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Phylogenetic engineering at an interface between large and small subunits imparts land-plant kinetic properties to algal Rubisco

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Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) catalyzes the rate-limiting step of photosynthetic CO_2 fixation and, thus, limits agricultural productivity. However, Rubisco enzymes from different species have different catalytic constants. If the structural basis for such differences were known, a rationale could be developed for genetically engineering an improved enzyme. Residues at the bottom of the large-subunit α/β-barrel active site of Rubisco from the green alga *Chlamydomonas reinhardtii* (methyl-Cys-256, Lys-258, and Ile-265) were previously changed through directed mutagenesis and chloroplast transformation to residues characteristic of land-plant Rubisco (Phe-256, Arg-258, and Val-265). The resultant enzyme has decreases in carboxylation efficiency and CO_2/O_2 specificity, despite the fact that land-plant Rubisco has greater specificity than the *Chlamydomonas* enzyme. Because the residues are close to a variable loop between β-strands A and B of the small subunit that can also affect catalysis, additional substitutions were created at this interface. When large-subunit Val-221 and Val-235 were changed to land-plant Cys-221 and Ile-235, they complemented the original substitutions and returned CO_2/O_2 specificity to the normal level. Further substitution with the shorter βA–βB loop of the spinach small subunit caused a 12–17% increase in specificity. The enhanced CO_2/O_2 specificity of the mutant enzyme is lower than that of the spinach enzyme, but the carboxylation and oxygenation kinetic constants are nearly indistinguishable from those of spinach and substantially different from those of *Chlamydomonas* Rubisco. Thus, this interface between large and small subunits, far from the active site, contributes significantly to the differences in catalytic properties between algal and land-plant Rubisco enzymes.

catalysis | *Chlamydomonas* | chloroplast | photosynthesis | ribulosebisphosphate carboxylase/oxygenase

CO_2 and O_2 compete at the active site of ribulose-1,5-bisphosphate (RuBP) carboxylase/oxygenase [Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), Enzyme Commission 4.1.1.39] for either the carboxylation or oxygenation of RuBP (reviewed in refs. 1–3). Whereas carboxylation is responsible for the accumulation of carbon in the biosphere, oxygenation is a nonessential reaction that leads to the loss of fixed carbon via the photorespiratory pathway. The ratio of the catalytic efficiencies (V_{max}/K_m) of carboxylation (V_c/K_c) and oxygenation (V_o/K_o) defines the CO_2/O_2-specificity kinetic constant Ω (4), which is determined by the differential stabilization of the carboxylation and oxygenation transition states for the rate-limiting partial reactions (5). However, net CO_2 fixation is determined by the difference between the velocities of carboxylation and oxygenation at the CO_2 and O_2 concentrations that occur *in vivo* (4, 6). Because of its pivotal role in catalyzing the rate-limiting step of photosynthesis, genetic engineering of Rubisco aimed at increasing net CO_2 fixation remains a worthy goal for increasing the agricultural production of food and energy (1–3, 7, 8).

Rubisco enzymes from different species have different kinetic constants (9–11). For example, the dimeric large-subunit enzymes of some prokaryotes have large V_c values and small Ω values, whereas the hexadecameric enzymes of land plants, comprised of eight chloroplast-encoded ≈55-kDa large subunits and eight nuclear-encoded ≈15-kDa small subunits, have smaller V_c values and larger Ω values. Thus, there appears to be an inverse relationship between V_c and Ω (9), and it is difficult to conclude that any Rubisco enzyme in nature is better than any other when the concentrations of CO_2 and O_2 surrounding the enzyme are taken into account (1, 8, 11). Nonetheless, if the structural basis for the differences in the kinetic constants could be determined, targets may be identified for facilitating the engineering of an improved Rubisco. Numerous x-ray crystal structures of divergent and mutant Rubisco enzymes have been solved (2, 12, 13), but the reason for differences in the differential stabilization of the carboxylation and oxygenation transition states remains elusive.

In a previous study, only 34 residues in the 475-residue large subunit of the green alga *Chlamydomonas reinhardtii* were identified that are present in <5% of 500 large-subunit sequences of land-plant Rubisco (14). Because the large subunit contains the active site, some of these "phylogenetic" residues may be responsible for the differences in the catalytic properties between *Chlamydomonas* and land-plant Rubisco enzymes. Whereas *Chlamydomonas* Rubisco has a higher V_c and an Ω value of ≈60, land-plant enzymes have lower V_c values and Ω values of ≈80–100 (9). When three of the *Chlamydomonas* residues (methyl-Cys-256, Lys-258, and Ile-265) were changed by directed mutagenesis and chloroplast transformation to those most often observed in land plants (Phe-256, Arg-258, and Val-265), the resulting C256F/K258R/I265V triple-mutant enzyme had a 70% decrease in V_c and a 10% decrease in Ω (14). One must conclude that there are other residues in land-plant Rubisco that complement these deleterious effects on V_c and Ω, and these residues must also be different from those in *Chlamydomonas* Rubisco.

Residues 256, 258, and 265 were originally chosen for analysis because they are near large-subunit Leu-290 (Fig. 1) (12, 14). A temperature-conditional, photosynthesis-deficient (acetate-requiring) L290F mutant had been recovered by mutant screening in *Chlamydomonas*, and the purified mutant enzyme was found to have a decreased Ω value (17). Furthermore, when the L290F mutant was subjected to genetic selection for photosynthetic growth at the restrictive temperature (35°C), large-subunit A222T and V262L suppressor substitutions were recovered that restored thermal stability and also increased Ω back to the

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Abbreviations: Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; RuBP, ribulose 1,5-bisphosphate; Ω, CO_2/O_2 specificity factor.

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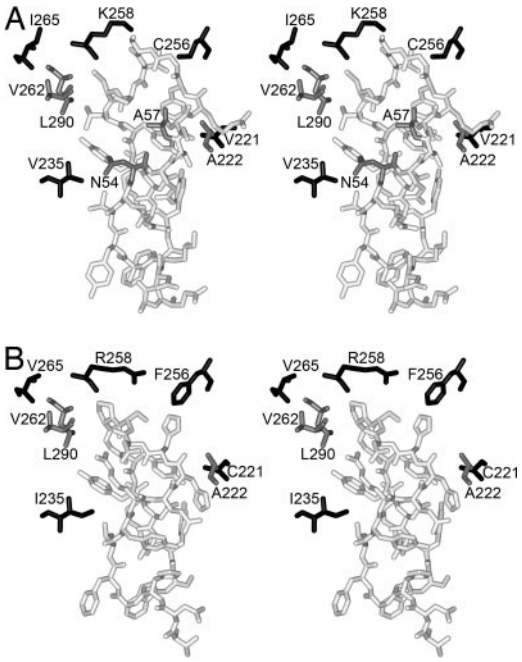

Fig. 1. Stereo images of large-subunit phylogenetic (in black) or mutant/suppressor (in gray) residues that surround the small-subunit βA–βB loop (in white) in the x-ray crystal structures of *Chlamydomonas* (*A*) and spinach (*B*) Rubisco (1GK8 and 8RUC, respectively) (15, 16). The central solvent channel of the holoenzyme is in front of the displayed structures. Only large-subunit phylogenetic residues 221, 235, 256, 258, and 265 differ between *Chlamydomonas* and spinach Rubisco in this region (14). In *Chlamydomonas*, an L290F substitution caused a decrease in Ω (17), and suppressor substitutions in the large subunit (A222T or V262L) or small subunit (N54S or A57V, colored gray in only the *Chlamydomonas* structure) returned Ω to the normal value (18–20). The small-subunit βA–βB loop contains 28 residues in *Chlamydomonas* and 22 residues in spinach Rubisco (7).

wild-type value (18, 19). Small-subunit N54S and A57V suppressor substitutions have also been recovered that improve the thermal stability and catalytic properties of the L290F enzyme (20). These residues reside in a loop between β strands A and B, the most variable structural feature among divergent Rubisco enzymes (Fig. 1) (reviewed in ref. 7). The βA–βB loops of four small subunits encircle each end of the central solvent channel that traverses the holoenzyme. When the 28-residue loop of the *Chlamydomonas* small subunit was recently replaced by the 22-residue loop of land plants (*Spinacea oleracea*) (Fig. 1) or the 10-residue loop of cyanobacteria (*Synechococcus*), no extensive structural alterations occurred in the large subunit (13). However, both foreign loops altered the catalytic properties of Rubisco, and the cyanobacterial loop caused an 11% decrease in Ω (13).

In this study, a focus has been placed on the small-subunit βA–βB loop in an attempt to identify other residues that may complement the deleterious effects of the *Chlamydomonas* C256F/K258R/I265V triple-mutant enzyme. Two additional large-subunit phylogenetic substitutions (V221C and V235I) that flank the small-subunit loop (Fig. 1) have now been found to increase the Ω value of the triple-mutant enzyme back to the wild-type value. Further addition of the small-subunit βA–βB loop of spinach produces an enzyme with catalytic properties that mimic those of the spinach enzyme. Despite being 20 Å away from the active site, the large/small-subunit interface at the βA–βB loop appears to contribute to the differences in the catalytic properties that define algal and land-plant Rubisco enzymes.

Materials and Methods

Strains and Culture Conditions. *C. reinhardtii* 2137 mt^+ is the wild-type strain (21). Photosynthesis-deficient, acetate-requiring mutants MX3312 and CAL005.01.13 were used for chloroplast and nuclear gene transformation, respectively. Mutant MX3312, which was created and provided by Dr. Genhai Zhu (Verdia, Redwood City, CA), lacks the large-subunit *rbcL* gene because of precise replacement with the bacterial *aadA* gene that confers spectinomycin resistance (22). Mutant CAL005.01.13 was kindly provided by Drs. Rachel M. Dent and Krishna K. Niyogi (University of California, Berkeley, CA). This mutant lacks the full-length sequences of the small-subunit *rbcS1* and *rbcS2* genes, which reside in a single ≈8-kbp locus (23) because of a 36-kbp deletion caused by insertional-mutagenesis transformation (24). For comparative purposes, small-subunit chimeric-mutant ABSO (13), which contains the βA–βB loop of spinach (*S. oleracea*), was also used in this study. All strains were maintained at 25°C in darkness with 10 mM acetate medium containing 1.5% Bacto-agar (Difco) (21). For biochemical analysis, cells were grown with 250–500 ml of liquid acetate medium at 25°C on a rotary shaker (220 rpm) in darkness.

Molecular and General Genetics. By using a plasmid containing the *Chlamydomonas rbcL* gene (19), directed mutagenesis was performed with synthetic oligonucleotides and a QuikChange site-directed mutagenesis kit (Stratagene) (25). To create the V235I single substitution, the sequence GTT was changed to ATT. To create the V221C/V235I double substitution, the directed-mutant V235I plasmid was used for the addition of the V221C substitution by changing the sequence GTT to TGT. Restriction digestion and ligation were used to combine the V221C/V235I double-mutant gene with the C256F/K258R/I265V triple-mutant plasmid described in ref. 14 to produce the pentamutant V221C/V235I/C256F/K258R/I265V plasmid. The C256F/K258R/I265V triple-mutant plasmid was also used for transformation of *rbcL* mutant MX3312 in this study, so that all mutant *rbcL* genes would be present in the same host strain, and all *rbcL*-mutant strains would be isogenic with the wild type (14).

Chloroplast and nuclear-gene transformation was performed by using a helium-driven biolistic device (26, 27). In all cases, photosynthesis-competent colonies were selected on minimal medium in the light (80 μmol of photons per m^2/s). Colonies were cloned out and screened by DNA purification, PCR amplification, restriction-enzyme analysis, and/or DNA sequencing (19, 27, 28). The engineered gene from each mutant strain was then PCR amplified and completely sequenced at the DNA sequencing facility of the University of Nebraska to confirm that only the expected mutations were present.

Genetic crosses were performed by established methods (20, 21). The centromere marker *pf-2* (paralyzed flagella) was included in all crosses to ensure that designated tetrads were the result of meiosis (21).

Biochemical Analysis. Dark-grown *Chlamydomonas* cells were sonicated at 0°C for 3 min in 50 mM *N*,*N*-bis(2-hydroxyethyl)glycine (bicine), pH 8.0/10 mM $NaHCO_3$/10 mM $MgCl_2$/1 mM DTT. Cell debris was removed by centrifugation at 30,000 × *g* for 15 min, and the amount of protein in the supernatant (cell extract) was quantified (29). Samples were subjected to SDS/PAGE with a 7.5–15% polyacrylamide gradient in the running gel (30, 31). Proteins were transferred from the gel to nitrocellulose, probed with rabbit anti-*Chlamydomonas* Rubisco IgG (0.5 μg/ml) (13), and detected by enhanced chemiluminescence (Amersham Pharmacia Biotech) (31).

Soluble proteins were extracted from spinach (purchased locally) by grinding 2 g of leaves in 5 ml of 50 mM bicine, pH 8.0/10 mM $NaHCO_3$/10 mM $MgCl_2$/1 mM DTT/2% (weight/vol) polyvinylpyrrolidone 40 (Sigma) for 1 min in a glass homogenizer at 0°C.

Cell/tissue debris was removed by centrifugation at 30,000 × *g* for 15 min.

Rubisco holoenzyme was purified from *Chlamydomonas* and spinach extracts by sucrose-gradient centrifugation in 50 mM bicine, pH 8.0/10 mM $NaHCO_3$/10 mM $MgCl_2$/1 mM DTT (32). RuBP carboxylase and oxygenase activities were measured by the incorporation of acid-stable ^{14}C from $NaH^{14}CO_3$ (17). The Ω of purified and activated Rubisco (20 μg/0.5-ml reaction) was determined by assaying carboxylase and oxygenase activities simultaneously with 88 μM [1-^{3}H]RuBP (15.8 Ci/mol) (1 Ci = 37 GBq) and 2 mM $NaH^{14}CO_3$ (5.0 Ci/mol) in 30-min reactions at 25°C (33, 34). [1-^{3}H]phosphoglycolate was dephosphorylated with tobacco phosphoglycolate phosphatase and separated from 3-phosphoglycerate by anion exchange with AG 1-X8 resin (Bio-Rad). [1-^{3}H]RuBP and phosphoglycolate phosphatase were synthesized/purified by standard methods (33, 35).

Rubisco thermal stability was assayed by incubating purified enzymes (5 μg) in 0.5 ml of 50 mM bicine, pH 8.0/10 mM $NaH^{14}CO_3$ (2 Ci/mol)/10 mM $MgCl_2$ at various temperatures for 10 min (36). The samples were cooled on ice for 5 min, and carboxylase activity was initiated at 25°C by adding 20 μl of 10 mM RuBP. Reactions were terminated after 1 min with 0.5 ml of 3 M formic acid in methanol.

Results

Recovery and Phenotypes of the Mutant Strains. The *Chlamydomonas* C256F/K258R/I265V triple-mutant strain was previously observed to have a reduced level of Rubisco holoenzyme, and the purified enzyme had a substantial increase in K_c and decrease in Ω (14). The substituted residues are characteristic of land-plant Rubisco enzymes, but the altered catalytic properties of the triple-mutant enzyme are not. Because the substituted large-subunit residues are at the interface with the small-subunit βA–βB loop (Fig. 1), our interest in identifying other phylogenetic residues that may improve the function of the triple-mutant enzyme has been drawn to this region. There are only two other residues close to the small-subunit βA–βB loop that differ between *Chlamydomonas* and land plants (Fig. 1) (14). Whereas the *Chlamydomonas* large subunit contains Val-221 and Val-235, >97% of land-plant large subunits contain Cys-221 and Ile-235 (14). To test the significance of these residues, single-mutant V235I, double-mutant V221C/V235I, triple-mutant C256F/K258R/I265V, and pentamutant V221C/V235I/C256F/K258R/I265V *rbcL* genes were transformed into the MX3312 *rbcL*-deletion mutant strain. In every case, photosynthesis-competent transformants were recovered at frequencies comparable to those obtained with a wild-type *rbcL* gene (19, 28). The growth phenotypes of the V235I single-mutant and V221C/V235I double-mutant strains were indistinguishable from wild type under all culture conditions. However, whereas the C256F/K258R/I265V triple mutant grew poorly on minimal medium at 35°C, the V221C/V235I/C256F/K258R/I265V pentamutant strain grew significantly better (Fig. 2), indicating that the V221C and V235I substitutions may improve the catalytic properties or amount of the triple-mutant enzyme.

To see whether the growth of the V221C/V235I/C256F/K258R/I265V pentamutant could be further improved by introducing a land-plant small-subunit βA–βB loop, the mt^+ pentamutant strain was first crossed with an mt^- strain of the CAL005.01.13 *rbcS*-deletion mutant (24). Because chloroplast genes are inherited uniparentally from the *Chlamydomonas* mt^+ parent to all progeny in a cross, and nuclear genes segregate 2:2, according to Mendel's laws, every acetate-requiring progeny clone would lack the *rbcS* genes but contain the *rbcL* V221C/V235I/C256F/K258R/I265V pentamutant gene. An acetate-requiring mt^+ progeny clone of this genotype was recovered, confirmed by DNA sequencing, and used as a host for transformation with the pABSO plasmid described in ref. 13. pABSO contains a *Chlamydomonas rbcS1* gene in which the region encoding the 28-residue βA–βB loop of the small subunit has been replaced precisely by a sequence encoding the 22-residue βA–βB loop of spinach. Photosynthesis-competent transformants were recovered (and confirmed by *rbcS* DNA sequencing), but the penta/ABSO strain displayed little or no improvement of photosynthetic growth at 35°C relative to that of the V221C/V235I/C256F/K258R/I265V pentamutant (Fig. 2).

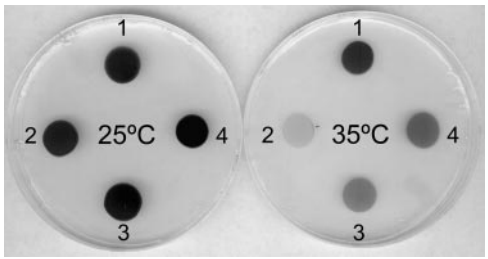

Fig. 2. Spot tests to assess the photoautotrophic growth of wild type (spot 1), large-subunit triple-mutant C256F/K258R/I265V (spot 2), large-subunit pentamutant V221C/V235I/C256F/K258R/I265V (spot 3), and penta/ABSO-mutant V221C/V235I/C256F/K258R/I265V/ABSO (spot 4), which contains the small-subunit βA–βB loop of spinach (13). Equal numbers of dark-grown cells were plated on minimal medium in the light (80 μmol of photons per m^2/sec), at either the normal growth temperature of 25°C or elevated temperature of 35°C.

Effect of Temperature on the Level of Rubisco Protein. To assess the biochemical basis for the influence of temperature on growth phenotype, extracts of cells grown at 25°C and 35°C were subjected to SDS/PAGE and Western analysis. Because unassembled small subunits are rapidly degraded (37) and large-subunit expression requires the presence of small subunits (23), the amount of subunits accurately reflects the level of holoenzyme *in vivo*. The V235I single-mutant and V221C/V235I double-mutant strains have levels of Rubisco holoenzyme equal to that of wild type (data not shown). As described in ref. 14, the C256F/K258R/I265V triple mutant has less Rubisco than wild type when grown at 25°C (Fig. 3, compare lanes 1 and 2). A further decrease in the amount of holoenzyme is observed when C256F/K258R/I265V triple-mutant cells are grown at 35°C (Fig. 3, compare lanes 2 and 7), which likely accounts for the temperature-conditional phenotype of the triple mutant (Fig. 2). The V221C/V235I/C256F/K258R/I265V penta- and penta/ABSO mutants also have less holoenzyme than wild type, but more than the triple mutant at both 25°C and 35°C (Fig. 3), which may

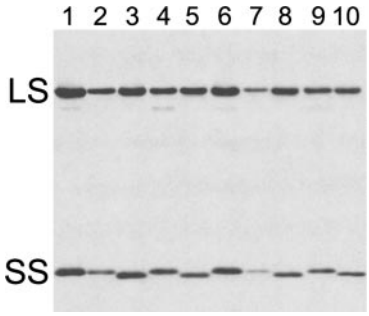

Fig. 3. Western blot analysis of total soluble proteins from wild type (lanes 1 and 6), large-subunit triple-mutant C256F/K258R/I265V (lanes 2 and 7), small-subunit chimeric-mutant ABSO (lanes 3 and 8) (13), large-subunit pentamutant V221C/V235I/C256F/K258R/I265V (lanes 4 and 9), and penta/ABSO-mutant V221C/V235I/C256F/K258R/I265V/ABSO (lanes 5 and 10). Extracts (30 μg per lane) of cells grown at either 25°C (lanes 1–4) or 35°C (lanes 6–10) in darkness were fractionated by SDS/PAGE (7.5–15%) (30, 31). The Rubisco large (LS) and small (SS) subunits were detected with anti-*Chlamydomonas* Rubisco IgG (31).

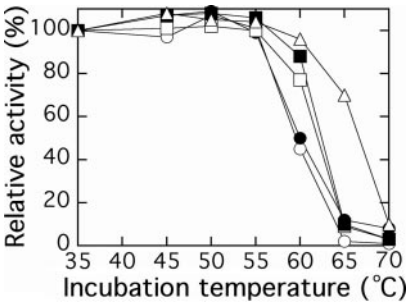

Fig. 4. Thermal inactivation of purified Rubisco from wild type (○), large-subunit triple-mutant C256F/K258R/I265V (●), small-subunit chimeric-mutant ABSO (□) (13), large-subunit pentamutant V221C/V235I/C256F/K258R/I265V (■), and penta/ABSO-mutant V221C/V235I/C256F/K258R/I265V/ABSO (△). Rubisco was incubated at each temperature for 10 min, cooled on ice, and assayed for RuBP carboxylase activity at 25°C (36). Activities were normalized to the specific activities measured after the 35°C incubation. Illustrated values did not differ by more than 10% of maximal activities in three independent experiments with separate enzyme preparations.

account for their improved photosynthetic growth relative to that of the triple mutant at 35°C (Fig. 2). Despite having only one *rbcS* gene (13, 23), the ABSO small-subunit mutant contains more Rubisco than the penta- and penta/ABSO mutants (Fig. 3). However, the penta/ABSO mutant has little or no improvement in the amount of holoenzyme relative to that of the pentamutant, indicating that the increased amount of holoenzyme in both the penta- and penta/ABSO mutants, relative to the triple mutant, arises primarily from the introduction of the V221C and V235I large-subunit substitutions.

The decrease in the amount of holoenzyme in the C256F/K258R/I265V triple mutant relative to that of wild type is not a result of protein instability in general. As shown in ref. 14, the triple-mutant and wild-type enzymes have similar thermal stability *in vitro* (Fig. 4). In contrast, the large-subunit V221C/V235I/C256F/K258R/I265V pentamutant and small-subunit chimeric-mutant ABSO enzymes have small increases in thermal stability at 60°C, and these effects appear to be additive in the penta/ABSO enzyme (Fig. 4). Whereas the wild-type enzyme is completely inactivated at 65°C, the penta/ABSO enzyme retains ≈70% of its initial activity (Fig. 4). Despite these increases in holoenzyme thermal stability *in vitro*, the penta and penta/ABSO mutant strains have decreased levels of Rubisco protein *in vivo* (Fig. 3). Thus, decreases in the amount of holoenzyme in the triple-, penta-, and penta/ABSO mutant strains may arise from either a defect in the assembly of the holoenzyme or from the introduction of a stable structural alteration that makes Rubisco more prone to proteolysis *in vivo*. These defects are only partially alleviated by the addition of the V221C and V235I large-subunit substitutions to the C256F/K258R/I265V triple-mutant enzyme (Fig. 3). Nonetheless, the stability of the holoenzymes *in vitro* (Fig. 4) allows sufficient quantities of the mutant enzymes to be purified for further biochemical analysis.

Catalytic Properties of the Mutant Enzymes. Except for a small decrease in K_o of the V221C/V235I double-mutant enzyme, the kinetic constants of the V235I and V221C/V235I enzymes are not significantly different from those of the wild-type enzyme (Table 1). However, when the V221C and V235I substitutions are added to the C256F/K258R/I265V triple-mutant enzyme, which was previously shown to have a decreased Ω value (14), the value of Ω is increased by 13% in the V221C/V235I/C256F/K258R/I265V pentamutant enzyme back to the wild-type value (Table 2). Because the V_c and K_c values of the triple- and pentamutant enzymes are the same, the increase in Ω likely results from an increase in K_o (Table 2). When the ABSO chimeric small subunit is added to the pentamutant large subunit to form the penta/ABSO enzyme, Ω is increased to a value 12% greater than that of the wild-type enzyme (Table 2). The increase in Ω relative to that of the pentamutant enzyme results from a decrease in K_c, and increase in V_c/K_c, despite a 70% decrease in K_o (Table 2). Relative to the wild-type enzyme, the penta/ABSO enzyme is characterized by a 45% decrease in V_c, a 26% decrease in K_c, and a 43% decrease in V_o, with little or no difference in K_o. The resulting kinetic properties are quite similar to those of land-plant Rubisco enzymes (9).

Table 1. Kinetic properties of Rubisco purified from *Chlamydomonas* wild type and large-subunit single (V235I) and double (V221C/V235I) mutants

Enzyme	Ω V_cK_o/V_oK_c	V_c μmol/h/mg	K_c μM CO_2	K_o μM O_2
Wild type	63 ± 2	111 ± 6	35 ± 2	501 ± 18
V235I	58 ± 3	122 ± 4	46 ± 11	469 ± 26
V221C/V235I	62 ± 3	127 ± 34	32 ± 3	371 ± 13

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

A direct comparison between penta/ABSO and land-plant Rubisco was made by performing another, independent series of kinetic measurements relative to the spinach enzyme (Table 3). The Ω value of the penta/ABSO enzyme was found to be 17% greater than that of the wild-type *Chlamydomonas* enzyme but 14% lower than that of the spinach enzyme. However, no measurable differences can be concluded to occur between the K_c, K_o, V_c, and V_o kinetic constants of the penta/ABSO and spinach Rubisco enzymes.

Discussion

By changing five large-subunit residues to those characteristic of land plants (V221C/V235I/C256F/K258R/I265V) (14) and introducing the shorter small-subunit βA–βB loop of spinach (Fig. 1) (13), an engineered *Chlamydomonas* Rubisco enzyme has been recovered that mimics the catalytic properties of land-plant Rubisco (spinach) (Table 3). With a 12–17% increase in Ω, a 45–53% decrease in V_c, and a 26–30% decrease in K_c, the engineered penta/ABSO enzyme is much different from wild-type *Chlamydomonas* Rubisco but quite similar to spinach Rubisco (Tables 2 and 3). Although the Ω value of the penta/ABSO enzyme is still lower than that of the spinach enzyme by 14%, only minor differences in the other kinetic constants (too small to be accurately measured) are responsible for this difference (Table 3). Thus, differences at the interface between large and small subunits at the entrance to the central solvent channel of the holoenzyme contribute significantly to the differences in catalytic properties between the algal and land-plant enzymes.

A higher Ω value is much the same as a lower compensation point (4). The penta/ABSO enzyme is less inhibited by O_2 and would continue to fix carbon at CO_2 concentrations too low to support net CO_2 fixation by the wild-type enzyme. However, despite an increase in Ω that arises from decreases in K_c and V_o (Tables 2 and 3), the penta/ABSO enzyme is not a "better" enzyme in *Chlamydomonas*. *Chlamydomonas*, like many photosynthetic microorganisms (38, 39), contains a mechanism that concentrates CO_2 at the site of Rubisco. Thus, the decrease in K_c may not be beneficial, and the lower V_c of penta/ABSO Rubisco would result in a decrease in net CO_2 fixation at physiological concentrations of CO_2 and O_2 (4, 6). Nonetheless, because Ω is defined by the difference between the free energies of activation for carboxylation and oxygenation at the rate-determining partial reactions (5), an engineered increase in Ω may be of significance for future genetic-engineering strategies aimed at improving the enzyme. In previous studies of directed-mutant cyanobacterial and dimeric prokaryotic Rubisco, substantial increases in Ω were also observed when substitutions were made

Table 2. Kinetic properties of Rubisco purified from *Chlamydomonas* wild type, large-subunit triple-mutant C256F/K258R/I265V, large-subunit pentamutant V221C/V235I/C256F/K258R/I265V, small-subunit chimeric-mutant ABSO, and penta/ABSO-mutant V221C/V235I/C256F/K258R/I265V/ABSO

Enzyme	Ω* V_cK_o/V_oK_c	V_c* μmol/h/mg	k_{cat} carboxylation* per sec	K_c* μM CO_2	K_o* μM O_2	V_o† μmol/h/mg	k_{cat} oxygenation† per sec	V_c/K_c†	V_o/K_o†	K_o/K_c†	V_c/V_o†
Wild type	60 ± 1	103 ± 6	2.32 ± 0.14	39 ± 2	478 ± 20	21	0.47	2.6	0.04	12	4.9
Triple	54 ± 2	46 ± 14	1.04 ± 0.32	84 ± 19	999 ± 359	10	0.23	0.5	0.01	12	4.5
Penta	61 ± 2	46 ± 6	1.04 ± 0.14	84 ± 6	1,400 ± 172	13	0.29	0.5	0.01	17	3.7
ABSO	61 ± 1	53 ± 9	1.19 ± 0.20	27 ± 4	424 ± 108	14	0.32	2.0	0.03	16	3.9
Penta/ABSO	67 ± 1	57 ± 4	1.28 ± 0.09	29 ± 6	422 ± 53	12	0.27	2.0	0.03	15	4.6

*Values are the means (±SD) ($n - 1$) of three separate enzyme preparations.
†Calculated values.

at active-site residues 340 and 379 (40, 41). However, in those cases, the increased Ω values were still lower than that of wild-type *Chlamydomonas* Rubisco.

The catalytic properties of the penta/ABSO enzyme are more like those of spinach Rubisco than *Chlamydomonas* Rubisco (Table 3), but the enzyme has an Ω value lower than that of spinach Rubisco and an associated structural alteration that causes a decrease in the amount of holoenzyme *in vivo* (Fig. 3). The decreased amount of Rubisco may not be surprising if one considers the difficulties encountered (42–44) or anticipated (45) in the interspecific expression of Rubisco enzymes or subunits. Because *Chlamydomonas* Rubisco is engineered to be more like land-plant Rubisco, structural determinants required for protein–protein interactions during assembly, targeting, or regulation in the algal-cell environment may be lost, leading to premature degradation. However, with regard to the enzyme *in vitro*, where holoenzyme stability does not appear to be an issue (Fig. 4), it would seem that additional substitutions may be required to increase the Ω of the penta/ABSO enzyme to the value of the spinach enzyme (Table 3). On the other hand, because the effects of the single-mutant substitutions on catalysis are not additive (Tables 1–3) (14), it is difficult to tell whether different combinations of fewer phylogenetic substitutions, with or without the land plant βA–βB loop, may better mimic the catalytic properties of land-plant enzymes. To completely assess the effects of all combinations of five large-subunit substitutions (V221C, V235I, C256F, K258R, and I265V) and the small-subunit βA–βB loop would require the creation and analysis of 2^6 enzymes (one of which would be wild type). Only 13 of these 64 possible enzymes have been investigated in this and previous studies (14).

An examination of the existing x-ray crystal structures may provide some clues as to which of the substituted residues in the penta/ABSO enzyme might have the greatest influence on Rubisco structure. When the structures of *Chlamydomonas* and spinach Rubisco are compared (15, 16), no substantial conformational differences are observed in the large-subunit residues surrounding residues 221, 235, and 265. However, whereas Arg-258 forms an ionic bond with Glu-259 from a neighboring large subunit in spinach Rubisco, Lys-258 and Glu-259 in the *Chlamydomonas* enzyme are displaced by residues of the larger small-subunit βA–βB loop (Ser-62 and Val-63) and interact through only a water-mediated hydrogen bond (Fig. 5*A*). In the crystal structure of the *Chlamydomonas* chimeric-mutant ABSO enzyme (13), which contains the shorter spinach βA–βB loop in place of the *Chlamydomonas* loop, the side chains of Lys-258 and Glu-259 have greater conformational flexibility and appear to move closer together (Fig. 5*B*). Thus, it is likely that Arg-258 and Glu-259 in the penta/ABSO enzyme also form an ionic bond between neighboring large subunits.

Large-subunit residue 256 also interacts with residue 258 and residues in the small-subunit βA–βB loop. In spinach Rubisco, the aromatic ring of Phe-256 is in contact with small-subunit His-56, which is replaced by Ala-57 in *Chlamydomonas* (Fig. 5*C*). The side chain of Phe-256 is also close to Glu-259 in a neighboring large subunit and may influence its conformation (Fig. 5*C*). The methyl-Cys-256 residue of *Chlamydomonas* is in van der Waals contact with small-subunit Val-63, which is absent from the shorter βA–βB loop of spinach Rubisco (Fig. 5*C*). In the ABSO enzyme, methyl-Cys-256 can no longer interact with the small-subunit βA–βB loop (Fig. 5*D*). Thus, the introduction of a C256F substitution in the *Chlamydomonas* enzyme would likely present a steric clash with small-subunit Val-63, but this could be compensated for by the introduction of the smaller spinach βA–βB loop.

In spinach Rubisco, the Cδ atom of Ile-235 is in van der Waals contact with the side chains of small-subunit Val-51 and Tyr-62, and one of the Cγ atoms is in van der Waals contact with the Tyr-62 side-chain oxygen (Fig. 5*E*). In the *Chlamydomonas* enzyme, Val-235 is also in contact with the homologous Tyr-68 oxygen. However, *Chlamydomonas* Val-235 is too far from the homologous small-subunit Val-52 to form a van der Waals contact, and the Val-52 side chain adopts a different conformation from that of Val-51 in spinach (Fig. 5*E*). In the ABSO enzyme, both of the Cγ atoms of Val-51 move into van der Waals contact with one of the side-chain atoms of large-subunit Val-235 (Fig. 5*F*). Although the conformation of the Val-51 side chain in ABSO is much like that of spinach Rubisco, small differences arise in contact distances between

Table 3. Kinetic properties of Rubisco purified from *Chlamydomonas* (wild type), spinach, and the *Chlamydomonas* penta/ABSO-mutant V221C/V235I/C256F/K258R/I265V/ABSO

Enzyme	Ω* V_cK_o/V_oK_c	V_c* μmol/h/mg	k_{cat} carboxylation* per sec	K_c* μM CO_2	K_o* μM O_2	V_o† μmol/h/mg	k_{cat} oxygenation† per sec	V_c/K_c†	V_o/K_o†	K_o/K_c†	V_c/V_o†
Chlamydomonas	60 ± 2	129 ± 7	2.91 ± 0.16	33 ± 1	422 ± 41	27	0.61	3.9	0.07	13	4.7
Spinach	81 ± 3	63 ± 6	1.42 ± 0.14	21 ± 1	496 ± 40	18	0.41	3.0	0.04	24	3.4
Penta/ABSO	70 ± 2	60 ± 9	1.35 ± 0.20	23 ± 1	449 ± 110	17	0.38	2.6	0.04	20	3.4

*Values are the means (±SD) ($n - 1$) of three separate enzyme preparations.
†Calculated values.

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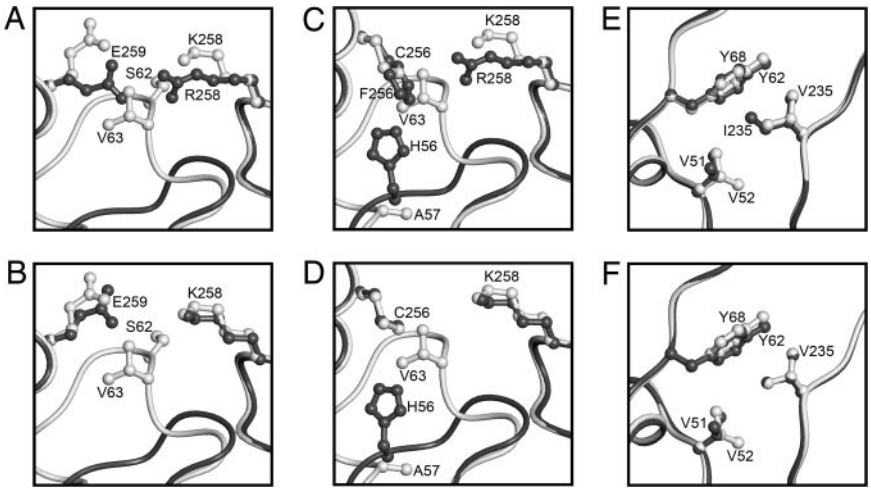

Fig. 5. Interactions of large-subunit phylogenetic residues 258 (*A* and *B*), 256 (*C* and *D*), and 235 (*E* and *F*) in the x-ray crystal structures of Rubisco from *Chlamydomonas* (1GK8) (13), spinach (8RUC) (10), and *Chlamydomonas* chimeric-mutant ABSO (1UZD), which contains the small-subunit βA–βB loop of spinach (13). The *Chlamydomonas* structure (in white) is aligned with the structures (in dark gray) of spinach (*A*, *C*, and *E*) or ABSO (*B*, *D*, and *F*). The central solvent channel of the holoenzyme is behind all of the displayed structures.

Val-51 and neighboring large and small residues. These perturbations may be complemented by the V235I substitution.

Only small differences in structure (Fig. 5) may arise from the phylogenetic differences at residues 221, 235, 256, 258, and 265, but the relationships between these residues and the small-subunit βA–βB loop are likely to be responsible for significant alterations in the kinetic properties of Rubisco (Tables 2 and 3) (13, 14). However, none of the single- or double-mutant phylogenetic substitutions substantially alters Rubisco catalysis (Table 1) (14). It is only when larger groups of the substitutions are formed that dramatic effects on catalysis are observed (Tables 2 and 3) (14). It thus remains a challenge to determine how these substitutions 20 Å from the active site can influence catalysis (12, 13). Because the substituted residues flank the central solvent channel of the holoenzyme (7), and conformational changes in the holoenzyme appear to be a required part of the catalytic mechanism (reviewed in refs. 1 and 2), the substituted residues in the penta/ABSO enzyme may also influence catalysis by altering structural dynamics (12). Nonetheless, the small phylogenetic differences in residues surrounding the small-subunit βA–βB loop can contribute to the differences in the catalytic properties of algal and land-plant enzymes. This interface between large and small subunits should be a prime target for the application of methods aimed at improving Rubisco.

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