Suppressor Mutations in the Chloroplast-encoded Large Subunit Improve the Thermal Stability of Wild-type Ribulose-1,5-bisphosphate Carboxylase/Oxygenase*

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A temperature-conditional, photosynthesis-deficient mutant of the green alga Chlamydomonas reinhardtii, previously recovered by genetic screening, results from a leucine 290 to phenylalanine (L290F) substitution in the chloroplast-encoded large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39). Rubisco purified from mutant cells grown at 25 °C has a reduction in CO2/O2 specificity and is inactivated at lower temperatures than those that inactivate the wild-type enzyme. Second-site alanine 222 to threonine (A222T) or valine 262 to leucine (V262L) substitutions were previously isolated via genetic selection for photosynthetic ability at the 35 °C restrictive temperature. These intragenic suppressors improve the CO2/O2 specificity and thermal stability of L290F Rubisco in vivo and in vitro. In the present study, directed mutagenesis and chloroplast transformation were used to create the A222T and V262L substitutions in an otherwise wild-type enzyme. Although neither substitution improves the CO2/O2 specificity above the wild-type value, both improve the thermal stability of wild-type Rubisco in vitro. Based on the x-ray crystal structure of spinach Rubisco, large subunit residues 222, 262, and 290 are far from the active site. They surround a loop of residues in the nuclear-encoded small subunit. Interactions at this subunit interface may substantially contribute to the thermal stability of the Rubisco holoenzyme.

Genetic screening for conditional lethal mutants identifies only those relatively few amino acid substitutions that are critical for protein structure or function (reviewed in Refs. 1 and 2). One such mutant of the green alga Chlamydomonas reinhardtii results from an L290F substitution in the chloroplast-encoded large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39) (3, 4). This mutant contains enough Rubisco holoenzyme at 25 °C to grow photoautotrophically but lacks holoenzyme and requires acetate for growth at the 35 °C restrictive temperature (3). Because the mutant enzyme subunits are synthesized and assembled into holoenzyme at apparently normal rates (4), the reduced level of Rubisco must result from increased degradation of an unstable holoenzyme in vivo (4–6). Rubisco purified from mutant cells grown at 25 °C has decreases in catalytic efficiency, CO2/O2 specificity, and holoenzyme thermal stability (4–6) despite the fact that residue 290 resides at the bottom of the α/β-barrel active-site domain, relatively far from the active-site residues (7). In an attempt to understand the nature of this distant effect, photosynthesis-competent revertants were selected from the L290F mutant strain at 35 °C (5, 8). Either an A222T or V262L large subunit substitution was found to complement the original mutant substitution (8). Comparison of enzymes purified from cells grown at 25 °C revealed that the A222T and V262L substitutions increased the CO2/O2 specificity of the L290F enzyme back to the wild-type level (8). Residues 222 and 262 are in van der Waals contact with each other, but both are more than 6 Å away from the atoms that comprise residue 290. However, based on the spinach Rubisco crystal structure (7), all three residues may be in contact with residues in a loop between β-strands A and B of the nuclear-encoded small subunit. Although the function of the small subunit is not well defined (reviewed in Ref. 9), it is apparent from the analysis of mutant L290F and its revertants that interactions between large and small subunits in the region of the small subunit βA/βB loop may contribute to both catalytic efficiency and thermal stability (8). Previous studies with isolated pea chloroplasts indicated that the small subunit βA/βB loop, which is 12 residues longer in land plants than in cyanobacteria, may be required for holoenzyme assembly (10, 11).

The chloroplast-localized Rubisco holoenzyme plays a central role in plant productivity because it catalyzes the rate-limiting step of photosynthesis. However, Rubisco is a bifunctional enzyme that catalyzes both the carboxylation and oxygenation of RuBP (reviewed in Refs. 9 and 12). The ratio of carboxylation to oxygenation at any specified concentration of CO2 and O2 is determined by the CO2/O2 specificity factor, Ω = Vmax CO2/Vmax O2, where V is the Vmax of carboxylation and oxygenation, and K is the Km for CO2 and O2, respectively (13). Because oxygenation initiates a fruitless photorespiratory pathway that leads to the loss of CO2 (14), Rubisco remains the major potential target for engineering an increase in net photosynthetic CO2 fixation (reviewed in Ref. 9).

Eukaryotic Rubisco does not assemble when its subunits are expressed in Escherichia coli (15, 16). However, directed mutations in the large subunit rbcL gene can be examined via chloroplast transformation of Chlamydomonas or tobacco (17–19). Mutations that do not eliminate Rubisco function are particularly easy to recover in Chlamydomonas by selecting for photosynthetic ability (20–22). No single mutant substitution is expected to substantially improve Rubisco (reviewed in Refs. 2 and 9). Nonetheless, because the A222T and V262L large...
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**RESULTS**

**Mutant Recovery and Phenotypes**—When the rbcL-25B1 insertion mutant was transformed with either pLS-A222T or pLS-V262L, photosynthesis-competent colonies were recovered on minimal medium (without acetate) in the light at a frequency of $1.2 \times 10^{-6}$ cells. Because this transformation frequency was comparable to the frequency obtained with wild-type rbcL (31), it was evident that the A222T and V262L suppressor mutations alone had no substantial deleterious effect on Rubisco function. In fact, the A222T and V262L mutants grew as well as wild type and, like wild type, grew somewhat better than the L290FA222T and L290F/V262L double mutants when spot tests (3, 23) were performed on minimal medium at 35 °C. Sucrose gradient fractionation of extracts of cells grown at 35 °C confirmed that the levels of Rubisco holoenzyme in the A222T and V262L suppressor strains (38 ± 6 and 34 ± 6 S.D. µg/mg total protein, respectively) were similar to that of wild type (39 ± 6 S.D. µg/mg total protein), which was about four times greater than the level of holoenzyme in the L290F mutant (8 ± 1 S.D. µg/mg total protein) (4–6).

**Catalytic Efficiency**—The suppressor mutations appear to improve $K_\text{c}$ of the L290F enzyme by increasing $V_\text{c}$ relative to $K_\text{c}$ (Table I, compare L290F with L290F/A222T and L290F/V262L) (38). However, analysis of the A222T and V262L suppressor-mutant enzymes revealed that they did not have $V_\text{c}$ or $K_\text{c}$ values greater than those of the wild-type enzyme. In fact, their $K_\text{c}/V_\text{c}$ values were substantially lower than that of the wild-type enzyme (Table I). The A222T and V262L Rubisco enzymes have wild-type $K_\text{c}$ values due to increases in $V_\text{c}/V_\text{o}$ relative to the wild-type enzyme. Because their $V_\text{o}$ and $K_\text{c}/V_\text{c}$ values were lower or unchanged, the improvements in $V_\text{c}/V_\text{o}$ must arise from decreases in $V_\text{o}$ (Table I, compare wild type with A222T and V262L). Despite their wild-type $K_\text{c}$ values, the A222T and V262L enzymes are not as good as the wild-type enzyme with respect to net carboxylation. These mutant enzymes have decreases in $K_\text{c}$ and $K_\text{c}/V_\text{c}$ (Table I). Although the A222T and V262L substitutions improve the L290F enzyme in similar ways (i.e. increased $V_\text{c}$, $V_\text{c}/K_\text{c}$, and $\Omega$), they appear to do so by somewhat different mechanisms. A222T caused a small decrease in $K_\text{c}$ but V262L substantially increased both $K_\text{c}$ and $\Omega$ (Table I).

**Rubisco Thermal Stability**—Because increased levels of Rubisco protein were observed in L290F/A222T and L290F/V262L revertant cells grown at 35 °C relative to the amount of holoenzyme in L290F mutant cells (8), it was reasonable to consider that the A222T and V262L suppressor substitutions improved the thermal stability of the original L290F mutant enzyme (4, 6). When purified enzymes were preincubated at elevated temperatures, revertant L290F/A222T Rubisco retained slightly more RuBP carboxylase activity at 55 °C than did the L290F enzyme, but revertant L290F/V262L enzyme

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**TABLE I**

| Enzymes | $\Omega^a$ | $V_\text{c}/V_\text{o}$ | $K_\text{c}$ | $K_\text{c}/V_\text{c}$ | $V_\text{c}/K_\text{c}$ | $K_\text{c}/K_\text{c}$ | $V_\text{c}/V_\text{o}$ |
|---------|------------|----------------------|-----------|-------------------|------------------|-----------------|------------------|
| Wild type | 63 ± 1 | 139 ± 15 | 32 ± 5 | 559 ± 71 | 4.3 | 17 | 3.7 |
| L290F | 57 ± 1 | 30 ± 7 | 20 ± 5 | 575 ± 76 | 7.4 | 23 | 4.0 |
| L290F/A222T | 60 ± 2 | 90 ± 16 | 36 ± 3 | 670 ± 15 | 9.4 | 23 | 4.0 |
| L290F/V262L | 60 ± 1 | 76 ± 9 | 10 ± 3 | 1279 ± 47 | 1.1 | 34 | 4.0 |
| A222T | 63 ± 1 | 115 ± 20 | 27 ± 3 | 507 ± 22 | 15 | 36 | 4.0 |
| V262L | 62 ± 2 | 102 ± 24 | 17 ± 2 | 728 ± 144 | 22 | 35 | 4.0 |

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*a* The values are the means ± S.D. of three separate enzyme preparations.

*b* Calculated values.
had a thermal stability comparable to that of the wild-type enzyme (Fig. 1). For example, whereas wild-type and L290F/V262L Rubisco retained 41 and 61% of their initial activities, respectively, at 60 °C, the mutant L290F and revertant L290F/A222T enzymes were completely inactivated. Much to our surprise, enzymes containing only the A222T or V262L suppressor substitutions had thermal stabilities greater than that of the wild-type enzyme. For example, whereas A222T and V262L Rubisco retained 58 and 93% of their initial activities, respectively, at 65 °C, the mutant, revertant, and wild-type enzymes were completely inactivated (Fig. 1).

**DISCUSSION**

One does not expect that a single amino acid substitution would improve the carboxylation efficiency of wild-type Rubisco, and the failure to select a better enzyme by using a variety of genetic approaches has illustrated this point (reviewed in Ref. 2). However, in the present study, we found that a single A222T or V262L substitution in the Rubisco large subunit, each recovered as a second-site suppressor of a deleterious L290F substitution (4, 8), could substantially improve the thermal stability of the otherwise wild-type holoenzyme (Fig. 1). This apparent inconsistency is explained by the fact that *Chlamydomonas* cannot grow at temperatures much above 35 °C, but its Rubisco enzyme is resistant to temperatures as high as 55 °C *in vitro* (6, 20) (Fig. 1). There is no natural selection for mutant enzymes like A222T and V262L Rubisco, which are resistant to even higher temperatures, 60 and 65 °C, respectively (Fig. 1). Although the suppressors increase the amount of thermally unstable L290F Rubisco *in vivo* (8), it is not surprising that they fail to increase the level of wild-type Rubisco *in vivo*. Wild-type Rubisco is already resistant to temperatures far exceeding those that *Chlamydomonas* encounters in nature.

Mutant L290F is the only *rbcL* mutant recovered by screening for a temperature-conditional phenotype (reviewed in Ref. 17). The A222T and V262L suppressor substitutions, which improve the thermal stability of L290F Rubisco, are likewise unique. They increase Rubisco thermal stability even in the absence of the original L290F mutant substitution (Fig. 1). Furthermore, residues 222, 262, and 290 are highly conserved among land plants and green algae. There is some divergence at these residues among non-green algae and prokaryotes, but it is striking that the well studied Rubisco from the thermophilic red alga *Galdieria psychrophila* (38, 39) has different residues at every one of these positions (Met-222, Ser-262, and Ile-290) among the cyanobacterium *Synechococcus* (7, 8), which is 12–18 residues longer in the Rubisco of plants and green algae than in the Rubisco of prokaryotes and non-green algae (2, 9). Insertion of the land plant loop into the small subunit of the cyanobacterium *Synechococcus* is sufficient for permitting the assembly of prokaryotic small subunits with eukaryotic large subunits in isolated chloroplasts (10) and an R33E substitution in the eukaryotic small subunit blocks holoenzyme assembly (11). Taken together, all of these obser-
improvements in the A222T and V262L substitutions increase the amount of hydrogen bond network, along with His-292 and His-325, that in van der Waals contact with Glu-158, which participates in a growth at 35 °C. Whereas the suppressor substitutions (Table I, compare L290F with L290F/V262L), are also necessary (and are select-
vate carboxylation (40, 41). Because of its proximity to CABP (Fig. 2). Leu-290, Leu-162, Leu-169, and Leu-375 are in van der Waals contact with Glu-158, which participates in a hydrogen bond network, along with His-292 and His-325, that terminates at the active-site residue His-327 (7). His-327, in β-strand 6 at the base of flexible loop 6, coordinates with one of the phosphate groups of the transition state analog CABP (Fig. 2) (7). Amino acid substitutions at His-327 in *Rhadospirillum rubrum* and *Synechococcus* Rubisco decrease but do not eliminate carboxylation (40, 41). Because of its proximity to CABP and loop 6 Lys-334, which discriminates between CO2 and O2 (29, 42, 43), His-327 may provide the means by which subtle alterations could be propagated to influence Ω. It was previously proposed that the L290F substitution caused thermal instability by disrupting the hydrogen bond network within the hydrophobic core of the α/β-barrel (2). Based on the work presented here, it would seem to be more likely that L290F and the A222T and V262L suppressor substitutions influence thermal stability by altering interactions at the small/large subunit interface. The L290F mutant and A222T and V262L suppressor-substituted residues may interact to influence catalysis via compensatory deflections of the small subunit βA/βB loop or by displacing a similar set of residues in the hydrophobic core of the large subunit α/β-barrel. However, to influence catalysis, and Ω in particular (Table I), the effects of the substitutions must be propagated to the active site. Directed mutagenesis of other residues along the hydrogen bond network may identify residues that mimic the effect of L290F on catalysis without necessarily affecting thermal stability.

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REFERENCES

1. Yanovsky, C. (1967) Annu. Rev. Genet. 1, 117–137
2. Spreitzer, R. J. (1993) Annu. Rev. Plant Physiol. Plant Mol. Biol. 44, 411–434
3. Spreitzer, R. J., Al-Abed, S. R., and Huester, M. J. (1988) Plant Physiol. (Rocky Mt.) 66, 773–777
4. Chen, Z., Chastain, C. J., Al-Abed, S. R., Chollet, R., and Spreitzer, R. J. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 4566–4569
5. Chen, Z., Green D., Westhoff, C., and Spreitzer, R. J. (1990) Arch. Biochem. Biophys. 283, 60–67
6. Chen, Z., Hong, S., and Spreitzer, R. J. (1993) Plant Physiol. (Rocky Mt.) 101, 1089–1104
7. Andersson, I. (1990) J. Biol. Chem. 265, 160–174
8. Hong, S., and Spreitzer, R. J. (1997) J. Biol. Chem. 272, 11114–11117
9. Spreitzer, R. J. (1999) Photosynth. Res. 60, 29–42
10. Wasmann, C. M., Ramage, T. R., Bohnert, H. J., and Ostrem, J. A. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 1198–1202
11. Flachmann, R., and Bohnert, H. J. (1992) J. Biol. Chem. 267, 10576–10583
12. Hartman, F. C., and Harpel, M. R. (1994) Annu. Rev. Biochem. 63, 197–234
13. Laing, W. A., Ogren, W. L., and Hageman, R. H. (1974) Plant Physiol. (Rocky Mt.) 54, 678–685
14. Ogren, W. L. (1984) Annu. Rev. Plant Physiol. 35, 415–442
15. Cloney, L. P., Bekkaoui, D. R., and Hemmingsen, S. M. (1982) Plant Physiol. 69, 235–239
16. Spreitzer, R. J. (1998) in *The Molecular Biology of Chloroplasts and Mitochondria in Chloramydomonas* (Roach, J. D., Goldschmidt-Clermont, M., and Merchant, S., eds) pp. 513–527, Klwer Academic Publishers Group, Dordrecht, Netherlands
17. Kanveski, I., Maliga, P., Rhodes, D. F., and Gutteridge, S. (1999) Plant Physiol. 122, 1285–1290
18. Greenby, A. A., van der Vies, S. M., and Rathstein, S. J. (1987) Eur. J. Biochem. 165, 227–231
19. Whitney, S. M., van Cammerer, S., Hudson, G. S., and Andrews, T. M. (1999) Plant Physiol. (Rocky Mt.) 121, 579–588
20. Zhu, G., and Spreitzer, R. J. (1996) J. Biol. Chem. 271, 18494–18498
21. Larsen, E. M., O'Brien, C. M., Zhu, G., Spreitzer, R. J., and Portis, A. R., Jr. (1997) J. Biol. Chem. 272, 17033–17037
22. Moreno, J., and Spreitzer, R. J. (1999) J. Biol. Chem. 274, 26789–26793
23. Spreitzer, R. J., and Al-Abed, S. R. (1981) Plant Physiol. (Rocky Mt.) 67, 555–569
24. Newman, S. M., Gillham, N. W., Harris, E. H., Johnson, A. M., and Boynton, J. E. (1991) Mol. Gen. Genet. 227, 65–74
25. Spreitzer, R. J., and Mets, L. (1981) Plant Physiol. (Rocky Mt.) 67, 1198–1202
26. Newcomer, S. M., Gilham, N. W., Harris, E. H., Johnson, A. M., and Boynton, J. E. (1991) Mol. Gen. Genet. 227, 65–74
27. Spreitzer, R. J., and Ogren, W. L. (1998) J. Biol. Chem. 273, 775–779
28. Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) *Meth. Enzymol.* 153, 203–208
29. Chen, Z., and Spreitzer, R. J. (1989) *Science* 247, 24734–24740
30. Chen, Z., Yu, W., Lee, J., Diao, R., and Spreitzer, R. J. (1991) Biochemistry 30, 8846–8850
31. Suzuki, R. K., Gefland, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, R. B., and Erlich, H. A. (1988) *Science* 239, 487–491
32. Bullock, W. O., Fernandez, J. M., and Short, J. M. (1987) *BioTechniques* 5, 376–379
33. Spreitzer, R. J., and Chastain, C. J. (1987) *Curr. Genet.* 11, 611–616
34. Bradford, M. M. (1976) *Anal. Biochem.* 72, 248–254
35. Spreitzer, R. J., and Mets, L. (1981) Plant Physiol. (Rocky Mt.) 67, 237–245
36. Spreitzer, R. J., Jordán, D. B., and Ogren, W. L. (1982) *FEBS Lett.* 144, 117–121
37. Kuehn, G. D., and Hsu, T. C. (1978) *Biochem.* 17, 809–912
38. Umura, K., Anwaruzzaman, Miyachi, S., and Yokota, A. (1997) *Biochem. Biophys. Res. Commun.* 233, 568–571
39. Sugawara, H., Yamamoto, H., Shibata, N., Inoue, T., Okada, S., Miyake, C., Yokota, A., and Kai, Y. (1996) *J. Biol. Chem.* 271, 15565–15566
40. Harpel, M. R., Larimer, F. W., and Hartman, F. C. (1991) *J. Biol. Chem.* 266, 24734–24740
41. Haining, R. L., and McFadden, B. A. (1994) *Photosynth. Res.* 41, 349–356
42. Chen, Z., and Spreitzer, R. J. (1988) *J. Biol. Chem.* 263, 3051–3053
43. Lorimer, G. H., Chen, Y. R., and Hartman, F. C. (1993) *Biochemistry* 32, 9018–9024
