of this region in catalysis, photosynthesis-competent revertants were selected. Two revertants, named R96-4C and R96-8E, were found to arise from second-site mutations that cause V262L and A222T substitutions, respectively. These intragenic suppressor mutations increase the CO$_2$/O$_2$ specificity and carboxylation $V_{\text{max}}$ back to wild-type values. Based on the crystal structure of the spinach holoenzyme, Leu-290 is not in van der Waals contact with either Val-262 or Ala-222. However, all three residues are located at the bottom of the α/β-barrel active site and may interact with residues of the nuclear encoded small subunits. It appears that amino acid residues at the interface of large and small subunits can influence both stability and catalysis.

Ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39, Rubisco)$^1$ initiates both photosynthetic carbon assimilation and photorespiration (see Refs 1 and 2 for review). The enzyme generates either two molecules of phosphoglycerate by carboxylation of RuBP or one molecule each of phosphoglycerate and phosphoglycolate by oxygenation of RuBP. Because phosphoglycolate enters the fruitless photorespiratory pathway and leads to the loss of CO$_2$ (see Ref 3 for review), an increase in carboxylation or a decrease in oxygenation would likely improve plant productivity (see Ref 1 for review). The ratio of carboxylation to oxygenation at any given concentrations of CO$_2$ and O$_2$ is defined by the CO$_2$/O$_2$ specificity factor, $\Omega = V_c/K_c/V_o/K_o$, where $V_c$ and $V_o$ are the $V_{\text{max}}$ values for carboxylation and oxygenation, and $K_c$ and $K_o$ are the Michaelis constants for CO$_2$ and O$_2$, respectively (4). Because CO$_2$ and O$_2$ are mutually competitive at the same active site, the differential stabilization of the carboxylation and oxygenation transition states ultimately determines $\Omega$ (5, 6).

The Rubisco holoenzyme in the chloroplasts of plants and green algae is composed of eight copies each of large and small subunits (see Refs 1 and 2 for review). A family of nuclear *rbcS* genes encodes the 15-kDa small subunits, whereas the chloroplast *rbcL* gene encodes the 55-kDa large subunits. The small subunit is synthesized as a 21-kDa precursor in the cytosol and processed to mature form upon entry into chloroplasts (see Refs 7 and 8 for review). Holoenzyme assembly is then facilitated by the action of chloroplast chaperonin 60 (see Ref 9 for review). The active site of Rubisco is formed at the interface between the carbonyl-terminal α/β-barrel domain of one large subunit and the amino-terminal domain of a second large subunit (10–12). Rubisco also requires an additional nuclear encoded protein, named Rubisco activase, for its activation in vivo (see Ref 13 for review).

In contrast to directed mutagenesis (2, 14, 15), random screening for chloroplast *rbcL* mutations in the green alga *Chlamydomonas reinhardtii*, followed by genetic selection, has identified a number of complementing large subunit substitutions that influence $\Omega$ (16–19). Because these substitutions are most often found in the secondary structure elements that comprise the cores of the α/β-barrel or amino-terminal domains their influence on $\Omega$ is not readily deduced from the existing x-ray crystal structures (11, 12, 20). In particular, one temperature-conditional mutant strain, named 68-4PP, results from an L290F substitution at the end of β-strand 5 at the bottom of the α/β barrel (21, 22). This substitution reduces $\Omega$ by 13%, and the mutant enzyme has decreased thermal stability at 35 °C in vivo and in vitro (22, 23). Genetic selection previously identified a nuclear suppressor mutation, named S52-2B, that restores the thermal stability and $\Omega$ value of the 68-4PP enzyme back to wild-type levels (23, 24). The S52-2B mutation does not reside in either of the two *rbcS* genes (24), and its gene product affects Rubisco at a posttranslational step (23, 25). Otherwise, the molecular basis for the S52-2B mutation is not yet known.

We reasoned that if suppressor mutations could arise in other nuclear genes or elsewhere in the 68-4PP *rbcL* gene, analysis of such suppressors might help to understand the mode of action of the 68-4PP L290F substitution and, perhaps, the means by which S52-2B acts as a suppressor. Therefore, additional photosynthesis-competent revertants were selected from mutant 68-4PP and analyzed. Two of these new revertants arose from *rbcL* second-site mutations that change residues at the interface between large and small subunits at the bottom of the α/β barrel.

**EXPERIMENTAL PROCEDURES**

*Strains and Culture Conditions—* *C. reinhardtii* wild-type 2137 mt$^+$ (26), mutant 68-4PP mt+ (21, 22), and revertant strains are maintained at 25 °C in darkness with 10 mM acetate medium containing 1.5% Bacto-agar (Difco) (26). The temperature-conditional 68-4PP mutant

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† Present address: Dept. of Biochemistry, Michigan State University, East Lansing, MI 48824.

§ To whom correspondence should be addressed. Tel.: 402-472-5446; Fax: 402-472-7842; E-mail: rjs@unlinfo.unl.edu.
Complementing Substitutions in Rubisco

Table I

| Strains                      | 25 °C | 35 °C |
|------------------------------|-------|-------|
| Wild type                    | 42 ± 4| 36 ± 2|
| 68-4PP (L290F)               | 30 ± 4| 6 ± 2 |
| R96-4C (L290F/V262L)         | 18 ± 2| 10 ± 4|
| R96-8E (L290F/A222T)         | 21 ± 4| 11 ± 3|

*The values are the means obtained from three different preparations and are reported with the sample (n = 1) standard deviations.

Table of contents

RESULTS

Molecular Genetics of Photosynthesis-Competent Revertants—In a previous study (24), photosynthesis-competent revertants were recovered at a frequency of 7 × 10⁻¹⁷ cells by directly plating rbcL mutant 68-4PP mt⁻ on minimal medium in the light at 35 °C. In the present study, revertants of 68-4PP mt⁻ were recovered spontaneously (at a frequency of 5 × 10⁻¹⁹) or they were recovered after methyl methanesulfonate or 5-fluoro-2'-deoxyuridine/methyl methanesulfonate treatment (at a frequency of 10⁻¹⁹) as a means for increasing the number of potential suppressor mutations. Genetic analysis of 11 revertants revealed that two of them, named R96-4C and R96-8E, were inherited in a uniparental pattern (10, 12). These enzyme activities measured at limiting CO₂ (0.53 mM NaHCO₃) under 100% N₂ and 100% O₂ is a function of the Kₗ and Kᵥ kinetic constants (22, 24). When these ratios were determined for purified Rubisco, wild-type and 68-4PP (L290F) mutant enzymes had values of 3.0 and 2.4, respectively. The R96-4C (L290F/V262L) and R96-8E (L290F/A222T) revertant enzymes had N₂/O₂ ratio values of 2.2 and 2.8, respectively, indicating that these enzymes had kinetic properties different from either the wild-type or 68-4PP mutant enzyme.

The 68-4PP (L290F) mutant enzyme is known to have an Ω value lower than that of the wild-type enzyme (22, 24). Detailed biochemical analysis of purified Rubisco confirmed that the 68-4PP enzyme has a 17% decrease in Ω and revealed that the revertant enzymes have Ω values restored to the wild-type value (Table II). The improved Ω values of both revertant enzymes arise from increases in Vₑ, Vₑ/Kᵥ, and Kᵥ/Kₗ relative to the original 68-4PP mutant enzyme (Table II). However, neither enzyme has a Vₑ, Vₑ/Kᵥ, or Kᵥ value as good as that of the wild-type enzyme. With regard to the revertant R96-4C (L290F/V262L) enzyme, the improved Ω occurs despite an increase in Kᵥ and decrease in Vₑ/Vₑ relative to the values of the 68-4PP (L290F) mutant enzyme.

DISCUSSION

Mutant 68-4PP Rubisco has a decreased Ω (22) and reduced thermal stability in vivo and in vitro (22, 23). These enzyme defects arise from an L290F substitution in the chloroplast-encoded Rubisco large subunit (22, 24). According to the crystal structure of spinach Rubisco (10, 12), Leu-290 is the first residue of β-strand 5 at the bottom of the α/β barrel. As such, it is relatively far from the active site residues that coordinate with the transition-state analog carboxyarabinitol 1,5-bisphosphate (10, 12). It was previously suggested (1) that the L290F substitution may disrupt the hydrophobic core of the α/β barrel by affecting a hydrogen bond network that extends from Glu-168 (at the bottom of the α/β barrel) to active-site His-327 (at the
neither of these phylogenetically conserved residues resides in the original L290F mutant substitution. Furthermore, based on the structural stability (Table I) and catalytic efficiency (Table II) of Rubisco. The values are the means of three separate enzyme preparations and are reported with the sample (n – 1) standard deviations.

Table II

Kinetic properties of Rubisco purified from wild type, mutant 68-4PP, revertant R96-4C, and revertant R96-8E

| Kinetic constants | Wild type | Mutant 68-4PP (L290F) | Revertant R96-4C (L290F/V262L) | Revertant R96-8E (L290F/A222T) |
|------------------|-----------|-----------------------|-------------------------------|-------------------------------|
| \( V_{c}/V_{o} \) | 65 ± 1    | 54 ± 4                | 64 ± 5                        | 63 ± 4                        |
| \( K_{c} \) (\( \mu \text{mol CO}_2/\text{mg} \)) | 34 ± 6   | 54 ± 6                | 75 ± 7                        | 44 ± 4                        |
| \( K_{o} \) (\( \mu \text{mol O}_2/\text{mg} \)) | 360 ± 39 | 709 ± 60              | 1198 ± 243                    | 630 ± 87                      |
| \( V_{c}/V_{o} \) | 100 ± 5  | 37 ± 3                | 67 ± 3                        | 72 ± 8                        |
| \( K_{c} \) | 11        | 13                    | 16                            | 14                            |
| \( V_{c}/V_{o} \) | 5.9       | 4.2                   | 4.0                           | 4.5                           |

\( a \) The values are the means of three separate enzyme preparations and are reported with the sample (n – 1) standard deviations.

\( b \) Calculated values.

Because the 68-4PP (L290F) mutation gives rise to a temperature-conditional, acetate-requiring phenotype in vivo (21, 22), it was possible to select for complementing mutations at the 35 °C restrictive temperature. Past genetic studies with *Chlamydomonas* Rubisco have identified compensatory substitutions at residues that are often in van der Waals contact with the original mutant residues (16–18). However, second-site substitutions that complement L290F were found to be relatively distant. Either a V262L or A222T substitution produces a moderate increase in the amount of L290F mutant Rubisco at 35 °C (Table I) and, more significantly, restores \( \Omega \) to the wild-type value (Table II). Because the V262L and A222T substitutions both increase the volume of the affected residues, it seems unlikely that they compensate for the increase in size caused by the original L290F mutant substitution. Furthermore, based on the x-ray crystal structure of spinach Rubisco (10, 12), neither of these phylogenetically conserved residues resides in the Rubisco active site. Ala-222 (in the middle of \( \alpha \)-helix 2) is more than 10 Å away from the atoms of Leu-290. Whereas Val-262 (below \( \beta \)-strand 4) appears to be close to Leu-290 (bottom of \( \beta \)-strand 5), the side chains of these two residues are oriented away from each other. Instead, Val-262 is in van der Waals contact with Ala-222 (10, 12). These “long-distance” interactions relative to residue 290 are interesting, especially considering that residues 222, 262, and 290 are close to residues of the Rubisco small subunit (10, 12).

In spinach Rubisco, a small subunit hairpin loop (residues 46–67 flanked by \( \beta \)-strands A and B) is in close contact with large subunit residues at the bottom of the \( \alpha/\beta \) barrel (10). Cyanobacterial Rubisco lacks 12 residues of this loop (residues 52–63), whereas *Chlamydomonas* Rubisco contains 6 additional residues (see Ref. 1 for review). Because \( \Omega \) values also diverge among these species, it is interesting to consider whether the small subunit hairpin loop could contribute to the enhanced catalytic efficiency of eukaryotic Rubisco (10, 20).

With regard to the crystal structure of spinach Rubisco (10, 12) and as shown in Fig. 1, the L290F substitution could place residue 290 in van der Waals contact with small subunit residues Gly-60 and Tyr-62 (Leu-66 and Tyr-68 in *Chlamydomonas* Rubisco, respectively). Furthermore, the backbone atoms of large subunit Val-262 are likely to be in van der Waals contact with the C\(_{\alpha}\), C\(_{\beta}\), and C\(_{\gamma}\) atoms of the spinach small subunit Pro-59 (Cys-65 in the *Chlamydomonas* enzyme), which is located at the tip of the small subunit hairpin loop (10) (Fig. 1). The C\(_{\beta}\) atom of large subunit Ala-222 is also in van der Waals contact with one of the C\(_{\gamma}\) atoms of spinach small subunit Tyr-61 (Tyr-67 in the *Chlamydomonas* enzyme). However, this tyrosyl residue resides in a second neighboring small subunit (10) (Fig. 1). In conclusion, it seems possible that the V262L and A222T large subunit substitutions could complement the L290F substitution via interactions transmitted through the Rubisco small subunit (Fig. 1). Perhaps these interactions at the interface between large and small subunits contribute to both the structural stability (Table I) and catalytic efficiency (Table II) of Rubisco.

Directional mutagenesis of pea rbcS followed by in vitro synthesis and transport into isolated chloroplasts indicated that Arg-53 in the small subunit hairpin loop is required for holoenzyme assembly (33). In spinach Rubisco (10, 12) this arginyl residue (Arg-59 in the *Chlamydomonas* enzyme) hydrogen bonds with large subunit Tyr-226, which is also in van der Waals contact with the side chain atoms of Ala-222 and Val-262. Such observations further support the idea that substitutions at Ala-222 and Val-262 may also influence the interactions between large and small subunits.

When a hybrid Rubisco enzyme comprised of cyanobacterial large subunits and diatom small subunits was expressed in *Escherichia coli*, it was found to have an \( \Omega \) value intermediate to the \( \Omega \) values of the native cyanobacterial and diatom holoenzymes (34). Thus, even though the hybrid enzyme had substantial decreases in \( V_{c} \) and \( V_{c}/K_{c} \) (34), these results indicate that small subunits can contribute to the catalytic efficiency of Rubisco. Considering that substitutions at large subunit residues 290, 222, and 262 can influence \( \Omega \) (Table II), perhaps the nearby small subunit residues (Fig. 1) could also play a role in determining the catalytic efficiency of Rubisco.

Most of the revertants recovered from mutant 68-4PP in this and a previous study (24) arose from nuclear suppressor mutations. One of these revertants has been analyzed in detail (23, 24), but the nuclear suppressor mutation does not reside in either of the two rbcS genes (24). Nonetheless, the idea that small subunits can act as a bridge between L290F and the complementing V262L and A222T substitutions would imply...
that substitutions in the small subunit may also complement the original 68-4PP (L290F) mutation. Further study of the existing nuclear suppressors will test this hypothesis.

Because the L290F, A222T, and V262L substitutions can influence Rubisco and may do so by influencing the structure of the large subunit/small subunit interface (Fig. 1), it would be interesting to examine the effect of nearby small subunit substitutions on Rubisco catalysis. However, it has not been possible to create such substitutions via directed mutagenesis because prokaryotic Rubisco enzymes lack either the small subunit or the small subunit hairpin loop (see Ref. 1 for review), and eukaryotes contain a family of \( rbcS \) genes that precludes transformation of mutant genes (see Refs. 1 and 8 for review). Only recently has a mutant strain of \( Chlamydomonas \) been recovered that lacks the \( rbcS \) gene family (35). Because the \( rbcS \) deletion mutant can be transformed with a single \( rbcS \) gene (35), it may soon be possible to directly investigate the potential role of small subunit residues 65, 66, 67, and 68 in Rubisco catalysis.

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