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 CO₂ 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 CO₂/O₂ 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 CO₂/O₂ 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 CO₂/O₂ 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-plantRubisco enzymes.

Conflict of interest statement: No conflicts declared.

Abbreviations: Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; bicine, N,N-bis(2-hydroxyethyl)glycine; RuBP, ribulose 1,5-bisphosphate; II, CO₂/O₂ specificity factor.

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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.

![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 V262I) 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).](https://www.pnas.org/content/108/52/20862)

**Materials and Methods**

**Strains and Culture Conditions.** *C. reinhardtii* 2137 mt was 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*) (20). 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 m²/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 NaHCO₃/10 mM MgCl₂/1 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 NaHCO₃/10 mM MgCl₂/1 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 NaHCO₃/10 mM MgCl₂/1 mM DTT (32). RuBP carboxylase and oxygenase activities were measured by the incorporation of acid-stable ¹⁴C from NaH¹⁴CO₃ (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 [¹³⁵]RuBP (15.8 Ci/mol) (1 Ci = 37 GBq) and 2 mM NaH¹⁴CO₃ (5.0 Ci/mol) in 30-min reactions at 25°C (33, 34).

Rubisco thermal stability was assayed by incubating purified enzymes (5 μg) in 0.5 ml of 50 mM bicine, pH 8.0/10 mM NaH¹⁴CO₃ [2 Ci/mol]/10 mM MgCl₂ 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 Ω, 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 B loop, the small-subunit Bα–β loop differs between *Chlamydomonas* and land plants (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, V221C, double-mutant V221C/K258R/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 strain could be further improved by introducing a land-plant small-subunit Bα–β loop, the mt+ pentamutant strain was 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 rbcS* _I* _gene in which the region encoding the 28-residue Bα–β loop of the small subunit has been replaced precisely by a sequence encoding the 22-residue Bα–β loop of spinach. Photosynthesis-competent transformants were recovered (and confirmed by _rbcS_ DNA sequencing), but the mt+/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).

**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 analyses. Because unassembled small subunits are rapidly degraded (37) and large-subunit expression requires the presence of small subunits (23), the amount of subunits accurately reflect 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 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 account for the reduced ability of the hexamutant strain to grow at 35°C (Fig. 1).

**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 Bα–β loop of spinach (13). Equal numbers of dark-grown cells were plated on minimal medium in the light (80 μmol of photons per m²/sec), at either the normal growth temperature of 25°C or elevated temperature of 35°C.

**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 ABISO (□) (13), large-subunit pentamutant V221C/V235I (△), and penta/ABISO-mutant V221C/V235I/C256F/K258R/I265V (⟨⟩). 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 ABISO small-subunit mutant contains more Rubisco than the penta- and penta/ABISO mutants (Fig. 3). However, the penta/ABISO 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/ABISO 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 ABISO enzymes have small increases in thermal stability at 60°C, and these effects appear to be additive in the penta/ABISO enzyme (Fig. 4). Whereas the wild-type enzyme is completely inactivated at 65°C, the penta/ABISO enzyme retains ≥70% of its initial activity (Fig. 4). Despite these increases in holoenzyme thermal stability in vitro, the penta and penta/ABISO 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/ABISO 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 $\Omega$ value (14), the value of $\Omega$ is increased by 13% in the V221C/V235I/C256F/K258R/I265V pentamutant enzyme back to the wild-type value (Table 2). Because the $V_o$ and $K_o$ values of the triple- and pentamutant enzymes are the same, the increase in $\Omega$ likely results from an increase in $K_o$ (Table 2). When the ABISO chimeric small subunit is added to the pentamutant large subunit to form the penta/ABISO enzyme, $\Omega$ is increased to a value 12% greater than that of the wild-type enzyme (Table 2). The increase in $\Omega$ relative to that of the pentamutant enzyme results from a decrease in $K_o$, and increase in $V_o/K_o$, despite a 70% decrease in $K_o$ (Table 2). Relative to the wild-type enzyme, the penta/ABISO enzyme is characterized by a 45% decrease in $V_o$, a 26% decrease in $K_o$ 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).

A direct comparison between penta/ABISO and land-plant Rubisco was made by performing another, independent series of kinetic measurements relative to the spinach enzyme (Table 3). The $\Omega$ value of the penta/ABISO 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 $K_o$, $V_o$ and $V_o$ kinetic constants of the penta/ABISO 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 $\Omega$, a 45–53% decrease in $V_o$, and a 26–30% decrease in $K_o$, the engineered penta/ABISO enzyme is much different from wild-type Chlamydomonas Rubisco but quite similar to spinach Rubisco (Tables 2 and 3). Although the $\Omega$ value of the penta/ABISO 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 $\Omega$ value is much the same as a lower compensation point (4). The penta/ABISO 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 $\Omega$ that arises from decreases in $K_o$ and $V_o$ (Tables 2 and 3), the penta/ABISO 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 $K_o$ may not be beneficial, and the lower $V_o$ of penta/ABISO Rubisco would result in a decrease in net CO$_2$ fixation at physiological concentrations of CO$_2$ and O$_2$ (4, 6). Nonetheless, because $\Omega$ 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 $\Omega$ 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 $\Omega$ were also observed when substitutions were made.

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

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$\Omega\ V_o/K_o\ V_o\ K_o\ \mu$M CO$_2$</th>
<th>$K_o\ \mu$M O$_2$</th>
</tr>
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<tbody>
<tr>
<td>Wild type</td>
<td>63 ± 2</td>
<td>111 ± 6</td>
</tr>
<tr>
<td>V235I</td>
<td>58 ± 3</td>
<td>122 ± 4</td>
</tr>
<tr>
<td>V221C/V235I</td>
<td>62 ± 3</td>
<td>127 ± 39</td>
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</table>

The values are the means (±SD) of three separate enzyme preparations.
whether different combinations of fewer phylogenetic substitutions, hand, because the effects of the single-mutant substitutions on enzyme to the value of the spinach enzyme (Table 3). On the other
substitutions may be required to increase the interactions during assembly, targeting, or regulation in the algal-cell
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
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 \( \Omega \) of the penta/ABSO enzyme to the value of the spinach enzyme (Table 3). On the other
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 \( \beta A-\beta B \) 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 \( \beta A-\beta B \) loop would require the creation and analysis of 25 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 \( \beta A-\beta B \) loop (Ser-62 and Val-63) and interact through only a water-mediated hydrogen bond (Fig. 5d). In the crystal structure of the *Chlamydomonas* chimeric-mutant ABSO enzyme (13), which contains the shorter spinach \( \beta A-\beta B \) 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. 5b). 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 258 also interacts with residue 258 and residues in the small-subunit \( \beta A-\beta B \) 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. 5c). The side chain of Phe-256 is also close to Glu-259 in a neighboring large subunit and may influence its conformation (Fig. 5c). The methyl-Cys-256 residue of *Chlamydomonas* is in van der Waals contact with small-subunit Val-63, which is absent from the shorter \( \beta A-\beta B \) loop of spinach Rubisco (Fig. 5c). In the ABSO enzyme, methyl-Cys-256 can no longer interact with the small-subunit \( \beta A-\beta B \) loop (Fig. 5d). 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 \( \beta A-\beta B \) loop.

In spinach Rubisco, the C6 atom of Ile-255 is in van der Waals contact with the side chains of small-subunit Val-51 and Tyr-62, and one of the C\( \gamma \) atoms is in van der Waals contact with the Tyr-62 side-chain oxygen (Fig. 5e). In the *Chlamydomonas* enzyme, Val-255 is also in contact with the homologous Tyr-68 oxygen. However, *Chlamydomonas* Val-255 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. 5e). In the ABSO enzyme, both of the C\( \gamma \) atoms of Val-51 move into van der Waals contact with one of the side-chain atoms of large-subunit Val-235 (Fig. 5f). 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 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

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<tr>
<th>Enzyme</th>
<th>( \Omega^* )</th>
<th>( V_{\text{cat}}^* )</th>
<th>( K_{\text{cat}}^* )</th>
<th>( K_{o^*} )</th>
<th>( k_{\text{cat}}^* )</th>
<th>( k_{\text{cat}}^o )</th>
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<td>Wild type</td>
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<td>2.32 ± 0.14</td>
<td>39 ± 2</td>
<td>478 ± 20</td>
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<tr>
<td>Triple</td>
<td>54 ± 2</td>
<td>46 ± 14</td>
<td>1.04 ± 0.32</td>
<td>84 ± 19</td>
<td>999 ± 359</td>
<td>10</td>
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<tr>
<td>Penta</td>
<td>61 ± 2</td>
<td>46 ± 0</td>
<td>1.04 ± 0.14</td>
<td>84 ± 6</td>
<td>1,400 ± 172</td>
<td>13</td>
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<tr>
<td>ABSO</td>
<td>61 ± 1</td>
<td>53 ± 9</td>
<td>1.19 ± 0.20</td>
<td>27 ± 4</td>
<td>424 ± 108</td>
<td>14</td>
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<tr>
<td>Penta/ABSO</td>
<td>67 ± 1</td>
<td>57 ± 4</td>
<td>1.28 ± 0.09</td>
<td>29 ± 6</td>
<td>422 ± 53</td>
<td>12</td>
</tr>
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*Values are the means (±SD) (n – 1) of three separate enzyme preparations.

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

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<thead>
<tr>
<th>Enzyme</th>
<th>( \Omega^* )</th>
<th>( V_{\text{cat}}^* )</th>
<th>( k_{\text{cat}}^* )</th>
<th>( K_{o^*} )</th>
<th>( k_{\text{cat}}^o )</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Chlamydomonas</em></td>
<td>60 ± 2</td>
<td>129 ± 7</td>
<td>2.91 ± 0.16</td>
<td>33 ± 1</td>
<td>422 ± 41</td>
</tr>
<tr>
<td>Spinach</td>
<td>81 ± 3</td>
<td>63 ± 6</td>
<td>1.42 ± 0.14</td>
<td>21 ± 1</td>
<td>496 ± 40</td>
</tr>
<tr>
<td>Penta/ABSO</td>
<td>70 ± 2</td>
<td>60 ± 9</td>
<td>1.35 ± 0.20</td>
<td>23 ± 1</td>
<td>449 ± 110</td>
</tr>
</tbody>
</table>

*Values are the means (±SD) (n – 1) of three separate enzyme preparations.

*Calculated values.
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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