Highly Conserved Small Subunit Residues Influence Rubisco Large Subunit Catalysis*5

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The chloroplast enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) catalyzes the rate-limiting step of photosynthetic CO₂ fixation. With a deeper understanding of its structure-function relationships and competitive inhibition by O₂, it may be possible to engineer an increase in agricultural productivity and renewable energy. The chloroplast-encoded large subunits form the active site, but the nuclear-encoded small subunits can also influence catalytic efficiency and CO₂/O₂ specificity. To further define the role of the small subunit in Rubisco function, the 10 most conserved residues in all small subunits were substituted with alanine by transformation of a Chlamydomonas reinhardtii mutant that lacks the small subunit gene family. All the mutant strains were able to grow photosynthetically, indicating that none of the residues is essential for function. Three of the substitutions have little or no effect (S16A, P19A, and E92A), one primarily affects holoenzyme stability (L18A), and the remainder affect catalysis with or without some level of associated structural instability (Y32A, E43A, W73A, L78A, P79A, and F81A). Y32A and E43A cause without some level of associated structural instability (Y32A, E43A, W73A, L78A, P79A, and F81A). Y32A and E43A cause without some level of associated structural instability (Y32A, E43A, W73A, L78A, P79A, and F81A). Y32A and E43A cause without some level of associated structural instability (Y32A, E43A, W73A, L78A, P79A, and F81A) to impair Rubisco CO₂/O₂ specificity.

In plants and green algae, ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39)2 is comprised of eight chloroplast-encoded large subunits and eight nuclear-encoded small subunits (referred to as L8S8 Rubisco) (reviewed in Refs. 1–3). The ~55-kDa large subunit contains a carboxyl-terminal α/β-barrel domain that, along with residues from the amino-terminal domain of a neighboring large subunit, forms the active site of the enzyme. Carboxylation of ribulose 1,5-bisphosphate (RuBP) initiates the rate-limiting step of photosynthetic CO₂ fixation. However, O₂ is mutually competitive with CO₂, and oxygenation of RuBP is a nonessential side reaction that ultimately leads to the loss of CO₂ in the photosynthetic pathway. Thus, net CO₂ fixation is determined by the difference between the rates of carboxylation and oxygenation (4), which are ultimately determined by the Vₘₐₓ values for carboxylation (Vₖ) and oxygenation (Vₙ), the Kₘ values for CO₂ (Kₖ) and O₂ (Kₙ), and the concentrations of CO₂ and O₂ at the Rubisco active site. Another kinetic constant referred to as the CO₂/O₂ specificity factor (Ω) is equal to the catalytic efficiency of carboxylation (Vₖ/Kₖ) relative to the catalytic efficiency of oxygenation (Vₙ/Kₙ) (4). Because Ω is determined by the difference between the carboxylation and oxygenation free energies of activation at the rate-determining step of catalysis (5), changes in Ω may identify regions of Rubisco worthy of genetic engineering. There continues to be much interest in either engineering or selecting improvements in Rubisco as a means for increasing net CO₂ fixation and the production of food, fiber, and renewable energy.

The catalytic mechanism of the large subunit active site has been studied extensively by employing directed mutagenesis of either the dimeric large subunit Rubisco enzyme of Rhodospirillum rubrum (referred to as L2 Rubisco) or the plant-like L8S8 Rubisco of Synechococcus expressed in Escherichia coli (reviewed in Refs. 6 and 7). Numerous x-ray crystal structures are available to serve as a basis for such studies (2). However, as of yet, this information has not been exploited for designing a better Rubisco or for explaining the variation in kinetic constants observed for Rubisco enzymes from different species (8–11). Much less is known about the role of the ~15-kDa small subunit in Rubisco function (reviewed in Ref. 12). The small subunit is not in contact with any of the large subunit active site residues, but directed mutagenesis of prokaryotic and algal small subunits (13–15) or creation of hybrid enzymes comprised of large and small subunits from different species (16, 17) has indicated that the small subunit can also influence the value of Ω. Because there is greater divergence between eukaryotic and prokaryotic small subunits than between large subunits, one wonders whether the small subunits may, in part, be responsible for the higher Ω values of eukaryotic Rubisco enzymes (8).

The loop between β-strands A and B of the small subunit is the most variable structure among all Rubisco enzymes (12). It is comprised of 10 residues in prokaryotes and eukaryotic non-green algae, ~22 residues in green plants, and 28 residues in green algae (see Fig. 1). The βA-βB loops of four small subunits, which reside at opposite ends of the octameric large subunit core, surround the opening of a solvent channel that passes...
Rubisco Small Subunit Mutants

through the holoenzyme (1, 2, 12). As a means for determining the significance of the longer loop in plant Rubisco, Bohnert and co-workers (28, 33) used in vitro transcription/translation and uptake into isolated pea chloroplasts to study the influence of engineered small subunits on the holoenzyme. This complex procedure was necessary because eukaryotic holoenzymes cannot be expressed in E. coli (34) and because the small subunit is coded by a family of rbcS genes in plants that cannot be eliminated and replaced with engineered copies (35). When the longer βA-βB loop of plants was engineered into the Synecococcus small subunit, this cyanobacterial small subunit was then able to assemble with plant large subunits in the isolated chloroplasts (33). Thus, the βA-βB loop appeared to be an assembly domain (33), but detailed analysis of Rubisco catalysis was difficult to perform because of the low yield of holoenzyme in the isolated chloroplast system (28, 33).

In the green alga Chlamydomonas reinhardtii, N54S, A57V, and C65S substitutions in the small subunit βA-βB loop were recovered as genetic suppressors of a temperature-conditional large subunit L290F mutant strain (15, 31). Whereas the L290F substitution caused a decrease in Ω and holoenzyme stability (36), the substitutions in the βA-βB loop returned Ω to nearly normal values and improved thermal stability of the holoenzyme (15, 31). When the rbcS gene family (comprised of linked rbcS1 and rbcS2 genes) of Chlamydomonas was deleted via an insertional mutagenesis procedure (37), it became possible to investigate the role of the βA-βB loop in greater detail, as well as the importance of the eukaryotic small subunit in general. Like all photosynthesis-deficient mutants of this model genetic organism, the Chlamydomonas rbcS deletion strain can be maintained with acetate as an alternative carbon source, and it lacks photosynthesis and requires acetate for growth because of deletion of the 13-kb locus that contains rbcS1 and rbcS2 (37). All of the strains are maintained at 25 °C in darkness on medium containing 10 mM acetate and 1.5% Bacto agar (39). For biochemical analysis, the cells were grown in 250–500 ml of liquid acetate medium at 25 °C on a rotary shaker at 120 rpm in darkness.

Directed Mutagenesis and Transformation—Plasmid pSS1 (37), which contains the entire Chlamydomonas rbcS1 gene on a 5-kb EcoRI fragment, was used for site-directed mutagenesis and transformation. Mutagenesis was performed with synthetic oligonucleotides and a QuikChange kit from Stratagene (40). The mutant plasmids were propagated in E. coli XL1-Blue (Stratagene). To create the mutant substitutions, the sequences encoding Ser-16 (TCC), Leu-18 (CTG), Pro-19 (CCT), Tyr-32 (TAC), Glu-35 (GAG), Trp-73 (TGG), Leu-78 (CTG), Pro-79 (CCC), Phe-81 (TTC), and Glu-92 (GAG) were changed to the most common sequence that encodes alanine (GCC). PCR and restriction fragment analysis were used to screen for the presence of the mutations.

Transformation was performed by electroporation (41), and photosynthesis-competent colonies were selected on minimal medium (without acetate) in the light (80 microeinstein/m²/s). The engineered rbcS1 gene from each mutant strain was then PCR-amplified and completely sequenced at the University of Nebraska DNA sequencing facility to confirm that only the intended mutation was present. The rbcS1 mutant strains were named S16A, L18A, P19A, Y32A, E43A, W73A, L78A, P79A, F81A, and E92A.

The unaltered pSS1 plasmid was also transformed into rbcSΔ-T60-3, and photosynthesis-competent colonies were recovered as described above. One of these strains, named S1, was retained as a wild-type control strain. Previous studies have shown that there is no difference in growth phenotype or Rubisco function for Chlamydomonas strains containing either rbcS1 or rbcS2 alone (14).

Biochemical Analysis—Dark-grown cells were sonicated at 0 °C for 3 min in extraction buffer comprised of 50 mM Bicine (pH 8.0), 10 mM NaHCO₃, 10 mM MgCl₂, and 1 mM dithiothreitol. Cell debris was removed by centrifugation at 30,000 × g for 15 min, and the amount of protein in the supernatant was quantified (42). The cell extract was subjected to SDS-PAGE and Western blotting (43, 44) or fractionated on 10–30% sucrose gradients prepared in the same extraction buffer to isolate pure

rubS sequences were available, focused on conserved regions rather than residues, and substituted a variety of similar or quite dissimilar residues, no specific structural region was studied in any greater detail. In the present study, the 10 most conserved small subunit residues were substituted with alanine in Chlamydomonas Rubisco. This eukaryotic system allows the effect of the substitutions to be assessed in vivo and in vitro. Analysis has indicated that the conserved small subunit residues may influence a single structural element that contributes to the Rubisco large subunit active site.

EXPERIMENTAL PROCEDURES

Strains and Culture Conditions—Cell wall-less C. reinhardtii strain rbcSΔ-T60-3 mt- was used as the host for rbcS nuclear gene transformation. It lacks photosynthesis and requires acetate for growth because of deletion of the 13-kb locus that contains rbcS1 and rbcS2 (37). All of the strains are maintained at 25 °C in darkness on medium containing 10 mM acetate and 1.5% Bacto agar (39). For biochemical analysis, the cells were grown in 250–500 ml of liquid acetate medium at 25 °C on a rotary shaker at 120 rpm in darkness.

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Rubisco Small Subunit Mutants

Rubisco holoenzyme (45). The thermal stability of purified Rubisco was determined as described previously (14, 46).

The $V_c$ and $K_c$ kinetic constants of purified and activated Rubisco were measured by the incorporation of acid-stable $^{14}$C from NaH$^{14}$CO$_3$ in the absence of O$_2$. The ratio ($v_c/v_o$) of the rates of carboxylase ($v_c$) measured simultaneously with 88 resins/m$^2$/s) at either the normal growth temperature of 25 °C or an elevated temperature of 35 °C. Because temperature-conditional Rubisco mutants may be useful for selecting second site suppressor substitutions (50, 51), the phenotypes of multiple, independent transformants were compared in spot tests prior to choosing a mutant strain for further analysis. All the mutants grew as well as wild type on acetate medium in darkness (data not shown). However, as shown in Fig. 2, five of the mutant strains (L18A, Y32A, E43A, W73A, and L78A) grew slower than the wild-type SS1 control strain under photosynthetic conditions at the normal growth temperature of 25 °C. Because temperature-conditional Rubisco mutants may be useful for selecting second site suppressor substitutions (50, 51), the phenotypes of the mutant strains were also checked at 35 °C (Fig. 2). At this restrictive temperature, the L18A, E43A, W73A, and L78A mutants failed to grow, and the Y32A and F81A mutants displayed substantial growth inhibition. The S16A, L18A, P19A, Y32A, E43A, W73A, and E92A, and these genes were transformed into the Chlamydomonas rbcSΔ-T60-3 strain. Considering the high conservation of the engineered residues, it was surprising to find that all of these mutant genes restored photosynthetic growth to the rbcS knock-out strain. Thus, it was immediately apparent that none of these residues is essential for Rubisco function or assembly. Spot tests were performed (39) to better judge the phenotypes of the mutant strains (Fig. 2). Because nuclear transformation occurs by nonhomologous recombination in Chlamydomonas, there is concern that phenotypic variation may be the result of alterations in gene dosage or transcriptional regulation. To guard against these possibilities, the phenotypes of multiple, independent transformants were compared in spot tests prior to choosing a mutant strain for further analysis. All the mutants grew as well as wild type on acetate medium in darkness (data not shown). However, as shown in Fig. 2, five of the mutant strains (L18A, Y32A, E43A, W73A, and L78A) grew slower than the wild-type SS1 control strain under photosynthetic conditions at the normal growth temperature of 25 °C.

Analysis of Holoenzyme Stability—To see whether the growth phenotypes of the mutant strains (Fig. 2) reflected a decrease in the amount of Rubisco holoenzyme, extracts of cells grown at 25 and 35 °C in darkness were subjected to SDS-PAGE and Western analysis (Fig. 3). Because small subunits are rap
Rubisco Small Subunit Mutants

Temperature.

Reflect alterations in Rubisco catalysis at this elevated 35 °C (W73A, L78A, and F81A) retained significant levels (Fig. 2). However, other mutants that grew poorly if at all at 35 °C in darkness, had nearly normal levels of Rubisco holoenzyme. The most obvious reduction in growth when compared with wild type at 25 °C (Fig. 3), and these mutants also had decreases in holoenzyme were observed for the L18A, Y32A, and E43A mutants (25 °C; Fig. 3), and these mutants also had decreases in holoenzyme were observed for the L18A, Y32A, and E43A mutants (25 °C; Fig. 3), indicating that reduced growth at 25 °C may result from enhanced proteolysis of mutant enzymes (Fig. 2). One generally suspects that the decreases in holoenzyme structure or stability. Mutants W73A and L78A also had reduced growth at 25 °C, but, because its carboxylation specific activity was decreased 5-fold, further study of this enzyme was also warranted. Only three of the mutant enzymes had decreased in vitro thermal stability relative to the SS1 wild type (Fig. 4), but, because this instability is detected at temperatures far above growth or enzyme assay temperatures, it is unlikely to affect the accurate determination of Rubisco kinetic constants.

Analysis of Rubisco Function—As a first step for analyzing the catalytic properties of the mutant enzymes, Ω values, carboxylation specific activities, and the influence of O₂ on carboxylation were determined (Table 1). The Y32A and E43A enzymes were found to have 8–10% decreases in Ω. Y32A caused an increase in O₂ inhibition, but E43A caused a decrease. Because the ratio of carboxylase activities in the absence and presence of O₂ is determined by only the Kᵣ and Kₛ kinetic constants (36), one might assume that the decrease in Ω of the E43A enzyme results from the observed substantial decrease in carboxylation specific activity (assayed with saturating CO₂ provided by 12.4 mM NaHCO₃) (Table 1). The W73A, L78A, and F81A mutant enzymes also had decreases in O₂ inhibition, but their Ω values were the same as that of the SS1 wild-type enzyme. The P79A enzyme had a wild-type Ω value and normal level of O₂ inhibition, but, because its carboxylation specific activity was decreased ~5-fold, further study of this enzyme was also warranted. Only three of the mutant enzymes (S16A, P19A, and E92A) had nearly normal catalytic properties in these initial assays (Table 1). Considering that the S16A, P19A, and E92A mutant strains also have wild-type phenotypes (Fig. 2) and nor-
TABLE 1
Kinetic properties and oxygen inhibition of Rubisco purified from wild-type SS1 and small subunit mutants

| Enzyme          | \( \frac{\Omega}{(V_{c}K_{c}V_{o}K_{o})} \) | 100% \( N_{2} \) at \( 22 \) °C | 100% \( N_{2} \) at \( 27 \) °C | 100% \( O_{2} \) at \( 27 \) °C | Ratio (A/B) |
|-----------------|------------------------------------------|---------------------------------|---------------------------------|---------------------------------|-------------|
| Wild-type SS1   | 60 ± 1                                   | 127 ± 12                        | 31 ± 2                          | 527 ± 20                        | 4.1 17 3.5  |
| S16A            | 54 ± 2                                   | 23 ± 2                          | 30 ± 2                          | 383 ± 31                        | 0.8 13 4.2  |
| Y32A            | 52 ± 2                                   | 10 ± 1                          | 64 ± 3                          | 1624 ± 302                      | 0.2 25 2.2  |
| E43A            | 55 ± 2                                   | 18 ± 2                          | 67 ± 3                          | 1159 ± 249                      | 0.6 25 2.3  |
| W73A            | 58 ± 2                                   | 12 ± 2                          | 65 ± 3                          | 1195 ± 249                      | 0.6 25 2.3  |
| L78A            | 62 ± 2                                   | 16 ± 2                          | 69 ± 3                          | 219 ± 24                        | 0.3 18 3.3  |
| F81A            | 61 ± 2                                   | 17 ± 2                          | 73 ± 3                          | 1058 ± 24                      | 1.1 23 2.6  |

The values are the means ± S.D. (n = 3) of three separate enzyme preparations.

TABLE 2
Kinetic properties of Rubisco purified from wild-type SS1 and small subunit mutants

| Enzyme          | \( \frac{1}{K_{c}} \), \( V_{r} \), \( K_{c} \), \( K_{o} \), \( V_{r}/V_{o} \) | \( \frac{\Omega}{(V_{c}K_{c}V_{o}K_{o})} \) | \( \mu \text{mol CO}_2/\text{mg of protein} \) | \( \mu \text{mol O}_2/\text{mg of protein} \) |
|-----------------|------------------------------------------|------------------------------------------|------------------------------------------|------------------------------------------|
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| L78A            | 62 ± 2                                   | 16 ± 2                                   | 69 ± 3                                  | 219 ± 24                                | 0.3 18 3.3  |
| P79A            | 58 ± 1                                   | 19 ± 1                                   | 62 ± 3                                  | 839 ± 83                                | 1.4 16 3.6  |
| F81A            | 61 ± 2                                   | 17 ± 2                                   | 73 ± 3                                  | 1058 ± 24                               | 1.1 23 2.6  |

**Analysis of Rubisco Structure**—The small subunit residues analyzed in the present study were chosen because of their conservation in numerous small subunits (12). One might suppose that such highly conserved residues reside in the hydrophobic core of the small subunit and play some role in protein folding or assembly. However, analysis of the 1.4-Å x-ray crystal structure of *Chlamydomonas* Rubisco revealed that all but one (Glu-92) of the conserved residues are in contact with the Rubisco large subunit (Fig. 5 and supplemental Table 1), and as expected, the contacted large subunit residues are highly conserved (supplemental Table S2). Residues Ser-16, Leu-18, and Tyr-32 are in contact with the bottom half of large subunit α-helix 8 (Fig. 5), which is a structural element of the α/β-barrel that forms the active site. Leu-78 from a neighboring small subunit is in contact with the loop preceding large subunit α-helix 8 at the top of the α/β-barrel. Pro-79, Phe-81, and Glu-92 are closely associated with Leu-78 in this region (Fig. 5). Glu-43 and Trp-73 are in van der Waals contact with each other, and Glu-43 forms an ionic bond with Arg-187 in α-helix 1 of the...
Rubisco Small Subunit Mutants

large subunit α/β-barrel (Fig. 5). Considering that the E43A, W73A, L78A, and F81A mutant substitutions produce similar catalytic effects (Table 2), and the affected residues cluster near the top of α-helix 8 (Fig. 5), small subunit structure in this region may play a significant role in determining Rubisco function.

DISCUSSION

Analysis of hybrid (16, 17), chimeric (11, 38), and small subunit mutant enzymes (13–15, 31) has shown that Rubisco small subunits can influence both carboxylation catalytic efficiency and Ω. However, because small subunits do not interact directly with the large subunit active site (2), it remains difficult to determine the structural basis for this influence. A chimeric *Chlamydomonas* Rubisco enzyme containing the shorter small subunit BA-βB loop of *Synechococcus* has a decreased Ω value, but the x-ray crystal structure of the mutant enzyme failed to identify a direct structural path from the βA-βB loop to the large subunit active site (38).

It was interesting to find that the small subunit residues chosen for analysis in the present study, based solely on their high level of conservation (Fig. 1), associate in groups near α-helix 8 of the large subunit α/β-barrel (Fig. 5). Previous comparison of the x-ray crystal structure of L8S8 spinach Rubisco with the L2 subunit was responsible for the observed differences in conformation (53). Because small subunit residues interact with these large subunit regions, it was concluded that the small subunit was responsible for the observed differences in conformation (53). Furthermore, because the loop before α-helix 8 contains two active site residues (Gly-403 and Gly-404) that bind to one of the phosphate groups of the CABP transition state analog (20, 23), it was also proposed (53) that small subunits might influence Rubisco catalysis via these interactions and account for the differences in Ω values between L2 (Ω ≈ 15) and L8B (Ω ≈ 80) Rubisco enzymes (8).

At the bottom of large subunit α-helix 8, the conserved small subunit residue Tyr-32 appears to shield large subunit residues Arg-431 and Asn-432 from solvent (Fig. 5). These residues are the last two at the carboxyl-terminal end of α-helix 8 (Fig. 5). Nonetheless, the Y32A mutant enzyme has a 10% decrease in Ω (Table 2) in addition to an associated structural instability (Figs. 3 and 4). Because α-helices are dipoles because of resonance at the peptide bond, perhaps the nature of small subunit residue 32 at the carboxyl-terminal end of large subunit α-helix 8 can influence the partial positive charge at the amino-terminal end of α-helix 8.

Of the three conserved small subunit residues that reside near the middle of large subunit α-helix 8 (Ser-16, Leu-18, and Pro-19) (Fig. 5), only Leu-18 has a significant effect on cell growth when substituted with alanine (Fig. 2). Because the side chain of Leu-18 is in van der Waals contact with three large subunit α-helix-8 residues (Glu-425, Ala-426, and Gln-429), the loss of these interactions may account for both the holoenzyme instability observed in L18A cells grown at 35°C (Fig. 3) and the difficulty with purifying L18A mutant Rubisco. However, despite the fact that Ser-16 and Pro-19 are in contact with Leu-18, the S16A and P19A substitutions have only minor effects on carboxylation specific activity (Table 1) and in vitro holoenzyme thermal stability (Figs. 3 and 4). Perhaps less conservative substitutions at these residues would produce an effect. In previous studies with *Synechococcus* Rubisco expressed in *E. coli*, P19A and P19H substitutions also had no significant effect on carboxylation or holoenzyme assembly (13, 26). However, an S16D substitution disrupted holoenzyme stability (26), whereas an S16A substitution was reported to cause a decrease in Ω (13). In this latter study (13), an increased Ω value was also reported for a P20A substitution, but other Rubisco kinetic constants were not measured. Other substitutions were created in cyanobacterial small subunits at residues near to Ser-16, Leu-18, and Pro-19 (Fig. 1), but, except for one study (27), analysis of these mutant enzymes was incomplete. R10G and E13V substitutions blocked holoenzyme assembly (13, 25), and some substitutions at Thr-14, Tyr-17, and Leu-21 caused decreases in both carboxylation and holoenzyme stability (13, 26, 27). Perhaps Ser-16 and Pro-19 have provided some selective advantage during evolution that is not readily apparent from the completed biochemical analysis of *Chlamydomonas* Rubisco. Further study of the region defined by the conservative Ser-16, Leu-18, and Pro-19 residues may be necessary to determine whether this region is primarily responsible for holoenzyme stability, catalytic efficiency, or both.

The remainder of the conserved small subunit residues cluster near the top of α-helix 8 (Glu-43, Trp-73, Leu-78, Pro-79, Phe-81, and Glu-92) (Fig. 5). With respect to the evolution of Rubisco structure, it is interesting to note that these residues reside in a small subunit different from the one that comes in contact with the carboxyl-terminal end of large subunit α-helix 8. Although conserved Tyr-32 caps the carboxyl-terminal end of large subunit α-helix 8, small subunit Leu-78 is in contact with large subunit residues Pro-410 and Gly-412 just before the start of α-helix 8 at Asn-413 (Fig. 5). The L78A substitution does not alter Ω, but it does cause a decrease in Vc and increases in Kc and Kc" (Table 2). The loss of interactions between small subunit Leu-78 and large subunit residues Pro-410 and Gly-412 may alter the structure of the loop preceding α-helix 8. Catalysis may be affected because this loop contains the active site residues Gly-403 and Gly-404, which interact with the CABP transition state analog (Fig. 5). Leu-78 and Phe-81 are not in direct contact with each other, but the L78A and F81A substitutions cause similar changes in catalytic properties (Table 2).

The aromatic ring of Phe-81 is surrounded by large subunit residues Trp-70, Gly-73, and Leu-74 in the amino-terminal domain of a neighboring large subunit (Fig. 5). The loss of the Phe-81 side group might influence the position of Leu-78. However, the Leu-78 side group is also in van der Waals contact with large subunit Trp-70, and residues Trp-70, Gly-73, and Leu-74 are in the loop between large subunit α-helix B and β-strand C (αB-βC loop) that contains active site residues Thr-65 and Trp-66 (Fig. 5). It might be possible that L78A and F81A affect catalysis by altering the loop before α-helix 8, the amino-terminal-domain αB-βC loop, or both. Conserved small subunit residue Pro-79 is in contact with large subunit Trp-70 and small subunit residues Leu-78 and Phe-81, but the P79A substitution causes a relatively small decrease in only Vc. Perhaps a less conservative substitution would produce a different
Rubisco Small Subunit Mutants

...effect. In a previous study, when the homologous Pro-59 residue in the small subunit of *Anabaena* Rubisco was replaced with a larger and polar histidine (Fig. 1), holoenzyme assembly in *E. coli* was blocked (25). However, the *Chlamydomonas* L78A, P79A, and F81A mutant strains have normal amounts of Rubisco when grown at 25 °C, and the purified mutant enzymes have only a modest decrease in thermal stability *in vitro* (Fig. 4). The side chain of conserved residue Glu-92 resides at the surface of small subunit α-helix B (Figs. 1 and 5). Glu-92 is in contact with some of the same residues contacted by small subunit Leu-78, Pro-79, and Phe-81, but the E92A substitution causes only a small decrease in carboxylation (Table 2) with no effect on holoenzyme stability (Figs. 3 and 4). Altogether, it seems likely that the conserved Leu-78, Pro-79, Phe-81, and Glu-92 residues may have a greater role in function than in holoenzyme assembly or stability.

Whereas conserved small subunit residues Glu-43 and Trp-73 are also in the region near the top of large subunit α-helix 8, they are in direct contact with large subunit α-helix 1 of the α/β-barrel (Fig. 5). Glu-43 forms an ionic bond with large subunit Arg-187, and Trp-73 is in van der Waals contact with large subunit Tyr-190. The E43A and W73A enzymes both have decreases in $V_\text{c}$ and increases in $K_\text{c}$ and $K_\text{m}$, but the E43A enzyme also has a decreased $\Omega$ value (Table 2) and an associated structural instability *in vivo* (Fig. 3), which may result from the loss of the salt bridge to the large subunit. Analysis of small subunit Trp-55 and Trp-58 in *Synechococcus* Rubisco (which are homologous to Trp-73 and Trp-76 in *Chlamydomonas*; Fig. 1) indicated that W55F and W58F mutant enzymes also had a decrease in $V_\text{c}$, but $K_\text{m}$ was not changed, and $K_\text{c}$ was not measured (24, 26). When the residue homologous to Glu-43 of Rubisco (which...
Rubisco Small Subunit Mutants

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