Phylogenetic Engineering of the Ribulose-1,5-bisphosphate Carboxylase/Oxygenase Large Subunit in *Chlamydomonas Reinhardtii*

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PHYLOGENETIC ENGINEERING OF THE RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE/OXYGENASE LARGE SUBUNIT IN CHLAMYDOMONAS REINHARDTII

by

Boon Hoe Lim

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PHYLOGENETIC ENGINEERING OF THE RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE/OXYGENASE LARGE SUBUNIT IN CHLAMYDOMONAS REINHARDTII

Boon Hoe Lim, Ph. D.
University of Nebraska, 2012

Advisor: Robert J. Spreitzer

Thirty-four residues in the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) may account for the kinetic differences between Rubisco enzyme from green algae and land plants. By substituting these "phylogenetic residues" as groups and combinations of groups in the large subunit of the green alga Chlamydomonas reinhardtii with those of land-plant Rubisco, the functions and relationships of these "phylogenetic groups" were determined.

A phylogenetic-group substitution at the base of catalytic loop 6 of the large subunit decreases the CO₂/O₂ specificity of the enzyme, but function is restored by a further phylogenetic-group substitution at the carboxy-terminal tail. Therefore, these two regions of the large subunit, which sandwich loop 6, are complementary. In addition, combining substitutions at the base of loop 6 and the large/small-subunit interface region produces a mutant enzyme that has to be complemented by the land-plant small subunit for function in Chlamydomonas. On the other hand, substitutions in α-helix G of the large subunit reduce the holoenzyme level, and, because Chlamydomonas mutants with additional substitutions in α-helices 7 and 8 cannot be recovered as photosynthetic-transformants, the three α helices appear to influence holoenzyme assembly.

A previous study showed that substituting five large-subunit residues and a small-
subunit loop with land-plant identities produced an enzyme (termed "penta/ABSO") with land-plant catalytic properties. In the present study, through structural dissection, it is concluded that all the residues substituted in penta/ABSO are required for the shift towards land-plant catalysis. Among the residues substituted in penta/ABSO is methyl-Cys-256, which indicates that posttranslational modifications of the large subunit may also play a role in catalysis. Further study of cysteine methylation and proline hydroxylation showed that mutations of methyl-Cys-256 and hydroxy-Pro-104 influence catalysis.

The current study complements previous knowledge about Rubisco, and provides further structural targets for the beneficial engineering of Rubisco.
ACKNOWLEDGEMENTS

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ABBREVIATIONS USED

Bicine     N,N-bis(2-hydroxyethyl)glycine
CABP       2-carboxyarabinitol 1,5-bisphosphate
DPM        Disintegrations per minute
DTT         dithiothreitol
EDTA       ethylenediaminetetraacetate
$K_c$      Michaelis constant ($K_m$) for substrate $CO_2$
$K_o$      Michaelis constant ($K_m$) for substrate $O_2$
kDa        kilodalton
2-PG        2-phosphoglycolate
3-PGA       3-phosphoglycerate
Rubisco    ribulose-1,5-bisphosphate carboxylase/oxygenase
RUBP       ribulose 1,5-bisphosphate
SD          standard deviation
SDS-PAGE    sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Tris        tris[hydroxymethyl]amino-methane
$v_c$      reaction velocity for Rubisco carboxylation
$v_o$      reaction velocity for Rubisco oxygenation
$V_c$      $V_{max}$ for Rubisco carboxylation
$V_o$      $V_{max}$ for Rubisco oxygenation
$\Omega$    $CO_2/O_2$ specificity factor, $V_cK_o/V_oK_c$
INTRODUCTION

RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE/OXYGENASE

Biological importance and function

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco; EC 4.1.1.39) is the most abundant protein on earth (Ellis, 1979) and is found in all photosynthetic organisms within all three domains of life (Eukarya, Bacteria, Archaea) (Tabita, 1999) from unicellular photosynthetic bacteria and algae to multicellular C₃ and C₄ plants (Andersson and Backlund, 2008). In earlier experiments, Rubisco was identified as “Fraction 1 protein” because it was the first and only protein to precipitate out of leaf extracts at 35% ammonium sulfate saturation (Wildman and Bonner, 1947; Wildman, 2002). This is not surprising considering that as much as 50% of leaf nitrogen is used by plants to synthesize Rubisco (Ellis, 1979; Spreitzer and Salvucci, 2002). For autotrophic prokaryotes, as much as 40% of total soluble protein can be Rubisco (Ellis, 1979; Tabita et al., 2007). Overall, it is estimated that Rubisco makes up 0.2% of the total protein in our planet’s biomass (Ellis, 1979).

The primary function of Rubisco is the reduction of CO₂ via the Calvin-Benson-Bassham reductive pentose phosphate pathway (Tabita et al., 2008a). Rubisco captures CO₂ by fixing the gaseous molecule onto ribulose 1,5-bisphosphate (RuBP), which is further metabolized into other organic compounds. Although various organisms can reduce CO₂ via three other pathways, which are the reductive tricarboxylic acid cycle, the Wood-Ljungdahl acetyl coenzyme A pathway, and the hydroxypropionate pathway, most carbon on earth is fixed through the Calvin cycle (Tabita et al., 2007). Therefore,
Rubisco is also obviously the most important enzyme for all life on Earth (Ellis, 1979). However, the catalytic rate of Rubisco has been unfavorably described as sluggish (Ellis, 1979) because while most enzymes have catalytic rates, $k_{cat}$, of over thousands per second (Wolfenden and Snider, 2001), Rubisco has a carboxylation $k_{cat}$ of only several per second, which is at least two orders of magnitude lower (Tabita et al., 2007). Moreover, the enzyme sometimes fixes $O_2$ instead of $CO_2$, leading to the nonessential, energy-expending and $CO_2$-losing photorespiratory pathway (Bowes et al., 1971). Therefore, there is much interest in understanding and improving the enzyme by overcoming its unusual limitations, especially because the potential benefits of such endeavors include greatly increasing crop and renewable-energy production, and decelerating the rapid rise of atmospheric $CO_2$ levels (reviewed by Spreitzer and Salvucci, 2002).

**CO$_2$/O$_2$ specificity factor and diversity of kinetic properties**

The $CO_2/O_2$ specificity factor of Rubisco is the ratio of the rate constants for carboxylation ($k_c$) and oxygenation ($k_o$), and is represented by the symbol $\Omega$ (Chen and Spreitzer, 1991; Spreitzer, 1993):

$$\Omega = \frac{k_c}{k_o}$$

$\Omega$ is also defined by the $V_{max}$ for carboxylation ($V_c$), $V_{max}$ for oxygenation ($V_o$), $K_m CO_2$ ($K_c$) and $K_m O_2$ ($K_o$) (Laing et al., 1974):

$$\Omega = \frac{V_c K_o}{V_o K_c}$$

In addition, $\Omega$ is related to the difference in the free energy of activation for oxygenation ($\Delta G_o^{\ddagger}$) and the free energy of activation for carboxylation ($\Delta G_c^{\ddagger}$) (Chen and Spreitzer, 1991):
\[ \Omega = e^{(\Delta G_o^\ddag - \Delta G_c^\ddag)/RT} \]

where \( R \) is the gas constant and \( T \) is the absolute temperature. The \( \Delta G_o^\ddag - \Delta G_c^\ddag \) term is directly related to the difference in the free energy of the transition states of the oxygenation and carboxylation reactions of Rubisco (Chen and Spreitzer, 1991, 1992). Consequently, altering the relative stabilities of the Rubisco transition-state complex of the two reactions through amino-acid modifications or substitutions, environmental pH changes, or metal-cofactor replacements are potential means of altering \( \Omega \) (Chen and Spreitzer, 1992). Specifically, \( \Omega \) could be increased by increasing the stability of the Rubisco transition-state complex for carboxylation or decreasing the stability of the complex for oxygenation (Chen and Spreitzer, 1991). Because the highest-resolution x-ray crystal structures of Rubisco from various species and Rubisco mutants can be obtained only when the holoenzyme is in complex with 2-carboxyarabinitol 1,5-bisphosphate (CABP), which is a carboxylation transition-state analog, the structure-function relationships of the holoenzyme are mapped by relating differences in measured \( \Omega \) values to subtle structural changes that alter the holoenzyme-CABP interactions in these crystal structures (Andersson and Backlund, 2008). Unsurprisingly, there is also a direct relation between \( \Omega \) and tightness of CABP binding by Rubisco (Satagopan and Spreitzer, 2004). Also, from the \( \Omega \)-and-free-energy equation, \( \Omega \) decreases with increased temperature (\( T \)) (Chen and Spreitzer, 1992), so increased global temperature would reduce the \( \Omega \) value of Rubisco in plants.

Even though \( \Omega \) is a useful measurement in studying the structure-function relationships in Rubisco, the only measurement to model plant growth based on the
kinetic properties of Rubisco is provided by net CO₂ fixation, denoted \( P_n \) (Laing et al., 1974; Spreitzer, 1993):

\[
P_n = V_c K_o ([CO_2]-t/\Omega [O_2]) / (K_c K_o + K_c [O_2] + K_o [CO_2])
\]

where \( t \) is the fraction of CO₂ released in photorespiration. From the \( P_n \) equation, net CO₂ fixation is not dependent on only \( \Omega \), but also on \( V_c \), \( K_c \), and \( K_o \) (Spreitzer, 1993). More importantly, varying the kinetic properties of Rubisco optimizes net CO₂ fixation in different photoautotrophic organisms because of the differences in intracellular gaseous CO₂ and O₂ concentrations around Rubisco (Spreitzer, 1999). Some plants, such as C₄ and Crassulacean acid metabolism (CAM) plants, and some photoautotrophic microbes, such as microalgae and cyanobacteria, have biochemical and physiological adaptations that increase the CO₂ concentration around Rubisco.

There is an observed trade-off between \( V_c \) and \( \Omega \) in Rubisco from different species, which is like the inverse relationship between activity and specificity that is common among other enzymes (Table 1). Generally, \( \Omega \) is higher but \( V_c \) is lower among eukaryotic Rubiscos compared to the prokaryotic enzymes (Table 1) (Jordan and Ogren, 1981b; Spreitzer and Salvucci, 2002). Therefore, even if a Rubisco with increased \( \Omega \) was engineered, the resulting decreased \( V_c \) might be less than optimal for photosynthetic growth (Spreitzer and Salvucci, 2002). A better understanding of the structure-function relationships of Rubisco is required before attempting to improve the enzyme, bearing in mind that the ultimate measurement for any beneficial improvement is \( P_n \) (Spreitzer, 1993).
Table 1: Diversity of Rubisco kinetic properties. Values are from Jordan and Ogren (1981b), Andrews and Lorimer (1985), Read and Tabita (1994), Whitney et al. (2001), Kubien et al. (2008), Genkov et al. (2010), and Savir et al. (2010).

<table>
<thead>
<tr>
<th>Species</th>
<th>Ω</th>
<th>( V_c )</th>
<th>( K_c )</th>
<th>( K_o )</th>
<th>( V_c K_o / V_o K_c )</th>
<th>( \mu \text{mol/hr/mg} )</th>
<th>( \mu \text{M CO}_2 )</th>
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Holoenzyme structure

There are three forms of Rubisco, which are usually found in different species (Fig. 1) (Tabita, 1999; Tabita et al., 2007, 2008a). Form-I Rubisco, which is the focus of this research, has the highest $\Omega$ value, and is the common type found in plants, algae, and most autotrophic prokaryotes (Tabita, 1999; Tabita et al., 2007, 2008a; Andersson, 2008, Andersson and Backlund, 2008). The presence of small subunits, and the heterohexadecameric holoenzyme structure composed of eight large subunits and eight small subunits, distinguish form-I Rubisco from the other forms (Fig. 1) (Knight et al., 1990; Schneider et al., 1990; Taylor et al., 2001). The form-I holoenzyme is a ring of four large-subunit dimers, with each open end of the ring capped by four small subunits. A solvent channel runs through the fourfold axis in the middle of the ring (Fig. 1) (Knight et al., 1990).

Unlike form-I Rubisco, forms-II and III Rubisco enzymes lack small subunits. Form II Rubisco, which is found in some photosynthetic prokaryotes and dinoflagellates, is composed of large-subunit dimers (Fig. 1) (Tabita et al., 2008a). The first Rubisco crystal structure solved was for the form-II Rubisco from *Rhodospirillum rubrum* (Schneider et al., 1986). On the other hand, form-III Rubisco, which is found only in archaea, is composed of large-subunit octamers (four dimers in a ring) or decamers (five dimers in a ring), depending on species (Fig. 1) (Andersson, 2008). The unique multidimeric structure of form-III Rubisco contributes to the thermostability of the holoenzyme, especially important for the extreme environments where thermophilic archaea would thrive (Maeda et al., 2002). The carboxylase activity of the form-III Rubisco is highly susceptible to $O_2$ inhibition because most archaea with the form-III
Figure 1: Rubisco holoenzyme structures for form-I, II, and III Rubisco. Form-I Rubisco is represented by that of *Chlamydomonas* (PDB 1GK8), form-II by that of *R. rubrum* (PDB 9RUB) and form-III by that of *Thermococcus kodakaraensis* (PDB 1GEH). The areas in red denote the active-site regions. For *Chlamydomonas* Rubisco, small subunits are in yellow and orange, with the variable βA-βB loop that surrounds the central solvent channel also shown as ribbons.
Rubisco are adapted to anaerobic habitats. Based on phylogenetic analysis, form-III Rubisco is the putative ancestor for all Rubisco proteins (Tabita et al., 2008a). Another class of proteins, known as Rubisco-like proteins or form-IV Rubisco, is similarly derived from the form-III ancestral Rubisco, but these proteins lack half of the active-site residues of bona fide Rubisco, and do not catalyze carbon fixation (Saito et al., 2009).

In all forms of Rubisco, the active sites are located in the large subunits (Fig. 1) (Knight et al., 1990). Rubisco large subunits are 440-480 amino acids in length (depending on species) and 50-55 kDa in molecular mass (Andersson and Backlund, 2008). The large subunit has an amino-terminal domain composed of a five-stranded mixed β-sheet packed by two α helices on one face of the sheet, and a longer carboxy-terminal domain composed of an eight-stranded parallel α/β-barrel structure (Knight et al., 1990; Andersson and Backlund, 2008).

Despite the structural similarities of the large subunits and active sites, the different forms of Rubisco share as little as 30% protein-sequence identity (Tabita et al., 2008a). However, some photosynthetic prokaryotes such as Rhodobacter sphaeroides, R. capsulatus, and Hydrogenovibrio marinus harbor both form-I and II Rubisco, but preferentially synthesize the higher-Ω form-I Rubisco under CO₂-limiting conditions (Gibson and Tabita, 1977; Tabita, 1999; Tabita et al., 2008a; Andersson, 2008; Andersson and Backlund, 2008).

Among plants and green algae, the Rubisco holoenzyme is localized in the chloroplast stroma, where one large subunit rbcL gene is found on each copy of the multicopy chloroplast genome. On the other hand, a family of Rubisco small-subunit rbcS genes, ranging from 2 to over 22 genes depending on the species, are found in the
nucleus (Spreitzer, 2003). Nuclear-encoded Rubisco small-subunit proteins have cleavable transit peptides to target and translocate the precursors into the chloroplast for assembly (Schmidt and Mishkind, 1986). The only exception among eukaryotes for separate compartmentalization of the rbcL and rbcS genes is among non-green algae, which have both small and large subunit genes in a chloroplast rbcLS operon (Tabita, 1999; Spreitzer, 2003). Among prokaryotes with form-I Rubisco, the two subunits are cotranscribed in a larger operon containing genes of several other Calvin cycle enzymes (Tabita, 1999).

Most Rubisco small subunits, which are 110-180 amino acids in length and 12-18 kDa in molecular mass, are composed of a four-stranded anti-parallel β-sheet (denoted strands A to D) with two α helices packed on one face of the sheet (Fig. 2). Small subunits from non-green algae and certain prokaryotes also have a longer carboxy-terminal extension with two additional β-strands, which are denoted strands E and F (Fig. 2) (Spreitzer, 2003; Andersson, 2008). A loop between β-strands A and B of the small subunit, known as the βA-βB loop, and a loop between β-strands E and F (when present), known as the βE-βF loop, surround the opening of the the central solvent channel and determine the channel’s aperture based on the bulkiness and length of the loops (Fig. 1) (Knight et al., 1990; Spreitzer, 2003). Because the highest measured Ω value is for red-algal Rubisco with the βE-βF loop, and because form-I Rubisco, which has small subunits, has higher Ω than the other Rubisco forms, the architecture of the central solvent channel and the small subunits can influence catalysis (Uemura, 1997; Spreitzer, 2003; Genkov et al., 2010). In addition to playing a role in catalysis, the small subunit is also responsible for aggregating Rubisco molecules into proteinaceous structures known
Figure 2: Alignment of Rubisco small subunits from *Chlamydomonas* (yellow) (PDB 1GK8), spinach (blue) (PDB 8RUC) and non-green alga *Galdieria partita* (green) (PDB ID 1BWV). In the structural alignment, the loops between β-strands A and B, and between C and D, and between E and F, which is only in non-green algae, are labeled. In the sequence alignment, the residue positions at the top are based on the *Chlamydomonas* small-subunit sequence, and the secondary structures are shaded and labeled at the bottom.
as pyrenoids in algae where CO₂ is concentrated to be more efficiently fixed by Rubisco (Genkov et al., 2010).

**Posttranslational modifications**

Posttranslational modifications of the Rubisco holoenzyme have mainly been observed and studied in form-I Rubisco (Houtz et al., 2008). The most important posttranslational modification of the Rubisco large subunit is the carboxymethylation of catalytic Lys-201 by non-substrate CO₂ to activate the enzyme (Lorimer, 1981; Cleland et al., 1998). Besides that, the first two residues of the Rubisco large subunit from plants and green algae are cleaved off by a dipeptidase, and the third residue (Pro-3) is N-acetylated (Houtz et al., 1989). The putative role of this amino-terminal modification is the protection of the protein from proteolysis (Houtz et al., 2008). Amino-terminal modifications of the Rubisco small-subunit also occur, the most obvious being the removal of the transit peptide, which is followed by N-methylation of Met-1 in the mature small subunit (Schmidt and Mishkind, 1986; Houtz et al., 2008).

Other posttranslational modifications occur in the large subunit. Lys-14 of some plant large subunits is Nε-trimethylated by a methyltransferase, but the role of this modification is unknown (Houtz et al., 1989, 2008). In the high-resolution crystal structure of Rubisco from the green alga *Chlamydomonas reinhardtii*, four additional posttranslational modifications of the large subunit were observed (Taylor et al., 2001). These include S-methylation of Cys-256 and Cys-369, and 4-hydroxylation of Pro-104 and Pro-151 (Taylor et al., 2001). Recently, determination of lysine acetylation in *Arabidopsis* proteins using generic anti-LysAc antibody immunodetection followed by
liquid chromatography-tandem mass spectrometry discovered nine acetylated lysine residues in the Rubisco large subunit and one acetylated lysine residue in the small subunit (Finkmeier et al., 2011), which is surprising considering that acetylation of lysines have never been observed in any of the Rubisco crystal structures from diverse species (Schneider et al., 1986; Knight et al., 1990; Taylor et al., 2001; Andersson, 2008).

Despite the vast knowledge on Rubisco posttranslational modification, much is still unknown about the functions or proteins involved. For example, several nuclear mutations in Chlamydomonas change the kinetic constants of Rubisco, and decrease $\Omega$, but the genetic loci of these mutations are yet to be identified. These mutations must affect Rubisco posttranslationally (Spreitzer et al., 1988a; Spreitzer et al., 1992; Gotor et al., 1994).

**Holoenzyme assembly**

Assembly of Rubisco is initiated by the folding of large-subunit monomers in an ATP-dependent process, which is mediated by the GroEL/GroES-type chaperonins (Goloubinoff et al., 1989). For form-II Rubisco, the properly-folded large subunits will spontaneously dimerize to form active Rubisco upon release from the chaperonin cage (Goloubinoff et al., 1989). For the hexadecameric form-I Rubisco, an additional chaperone, RbcX, is also involved (Saschenbrecker et al., 2007). The RbcX chaperone binds to the carboxy-terminal tail of the Rubisco large subunit, and facilitates and stabilizes the formation of octameric large subunits after the GroEL/GroES-mediated step (Saschenbrecker et al., 2007; Liu et al., 2010). RbcX is then displaced from the Rubisco
large-subunit octamers by Rubisco small subunits (Liu et al., 2010). In cyanobacteria, the RbcX gene is found in the same operon as the Rubisco large and small-subunit genes (Tabita, 1999) whereas, in plants, the RbcX gene is found in the nucleus (Kolesinski et al., 2011).

**Reaction mechanism and active-site residues**

The carboxylation of RuBP by Rubisco involves five steps, which are enolization, carboxylation, hydration, C2-C3 bond scission, and stereospecific protonation (Fig. 3). The sequence of steps and the nature of the reaction intermediates were mainly deduced by radiolabeling and borohydride trapping experiments, and, more recently, by quantum-chemical modelling, whereas the positions and roles of the active-site residues were identified by chemical affinity labeling, site-directed mutagenesis, and crystal-structure analysis (Miziorko and Lorimer, 1983; Knight et al., 1990; Taylor and Andersson, 1997; Kannappan and Gready, 2008). The active sites are located in the interface regions between two large subunits that are assembled head-to-tail. The twenty active-site residues, which are Glu-60, Thr-65, Trp-66, Asn-123, Thr-173, Lys-175, Lys-177, Lys-201, Asp-203, Glu-204, His-294, Arg-295, His-327, Lys-334, Leu-335, Ser-379, Gly-380, Gly-381, Gly-403 and Gly-404, are mainly located in flexible loops in the Rubisco large-subunit. They form electrostatic interactions with the carboxylation transition-state analog CABP, as observed in x-ray crystal structures of CABP-bound Rubisco (Knight et al., 1990; Taylor et al., 2001). Active-site residues Glu-60, Thr-65, Trp-66 and Asn-123, which are from the amino-terminal domain of one large subunit, and the other active-site residues, which are from the carboxy-terminal domain of an adjacent large subunit, form
Figure 3: Reaction mechanism for Rubisco-catalyzed carboxylation or oxygenation of ribulose 1,5-bisphosphate (Chen and Spreitzer, 1992; Andersson, 2008; Kannappan and Gready, 2008). Carbon positions are indicated for the substrate ribulose bisphosphate. Reaction steps are in italics and bold.
each active-site pocket. Thus, each dimer of large subunits has two active sites, and each hexadecameric holoenzyme has eight active sites (Knight et al., 1990; Taylor et al., 2001).

Before catalysis, Rubisco has to be activated by CO$_2$ through the carbamylation of N$\varepsilon$ of Lys-201 (numbering based on form-I Rubisco). The activator CO$_2$ is distinct from the substrate CO$_2$ used for RuBP carboxylation. After Lys-201 carbamylation, which is reversible, Mg$^{2+}$ binds in the Rubisco active-site, stabilized by the negatively-charged carbamate (Lorimer, 1981). This is followed by RuBP binding to the active-site. If RuBP binds to the unactivated enzyme (i.e. before the carbamylation of Lys-201), a dead-end enzyme-substrate complex is formed that has to be opened by a protein known as Rubisco activase to release the unreacted RuBP (Salvucci et al., 1985).

Lys-201 is essential for the first enolization step in Rubisco catalysis (Fig. 3). To initiate the enolization of RuBP, Lys-201 is carbamylated to enable the abstraction of a proton from the C-3 carbon of RuBP by the negatively-charged carbamate side-group. In addition to that, carbamylation of Lys-201 enables binding of Mg$^{2+}$ at the active site. Two other active-site residues, Asp-203 and Glu-204, also provide negatively-charged side-chains for coordination with the Mg$^{2+}$ cofactor (Gutteridge et al., 1988). Lys-175 is also essential for enolization (Taylor and Andersson, 1997). Based on the atomic coordinates of Lys-175 in the x-ray crystal structures of Rubisco, the position of Lys-175 is close enough to reprotoonate the oxygen carbonyl at the C-2 position of RuBP, concurrent with the deprotonation of the C-3 carbon by Lys-201 during enolization (Taylor and Andersson, 1997). Also, Lys-175 forms electrostatic interaction with another active-site residue, Asp-203, which in turn coordinates with the Mg$^{2+}$ cofactor.
The second step in Rubisco catalysis is carboxylation (or oxygenation) of the 2,3-enediol intermediate (Fig. 3). Lys-334 is essential for polarizing the substrate CO₂ to facilitate the nucleophilic addition of the gaseous substrate to the C-2 carbon of the enediol intermediate in the carboxylation step, or for polarizing O₂ in the corresponding oxygenation step (Gutteridge et al., 1993). Changing Lys-334 to arginine, which would still retain the positive charge on position 334, reduces carboxylation almost completely, but does not affect oxygenation significantly (Gutteridge et al., 1993).

The third and fourth step in Rubisco catalysis are hydration of the ketocarboxyarabinitol bisphosphate (or ketoperoxyarabinitol bisphosphate) intermediate to form a gem-diol, and C2-C3 bond scission of the gem-diol to form one substrate molecule of 3-PGA and one of an aci-acid intermediate (or, for oxygenation, 3-PGA and 2-PG) (Fig. 3). There is an additional fifth step in Rubisco catalysis for carboxylation, which is the stereospecific protonation of the three-carbon aci-acid intermediate to form another 3-PGA molecule, with Lys-175 as the proton donor (Fig. 3) (Harpel et al., 2002; Kannappan and Gready, 2008). Engineered Rubisco enzymes with mutations at Lys-175 are deficient in the enolization of RuBP, and produce pyruvate instead of 3-PGA in the final stereospecific protonation step (Harpel et al., 2002).

The precise roles of other active-site residues in Rubisco catalysis are less defined. For example, because the imidazole side-chain of His-294 is suitably positioned to either accept or donate a proton to the C-3 hydroxyl group of RuBP, His-294 could play a role in the gas addition, hydration, or C2-C3 bond scission steps (Kannappan and Gready, 2008). However, mutations of His-294 eliminate enolization of RuBP (Harpel et al., 1998). Therefore, through electrostatic interactions, His-294 could also affect the
spatial position and the pKa of carbamate-Lys-201 that is directly responsible for enolization (Kannappan and Gready, 2008). There are other electrostatic interactions between the active-site residues that could affect the pKas of the interacting side chains, such as between Glu-60 and Lys-334, Thr-173 and Lys-201, and Lys-175 and Lys-177. Also, a number of active-site residues, which are Thr-65, Trp-66, Asn-123, Arg-295, His-327, Ser-379, Gly-380, Gly-381, Gly-403 and Gly-404, interact with the negatively-charged terminal phosphates of RuBP (Knight et al., 1990).

**Structural rearrangements during catalysis**

The substrate-free Rubisco holoenzyme is in an initial "open" state whereby the catalytic loop 6 of the large subunit is retracted (Duff et al., 2000). Thus, in the open state, the active site of Rubisco is exposed to solvent (Duff et al., 2000). Substrates RuBP and CO$_2$, and the cofactor Mg$^{2+}$, can access the active site only in the open state (Schreuder et al., 1993; Duff et al., 2000).

Upon binding of substrate RuBP or any organophosphate inhibitor to the Rubisco active site, global conformational changes involving domain movements occur. First, catalytic loop 6 of Rubisco extends over the active site at the top of the large-subunit $\alpha/\beta$-barrel. The Rubisco large-subunit carboxy terminus (from residue 462 onwards) folds over loop 6. The amino-terminal domain (residues 1-150) from an adjacent large subunit also rotates toward the carboxy-terminal domain to cover the top of the $\alpha/\beta$-barrel, with a corresponding shift of the small subunit (Duff et al., 2000). As a result, the active site of Rubisco is shielded from solvent, and the active-site residues from two adjacent large
subunits are also near enough to substrate RuBP to catalyze the reaction (Schreuder et al., 1993; Duff et al., 2000).

The solvent-occluded state of Rubisco is known as the "closed" state. Catalysis takes place during the closed state where there is less chance of the reaction intermediates being released prematurely or being misprotonated by external solvent (Schreuder et al., 1993). Crystal structures of the closed state of Rubisco most often contain the carboxylation transition-state analog CABP, which is analogous to the ketocarboxyarabinitol-bisphosphate intermediate (Andersson and Backlund, 2008). Loop 6 and the carboxy-terminal tail are ordered in the closed state, but these structural elements are disordered in the open state. The conformational stabilization of loop 6 and the carboxy terminus in the closed state occurs because of interactions between loop 6, RuBP, reaction intermediates, the carboxy terminus, and residues from an adjacent large subunit. Specifically, Lys-334 in loop 6 forms electrostatic interactions with a terminal phosphate of RuBP, the carboxylate group in the ketocarboxyarabinitol bisphosphate intermediate, and also Glu-60 and Thr-65 from an adjacent large subunit. Glu-338 in loop 6 also interacts with Asp-473 in the carboxy-terminal tail of Rubisco (Schreuder et al., 1993; Duff et al., 2000). Mutations of Asp-473 produce functional Rubisco, but caused the carboxy terminus to be disordered in the crystal structures (Satagopan and Spreitzer, 2004; Karkehabadi et al., 2007).

The closed state of Rubisco becomes open only when the six-carbon ketocarboxyarabinitol-bisphosphate intermediate of RuBP is converted into two three-carbon 3-PGA carboxylation products or into the single three-carbon 3-PGA and two-carbon 2-PG oxygenation products within the active site. Duff et al. (2000) proposed that
the shift from a closed-state Rubisco to an open state is caused by the increased distance between the terminal phosphates after scission of the six-carbon ketocarboxyarabinitol bisphosphate, but this was disproved by subsequent mutagenesis experiments involving the carboxy terminus (Satagopan and Spreitzer, 2004).

DISSECTION OF RUBISCO

Random mutant screening and genetic selection

Early genetic studies of Rubisco were focused on attempts to select a better enzyme (Somerville and Ogren, 1982; Spreitzer et al., 1982). However, no Rubisco mutants were recovered by screening or selection in Arabidopsis (Somerville and Ogren, 1982). In contrast, screening of Chlamydomonas for mutants that were photosynthesis-deficient yielded the first Rubisco large-subunit mutant, which has a Gly-171-to-Asp (G171D) substitution (Spreitzer and Mets, 1980; Dron et al., 1983). The G171D substitution eliminates the activity of the holoenzyme without affecting structural stability (Spreitzer and Mets, 1980). A multitude of other mutations in Chlamydomonas Rubisco that negatively affect holoenzyme activity and/or stability were isolated using this prolific screen (Spreitzer, 1993). Mutants T173I and G237S lacked Rubisco activity, whereas G54D and R217S lacked the holoenzyme (Spreitzer et al., 1988b; Spreitzer, 1993; Thow et al., 1994). Several mutants with nonsense mutations at amino-acid positions 45, 66, and 451 were also isolated through this screen (Spreitzer and Chastain, 1987). However, unlike the other Chlamydomonas Rubisco mutants discovered by random mutant screening, mutants V331A and L290F, had measurable but decreased carboxylase activity and Ω (Chen et al., 1988; Chen and Spreitzer, 1989). Rubisco-
mutant V331A was isolated by screening for photosynthesis-deficient mutants, but L290F was isolated by screening for mutants that were photosynthesis-deficient only at elevated temperature (Chen et al., 1988; Chen and Spreitzer, 1989).

The *Chlamydomonas* missense mutations are distributed in four regions of the Rubisco large subunit. The first region, highlighted by the G54D mutation, is in the amino-terminal domain near the loop containing active-site residues Glu-60, Thr-65, and Trp-66 (Spreitzer et al., 1995). The second region, highlighted by the R217S and G237S mutations, is near the active-site residue Lys-201 that has to be activated for catalysis (Thow et al., 1994). The third region, highlighted by the V331A mutation, is near catalytic loop 6 (Chen and Spreitzer, 1989). Finally, the fourth region, highlighted by the L290F mutation, is at the bottom of the large-subunit α/β-barrel (Chen et al., 1988). Unlike the other three regions, which are in close proximity to the Rubisco active-site, the region at the bottom of the barrel is 20 Å from the active-site, thus providing the first indication that residues far from the active site could affect catalysis (Du and Spreitzer, 2000).

Reversion experiments were carried out on the photosynthesis-deficient Rubisco mutants by subjecting the mutant cells to photosynthetic selection (Spreitzer et al., 1982). The basis for the reversion experiments was that suppressor mutations in the Rubisco primary-structure would be isolated if these suppressor residues complement and interact with the original mutated residue to restore Rubisco function *in vivo* (Spreitzer et al., 1985). From the reversion experiments, it was discovered that the V331A substitution, which is at the amino-terminal end of loop 6 is complemented by suppressor mutations T342I and G344S, which are on the carboxy-terminal end of the loop (Chen and
Spreitzer, 1989; Chen et al., 1991). On the other hand, mutation R217S, which affects Rubisco assembly, is complemented by A242V, and, because both the mutation and suppressor residues are within the hydrophobic wall of the $\alpha/$$\beta$-barrel, the residues could influence the structure of the barrel (Thow et al., 1994). Another significant result from the reversion experiments is that Rubisco large-subunit mutation L290F, which causes a decrease in $\Omega$, can be complemented by A222T and V262L suppressor substitutions in the large subunit as well as by N54S, A57V, and C65S substitutions in the small subunit, indicating that the small subunit can affect $\Omega$ through interactions with the large subunit (Hong and Spreitzer, 1997; Du and Spreitzer, 2000; Du et al., 2000; Genkov et al., 2006). Unlike the other Rubisco mutations, G54D within the hydrophobic core of the amino-terminal domain cannot be complemented by other suppressor-residue substitutions. Instead, the mutated residue can pseudorevert to alanine or valine, which are non-polar residues, signifying the role of residue 54 in retaining the hydrophobicity of the structural core (Spreitzer et al., 1995).

Screening for photosynthesis-deficient Chlamydomonas mutants also found other non-Rubisco nuclear-gene mutations that could reduce the mRNA level and posttranslationally affect the kinetic properties of Rubisco (Spreitzer et al., 1988a; Gotor et al., 1994; Hong and Spreitzer, 1994). The mutated proteins likely play a role in Rubisco transcription and posttranslational modification, but, as of yet, only one of the mutations has been identified, and found to have occurred in an mRNA-stabilizing protein that has orthologs in plants (Johnson et al., 2010).
Directed mutagenesis

Site-directed mutagenesis studies of Rubisco active-site residues have been extensively carried out on prokaryotic Rubisco from *R. rubrum* and *Synechococcus*, but the prokaryotic enzymes were expressed only in *Escherichia coli* for study (Hartman and Harpel, 1994; Gutteridge *et al.*, 1993; Harpel *et al.*, 2002). Eukaryotic Rubisco cannot be expressed in *E. coli*, and the reason for that is still unclear (Cloney *et al.*, 1993). Mutations of the prokaryotic Rubisco active-site residues eliminate activity, which indicates that the active-site residues are immutable for proper function, consistent with the fact that these residues are almost 100% conserved among species (Hartman and Harpel, 1994). Similarly, mutating the active-site residues of eukaryotic Rubisco in *Chlamydomonas* decreases the carboxylase activity to less than 5% and Ω to less than 30% of the wild type value, and eliminates photosynthetic growth of the organism (Zhu and Spreitzer, 1994).

Directed mutagenesis of non-active-site residues, guided by phylogenetic data, has also been pursued to understand the structure-function relationships of Rubisco. Certain stretches of Rubisco amino-acid sequences were changed from one species to that of another, with the expectation of a corresponding shift in kinetic properties. The non-active-site residues in and around loop 6 were one of the earlier targets for these directed-mutagenesis experiments because earlier mutant screening in *Chlamydomonas* found that changes in the region, specifically V331A, could modify Ω (Chen and Spreitzer, 1989). A stretch of four amino acids, from residue 338 to 341, at the carboxy-terminal end of loop 6, was changed in Rubisco of the cyanobacterium *Synechococcus* to the sequence found in plants, but did not cause any alterations in kinetic properties, even though the
plant Rubisco should have higher $\Omega$ and lower $V_c$ (Gutteridge et al., 1993). On the other
hand, mutating residues that flank loop 6 in Chlamydomonas Rubisco to that of plants
decreased $\Omega$ and $V_c$ (Zhu and Spreitzer, 1996). Creating the suppressor substitutions of
V331A in Chlamydomonas Rubisco, which are T342I and G344S, alone decreased $\Omega$ and $V_c$, even though these substitutions improve the catalytic properties of the previous
V331A mutant enzyme (Karkehabadi et al., 2007). Therefore, residue engineering in the
region around loop 6 does not improve the net CO$_2$ fixation of the enzyme, but, instead,
is deleterious to catalysis.

Because substitutions in the amino-terminal domain of the Rubisco large subunit
affect both $\Omega$ and enzyme stability, evidenced by the G54D Rubisco-deficient
Chlamydomonas mutant and the pseudorevertant G54V, which has a 17% decrease in $\Omega$, a
couple of variable non-active-site residues in the region were targeted for directed-
mutagenesis studies (Spreitzer et al., 1995; Du et al., 2003). Met-42 and Cys-53 were
changed singly or as a pair to the Val-42 and Ala-53 residues of plants, but the mutant
enzymes had no significant changes in catalysis (Du et al., 2003). Thus, residue
differences in the amino-terminal domain alone cannot account for the differences in
Rubisco kinetic properties among species.

Because of the phylogenetic diversity in the Rubisco large-subunit carboxy-
terminus, which folds over loop 6 during catalysis, site-directed mutagenesis experiments
of this region have been pursued extensively (Gutteridge et al., 1993; Zhu et al., 1998;
Satagopan and Spreitzer, 2008). When the carboxy terminus, residues 470-475, of the
Chlamydomonas large subunit was substituted with the residues from spinach, there was
an increase in $\Omega$ by 10%, but a decrease in carboxylation catalytic efficiency (Satagopan
and Spreitzer, 2008). On the other hand, no significant increase in Ω was measured when the carboxy terminus of *Synechococcus* Rubisco was changed to that of plants (Gutteridge et al., 1993). Also, because red-algal Rubisco has 10 additional residues at the carboxy terminus and higher Ω when compared to *Synechococcus* Rubisco, the extra residues were engineered into the *Synechococcus* enzyme, but did not cause a marked increase in Ω (Zhu et al., 1998). Truncations of the carboxy-terminal region of the *Synechococcus* and *R. rubrum* enzymes eliminate enzymatic activity, and for the *R. rubrum* Rubisco, also alter the quarternary structure of the holoenzyme (Ranty et al., 1990; Gutteridge et al., 1993). Thus, the carboxy terminus most likely plays a role in Rubisco catalysis, possibly through interactions with loop 6, which is also evidenced by the fact that mutations of Asp-473, a latch residue that holds the carboxy terminus over loop 6, decrease Ω (Duff et al., 2000; Satagopan and Spreitzer, 2004; Karkehabadi et al., 2007).

Directed mutagenesis experiments of Rubisco should also consider the interactions between Rubisco and Rubisco activase (Spreitzer and Salvucci, 2002). Activase from Solanaceae species (e.g. tobacco) will not activate Rubisco from non-Solanaceae species (e.g. *Chlamydomonas* and spinach), and vice versa (Wang et al., 1992). In fact, substitutions P89R and D94K in the *Chlamydomonas* Rubisco large subunit produced enzymes that could be activated by activase from tobacco but not spinach, effectively switching the activase-recognition site from that of non-Solanaceae to Solanaceae (Larson et al., 1997; Ott et al., 2000). Because changes in other residues around this region may also affect activase interaction with Rubisco, future experiments should keep that possibility in mind.
The penta/ABSO mutant and the large/small-subunit interface

Another region of the Rubisco holoenzyme analyzed by site-directed mutagenesis experiments is the interface between the large and small subunit, at the bottom of the $\alpha/\beta$ barrel, which is on the opposite end from the active site (Du and Spreitzer, 2000; Spreitzer et al., 2005; Genkov et al., 2006). The large/small-subunit interface region became of interest when it was discovered that an L290F mutation in the region is the cause for photosynthesis deficiency at elevated temperatures in a Chlamydomonas mutant (Chen et al., 1988; Spreitzer et al., 1988a). Further reversion experiments and site-directed mutagenesis studies indicated that the region affects both catalysis and stability of Rubisco (Chen et al., 1988; Hong and Spreitzer, 1997; Du et al., 2000; Du and Spreitzer, 2000; Genkov et al., 2006). More importantly, a phylogenetic Chlamydomonas-to-plant substitution involving five residues of the Rubisco large-subunit, V221C/V235I/C256F/K258R/I265V, together with changing the small-subunit loop between $\beta$-strands A and B to that of spinach (Spinacia oleracea) (ABSO), all at the interface region, produces a Rubisco mutant in Chlamydomonas, named "penta/ABSO", with kinetic properties shifted towards the plant Rubisco properties, marked by an increase in $\Omega$ and decrease in $V_c$ (Table 1) (Spreitzer et al., 2005).

Hybrid enzymes

A more elaborate directed-engineering approach for studying Rubisco structure-function relationships is swapping the whole large or small subunit of Rubisco from different species, which have different catalytic properties (Table 1) (Jordan and Ogren, 1981b; Read and Tabita, 1992; Kanevski et al., 1999; Whitney et al., 2001, 2011;
Genkov et al., 2010). However, a limitation to swapping the whole subunit is that prokaryotic Rubisco large subunits cannot be expressed in eukaryotes and vice versa, with the exception of the prokaryotic *R. rubrum* Rubisco, which can be expressed in tobacco plants, but the transgenic plants require elevated CO$_2$ (5% v/v in air) for growth (Cloney et al., 1993; Whitney and Andrews, 2001). Even transgenic tobacco plants that express the sunflower or *Flaveria* Rubisco large subunits produce less than 50% of wild-type holoenzyme level, and mainly require elevated CO$_2$ or sucrose supplementation for growth (Kanevski et al., 1999; Whitney et al., 2011). Moreover, even though the Rubisco small subunit is not hindered by the eukaryotic/prokaryotic-expression barrier, plant Rubisco small subunits are encoded by a family of nuclear genes (Spreitzer et al., 2003). Thus, the native small subunits are still present in experiments where transgenic plants were transformed with foreign small subunits, which complicates the analysis of the experiments (Read and Tabita, 1992; Getzoff et al., 1998; Ishikawa et al., 2011). On the other hand, all the native Rubisco small-subunit genes in *Chlamydomonas* have been successfully knocked out and replaced by the foreign small-subunit genes from *Arabidopsis*, spinach, and sunflower, and the mutant Rubisco enzymes have increases in $\Omega$ by 3-11% (Genkov et al., 2010). When the cyanobacterial *Synechococcus* Rubisco small subunit was replaced with that from marine algae, an increase in $\Omega$ was also observed (Read and Tabita, 1992). Thus, even though the active-site region is located in the Rubisco large subunit, the small subunit also influences catalysis (Read and Tabita, 1992; Karkehabadi et al., 2005; Spreitzer et al., 2005; Genkov et al., 2010).
Directed evolution

The first directed-evolution experiment that specifically targeted the Rubisco genes was performed in the photosynthetic bacteria *Rhodobacter capsulatus* using a library of cyanobacterial *Synechococcus* Rubisco large and small-subunit genes that were mutagenized in the *E. coli* mutator-strain XL-1 Red (Smith and Tabita, 2003). Because the *R. capsulatus* mutant depends on *Synechococcus* Rubisco for photosynthetic growth at elevated CO$_2$ (5%), any *Synechococcus* Rubisco mutant enzyme that enabled the *R. capsulatus* strain to grow at lower CO$_2$ (1.5%) should harbor positive mutations that increased CO$_2$ fixation of the enzyme *in vivo* (Smith and Tabita, 2003). Instead of changes in kinetic properties with regard to CO$_2$ fixation, the positive Rubisco mutants had increased affinity for the substrate RuBP (Smith and Tabita, 2003). In other Rubisco selection experiments, Rubisco-dependent *E. coli* strains were engineered by expressing the enzyme phosphoribulose kinase, which diverted carbon to RuBP (Parikh *et al.*, 2006; Mueller-Cajar *et al.*, 2007; Mueller-Cajar and Whitney, 2008). Rubisco libraries created by PCR-based mutagenesis were transformed into the Rubisco-dependent *E. coli*, and the fastest-growing colonies were picked for analysis (Parikh *et al.*, 2006; Mueller-Cajar *et al.*, 2007; Mueller-Cajar and Whitney, 2008). Similar to the *R. capsulatus* selection system, the *E. coli* Rubisco selection system selected for enzymes with improved expression and RuBP affinity, but not catalysis (Parikh *et al.*, 2006; Mueller-Cajar *et al.*, 2007; Mueller-Cajar and Whitney, 2008). On the other hand, improvements in Ω and $V_c$ of Rubisco by 20% and 50%, respectively, were reported after screening 60,000 *Chlamydomonas* transformants that were transformed with a library of gene-shuffled Rubisco variants, but the residue-substitutions were not included in the report (Zhu *et al.*, 2018).
2005). Therefore, selection strategies with Rubisco have yet to produce a better plant enzyme.

**Chlamydomonas reinhardtii as a model organism**

The most well-developed and expedient genetic system for study of eukaryotic Rubisco is the green alga *Chlamydomonas*, with the goal of eventually transferring any identified improvements into crops (Spreitzer, 1998). Unlike plants, which are obligate photoautotrophs, *Chlamydomonas* can grow heterotrophically when supplemented with acetate as an alternate carbon source, thus allowing expression and analysis of defective Rubisco mutants (Spreitzer and Mets, 1980, 1981). More importantly, the Rubisco large and small-subunit genes have been knocked out in *Chlamydomonas* to create hosts for transformation with genetically-engineered Rubisco genes (Spreitzer and Mets, 1980; Newman et al., 1991; Khrebtukova and Spreitzer, 1996; Dent et al., 2005; Zhu et al., 2005). Chloroplast and nuclear transformation of the Rubisco large and small-subunit genes, respectively, have been routinely carried out for *Chlamydomonas* (Zhu and Spreitzer, 1994, 1996; Satagopan and Spreitzer, 2004; Genkov et al., 2006). Even though genetic studies of eukaryotic Rubisco have also been carried out in tobacco, there are several drawbacks to the tobacco system. There is a need to use a co-transformation system with a selectable antibiotic-resistance marker instead of direct selection for photoautotrophy, an inability to fully replace the family of native Rubisco small-subunit genes, and a longer transformation time of three weeks (Svab and Maliga, 1993; Whitney and Andrews, 2001). Also, more Rubisco mutants can be screened or selected for in
Chlamydomonas compared to plants (Spreitzer and Mets, 1980; Somerville and Ogren, 1982).

Mating of Chlamydomonas mutants is also an available tool to combine several non-allelic mutations into a single strain. Tetrad analysis can be done to determine genetic linkage, and, through the inheritance pattern, the organelle-localization of the mutations (Spreitzer and Mets, 1980; Hong and Spreitzer, 1994).

The wealth of bioinformatics resources also benefits Chlamydomonas Rubisco research. The availability of both the chloroplast and nuclear-genome sequences facilitates genetic analysis and engineering of mutants (Maul et al., 2002; Merchant et al., 2007). In addition, the availability of over ten x-ray crystal-structures of separate Chlamydomonas mutant Rubisco enzymes provides extensive data for structural studies (Andersson and Backlund, 2008).

Bioinformatics analysis

With the increased abundance of rbcL sequences, more elaborate and intricate computational phylogenetic analysis can be undertaken to define catalysis-influencing residue changes within the Rubisco large subunit. One of the studies, which coupled phylogenetic and crystal structure data, forms the basis for the present study and for the engineering of the penta/ABSO mutant (Du et al., 2003; Spreitzer et al., 2005).

In a recent phylogenetic analysis, Kapralov et al. (2011) sought to determine the residues that could be responsible for the differences in kinetic properties between Rubisco enzymes from the C3 and C4 species within the Flaveria genus. Changes in large-subunit residues 149 and 309 correlate with changes in Rubisco kinetic properties,
though large-subunit residue 265 and small-subunit residues 20, 24 and 57 are also changed in different *Flaveria* species (Kapralov *et al.*, 2011). To test the importance of residues 149 and 309, Whitney *et al.* (2011) expressed the *Flaveria rbcL* gene in tobacco, and changed residues 149 and 309 separately to that of either the C3 or C4 species, and found that only residue 309 is responsible for the switch in the kinetic properties.

However, most of the other phylogenetic studies of Rubisco did not necessarily focus on evolutionary forces acting on the kinetic properties of Rubisco (Nozaki *et al.*, 2002; Yu *et al.*, 2005; Kapralov and Filatov, 2006, 2007). Instead, the positively-selected residues from these studies could play a role in Rubisco stability, or interaction with activase or other molecules. For example, Nozaki *et al.* (2002) sought to determine the large-subunit residues that could explain the presence or absence of the pyrenoid within the green algal *Chloromonas* lineage. Kapralov and Filatov (2006) sought to determine the residues that could facilitate the environmental adaptation of the Hawaiian-plant genus *Schiedea* between rainforest and dry coastal cliffs. Yu *et al.* (2005) sought to determine the clusters of surface residues that differentiate green plants, cyanobacteria and non-green algae Rubiscos. Additional bioinformatics-based, site-directed mutagenesis and biochemical analysis of Rubisco is required.

**RATIONALE AND OBJECTIVES**

The structural basis for the differences in kinetic properties between Rubisco from diverse species has yet to be elucidated (Spreitzer, 1993, 1999; Spreitzer and Salvucci, 2002). Understanding the structure-function relationship of Rubisco would allow future engineering of the holoenzyme for improved photosynthesis in crops (Spreitzer and
Salvucci, 2002). In other words, defining the structural regions or residues in Rubisco to be targeted for engineering is crucial.

The active-site residues are almost 100% conserved among the ~2500 Rubisco large-subunit sequences in the NCBI Entrez Proteins database. Moreover, crystal-structure data indicate that all Rubisco holoenzymes adopt similar tertiary folds (Andersson and Backlund, 2008). Therefore, differences in the non-active-site residues of Rubisco, which cause subtle structural changes, must account for the differences in kinetic properties, and, because the small subunit is too divergent, it is reasonable to focus on differences within only the large subunit. Moreover, the catalytic subunit of Rubisco is the large subunit (Knight et al., 1990).

Because site-directed mutagenesis and transformation of Rubisco in *Chlamydomonas* is well-established, and the crystal structures of various mutant forms of *Chlamydomonas* Rubisco have been solved, including the highest-resolution structure, *Chlamydomonas* has been extensively used for structure-function and genetic-engineering studies (Zhu and Spreitzer, 1994, 1996; Taylor et al., 2001; Andersson and Backlund, 2008). The kinetic properties of *Chlamydomonas* Rubisco are different from plants, but most strikingly, for *Chlamydomonas* Rubisco, $\Omega \approx 60$, whereas for plants, $\Omega \approx 80-100$ (Jordan and Ogren, 1981b; Genkov et al., 2010). Therefore, knowing the phylogenetic-residue changes responsible for the differences in kinetic properties between *Chlamydomonas* and plant Rubiscos could be important for defining genetic-engineering targets.

Even though the phylogenetic residues changed in the penta/ABSO enzyme are suitable targets for genetic engineering, there are other diverse residues within the large
subunit that may also influence catalysis. Therefore, a global subunit-wide inquiry, that would encompass all the other phylogenetically-diverse residues of the Rubisco large subunit, might identify other residues that contribute to phylogenetic differences in catalysis. This is the first global structure-function study of the Rubisco large subunit by directed mutagenesis. The only other Rubisco study to attempt such a broad scope focused on substituting only conserved glycine residues with alanines and prolines (Cheng and McFadden, 1998).
MATERIALS AND METHODS

MATERIALS

Molecular biology

DNA-miniprep kits for plasmid purification and gel-extraction kits for agarose-gel-embedded DNA-fragment purification were purchased from Qiagen. Restriction endonucleases and T4 DNA ligase for plasmid recombination were from New England Biolabs. *Taq* DNA polymerase was from Invitrogen. *Pfu* Turbo DNA Polymerase and the Quikchange Mutagenesis Kit for site-directed mutagenesis were from Stratagene. Oligonucleotides for site-directed mutagenesis and for sequencing were from Sigma-Aldrich. Tungsten (M-10, 0.7 µm) for chloroplast transformation was from Biorad Laboratories.

Biochemistry

Most reagents, including 2-phosphoglycolate (tri(monocyclohexylammonium) salt) (2-PG), 3-phosphoglycerate (sodium salt) (3-PGA), and ribulose 1,5-bisphosphate (sodium salt hydrate) (RuBP), and enzymes for synthesis of D-[1-3H]ribulose 1,5-bisphosphate ([1-3H]RuBP), which are hexokinase, glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, phosphoribulokinase and pyruvate kinase, were purchased from Sigma-Aldrich. NaH\(^{14}\)CO\(_3\) was from ViTrax. D-[2-3H]glucose was from Amersham Bioscience.
STRAINS AND CULTURE CONDITIONS

Chlamydomonas reinhardtii 2137 mt+ was used as the wild-type strain (Spreitzer and Mets, 1981) and, in most cases, MX3312 mt+ was used as the host for chloroplast transformation with mutant rbcL genes (Satagopan and Spreitzer, 2004; Zhu et al., 2005). Chlamydomonas MX3312 has the chloroplast 1428-bp rbcL gene replaced by the 786-bp bacterial aadA gene, which confers spectinomycin resistance (Hollingshead and Vapnek, 1985). Except for the photosynthesis-deficient, acetate-requiring phenotype, the MX3312 strain is indistinguishable from wild type because the rbcL gene knock-out was created in Chlamydomonas 2137 mt+ through homologous recombination, preserving the rbcL 5' and 3' flanking sequences (Satagopan and Spreitzer, 2004; Zhu et al., 2005). For co-expression of engineered large subunits with the small-subunit βA-βB loop from spinach (Spinacia oleracea), the rbcLΔ/ABSO transformation host was used, which was created by replacing the rbcL gene of Chlamydomonas penta/ABSO with the aadA gene (Spreitzer et al., 2005; Genkov and Spreitzer, unpublished). For co-expression of mutant large subunits with the entire small subunit from Arabidopsis, the rbcLΔ/SSAT transformation host was used, which was created by replacing the rbcL gene with the aadA gene in a cell-walled SSAT strain (Genkov et al., 2010; Genkov and Spreitzer, unpublished). All strains were maintained in the dark at 25°C on medium containing 10 mM acetate solidified with 1.5% Difco Bacto-Agar (Spreitzer and Mets, 1981).

Electrocompetent Escherichia coli XL-1 Blue was used for propagating plasmid DNA (Stratagene). XL-1 Blue was prepared for electrocompetence in Biorad’s Gene Pulser Xcell system (Miller and Nickoloff, 1995). Briefly, a starter culture of cells was grown overnight at 37°C in 5 mL of LB medium with 10 µg/mL tetracycline, shaking at
280 RPM. The starter culture was then poured into 500 mL of LB medium, and the new culture was grown until the OD was between 0.4-0.7, which took about 4 hr. The cells were pelleted and washed twice with 10% ice-cold glycerol, and finally resuspended in 2 mL of 10% ice-cold glycerol. Aliquots of cells (50 µL) were stored at -80°C for at least 6 months.

MOLECULAR-BIOLOGY METHODS

Site-directed mutagenesis and mutant-plasmid construction

Site-directed mutagenesis was performed using the Quickchange Mutagenesis Kit (Papworth et al., 1996). A 25-µL PCR reaction mix was made consisting of 1.25 U Pfu Turbo DNA Polymerase, 1X Pfu Turbo buffer (20 mM Tris-HCl, pH 8.8, 10 mM KCl, 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 1.0% Triton X-100 and 1 mg/mL BSA), 25 ng of template DNA, 62.5 ng each of a pair of complementary 30-40 bp primers, which contain the desired base changes, and 0.4 mM of each dNTP. The mix was heated to 95°C for 30 sec, followed by 18 cycles of 95°C for 30 sec, 55°C for 1 min, and 68°C for 6 min. Then, 5 U of restriction-endonuclease DpnI was added to the PCR mix and incubated at 37°C for 1 hr to fully digest the template DNA. Finally, 1 µL of the reaction mix was electroporated into E. coli XL-1 Blue, and transformants were selected on LB medium containing 100 µg/mL ampicillin at 37°C overnight. E. coli colonies were inoculated into 7 mL of liquid LB medium for DNA miniprep using the Qiagen kit, which is based on the alkaline lysis procedure (Birnboim and Doly, 1979), and mutant plasmids were screened by restriction-enzyme digestion.

The template DNA used for site-directed mutagenesis is the pLS-H plasmid,
which consists of a 2670-bp *Hpa*I fragment of *Chlamydomonas* chloroplast DNA, including the Rubisco *rbcL* gene, cloned into the *Sma*I site of the pUC19 plasmid (Yanisch-Perron *et al*., 1985; Du and Spreitzer, 2000). Codon changes were strictly limited to only those codons commonly used in *rbcL*, and, when possible, introduced or removed a restriction site for ease of mutant-plasmid screening. In certain cases, a silent mutation was introduced at a second site, to alter the restriction pattern for mutant screening. Mutations were combined by restriction-enzyme digestion and ligation.

**Chloroplast transformation**

Chloroplast transformation was performed using a particle-inflow gun (Finer *et al*., 1992; Zhu and Spreitzer, 1994, 1996). Strains MX3312, *rbcLΔ/ABSO*, or *rbcLΔ/SSAT* were grown in 50 mL of liquid acetate medium in the dark at 25°C on a rotary shaker at 220 RPM until they reached the late-log phase of growth (~2.5 X 10^6 cells/mL). The cells were pelleted, resuspended at a concentration of 2.5 X 10^8 cells/mL in liquid minimal medium (without acetate), and 0.5 X 10^7 cells were plated on solid acetate medium. When the plates were dry, the cells were bombarded with DNA-coated tungsten. The tungsten particles were coated with DNA by mixing 2.5 µg of plasmid, 25 µL of tungsten (60 mg/mL in H_2O), 25 µL of 2.5 M CaCl_2 and 10 µL of 0.1 M spermidine (free base). The tungsten-DNA suspension was allowed to sedimt at room temperature for 30 min. Forty microliters of the supernatant was removed, and the tungsten-DNA sediment was resuspended in the remaining liquid and loaded for bombardment, which was performed at a vacuum of 28 inches Hg and helium pressure of 70 PSI (Boynton *et al*., 1988; Boynton and Gillham, 1993; Finer *et al*., 1992).
To select for photosynthetic *Chlamydomonas* transformants, cells were scraped off the plate and replated on six minimal-medium plates, which were incubated at 25°C under 80 microeinsteins/m\(^2\)/s fluorescent lamps. The *Chlamydomonas* SSAT strain required 5% CO\(_2\) for growth. For the other *Chlamydomonas* strains, incubation at 5% CO\(_2\) reduced transformation time to 6 days, from 2-4 weeks, before colonies were visible. Transformant colonies were picked and maintained on acetate medium in darkness. Because there are multiple copies of the chloroplast genome, all transformants were cloned to homoplasmicity by plating on acetate medium to obtain single colonies and then by replica-plating on minimal medium to screen for photosynthetic growth. Cells were usually homoplasmic after three cloning cycles, which was confirmed by PCR.

**DNA extraction from *Chlamydomonas***

Extraction of DNA from *Chlamydomonas* was carried out according to a previously-established protocol with slight modifications (Newman *et al.*, 1990). Briefly, a whole 100-mm acetate-medium plate of fresh *Chlamydomonas* cells, which had been growing for less than a week, was scraped and resuspended in 0.5 mL of 150 mM NaCl, 10 mM EDTA and 10 mM Tris-HCl, pH 8.0. Cells were pelleted by spinning at 14,000 RPM for 10 sec, and the supernatant was discarded. The pelleted cells were then completely lysed by vortexing in 0.45 mL of 1.3% SDS, 250 mM NaCl, 25 mM EDTA, and 66 mM Tris-HCl, pH 8.0. The cell lysate was phenol/chloroform extracted with 350 µL of 1:1 phenol:chloroform three times, keeping the aqueous layer each time. DNA from the final clear, aqueous solution was precipitated with 800 µL of 100% ethanol at -20°C overnight, washed with 200 µL of 70% ethanol, and dissolved in 40 µL of H\(_2\)O.
PCR and sequencing of the *Chlamydomonas rbcL* gene

PCR was carried out in a 100-µL reaction mix consisting of 5 µL of genomic DNA, 2.5 U *Taq* DNA polymerase, 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 0.2 mM of each dNTP, 3 mM of MgCl₂, and 0.5 µM each of a pair of primers flanking the *rbcL* gene (5'-GTAAGACGACCGACATATACCTAAAGGCC-3' and 5'-CGCACTCTACCGATTGAGTTACATCCGC-3'). The PCR steps were 94°C initial denaturation for 3 min, followed by 30 cycles of 94°C for 1 min, 56°C for 2 min, and 72°C for 2 min. A final 72°C extension was performed for 10 min. The 1907-bp PCR product was run on a 1% agarose TAE gel, purified with a Qiagen Gel Extraction kit, and sequenced by Eurofins MWG Operon or the University of Nebraska DNA sequencing facility.

BIOCHEMICAL-ANALYSIS METHODS

Protein extraction and Rubisco purification from *Chlamydomonas* (Spreitzer and Chastain, 1987)

*Chlamydomonas* for protein extraction was grown in 250-500 mL of acetate medium in the dark at 25°C on a rotary shaker at 220 RPM until late-log phase of growth (~2.5 X 10⁶ cells/ml). Cells were pelleted by centrifuging at 1,500 g at 4°C, washed once with 4°C H₂O, pelleted again, resuspended in 1.5 mL of ice-cold extraction buffer (2 mM DTT, 50 mM Bicine-NaOH, pH 8.0, 10 mM MgCl₂, 10 mM NaHCO₃), and sonicated for 3 min in an ice bath with 30-sec pulses. Sonicated cells were centrifuged at 30,000 g for 15 min at 4°C to sediment the cell debris, and the supernatant containing total soluble protein was transferred to a new 4°C pre-chilled microfuge tube and kept on ice. Protein
concentration was determined with the Coomassie Brilliant Blue binding assay with BSA as the standard (Bradford, 1976). Proteins were kept at -80°C.

To further purify Rubisco from the soluble cell protein, 1 mL of the protein solution was separated in a linear 10% to 30% 12-mL sucrose gradient (2 mM DTT, 50 mM Bicine-NaOH, pH 8.0, 10 mM MgCl₂, 10 mM NaHCO₃) at 37,000 RPM for 20 hr at 4°C using a SW40 Ti swinging-bucket rotor (Beckman Coulter). The protein gradient was fractionated with a Model 185 density-gradient fractionator (ISCO, Inc.) while scanning at 280 nm with a UA-5 absorbance/fluorescence monitor (ISCO, Inc.). The hexadecameric-Rubisco fraction, which forms a distinct peak, was collected. Rubisco was dialyzed overnight at 4°C in 2 mM DTT, 50 mM Bicine-NaOH, pH 8.0, 10 mM MgCl₂, and 10 mM NaHCO₃ (or 2 mM NaHCO₃ for enzyme assays), to remove the sucrose, and re-concentrated to ~100 µL using a Centricon YM-100 column (Amicon). Purified Rubisco was used directly in enzyme assays or stored at -20°C.

**Determination of Rubisco N₂/O₂ ratio**

The Rubisco N₂/O₂ ratio, is the ratio of the carboxylase activity in the absence of O₂ divided by the activity in the presence of O₂. The N₂/O₂ ratio is a measurement of the susceptibility of Rubisco carboxylation to inhibition by O₂ (Spreitzer and Chastain, 1987; Chen et al., 1988). Briefly, 20 µg of purified Rubisco in 10 µL of 1 mM DTT, 50 mM Bicine-NaOH, pH 8.0, 10 mM MgCl₂, and 2 mM NaHCO₃ was injected with a Hamilton syringe into a rubber-stopper-sealed 7-mL scintillation vial containing reaction buffer for a total of 0.5 mL of reaction mix (20 µg of purified Rubisco, 0.4 mM RuBP, 50 mM Bicine-NaOH, pH 8.0, 10 mM MgCl₂, 0.98 mM NaHCO₃). The reaction buffers were
gassed at 7 PSI for 15 min with either 100% N₂ (to create an environment without O₂) or 100% O₂, before adding NaHCO₃, which included trace amounts of radiolabeled NaH¹⁴CO₃ (7.2 Ci/mol). The reaction was allowed to proceed for 1 min in a 25°C water-bath, and then stopped with 400 µL of 3 M formic acid in methanol. The rubber stopper was removed from the scintillation vial, and the reaction mix was dried in a force-draft oven at 65°C overnight. Then, 250 µL of 0.25 M HCl was added to the dried product, and the acid-stable ¹⁴CO₂ was measured in a liquid scintillation counter (after addition of 5 mL of scintillation cocktail). A third reaction was also carried out to measure the specific activity of Rubisco, with a similar procedure, except that a saturating 12.4 mM NaHCO₃ concentration was used, and the reaction was pre-gassed with 100% N₂.

Determination of Rubisco specificity factor (Ω)

The Rubisco Ω assay is based on the simultaneous measurement of ¹⁴C DPM for carboxylation and ³H DPM for oxygenation of [¹-³H]RuBP (Jordan and Ogren, 1981a; Spreitzer et al., 1982). A reaction mix containing Rubisco, [¹-³H]RuBP, ¹⁴CO₂ from NaH¹⁴CO₃, and O₂ is incubated, and then the carboxylation product, [¹-¹⁴C]phosphoglycerate, and oxygenation product, [¹-³H]phosphoglycolate, are measured by liquid scintillation spectroscopy (Jordan and Ogren, 1981a).

Briefly, 10 µL (22 nmol, 0.34 µCi) of [¹-³H]RuBP was injected with a Hamilton syringe into a rubber-stopper-sealed 7-mL scintillation vial at 25°C containing reaction buffer for a total of 0.5 mL of reaction-mix (20 µg of purified Rubisco, 50 mM Bicine-NaOH pH 8.3, 10 mM MgCl₂, 2 mM of 5 Ci/mol NaH¹⁴CO₃). The reaction buffer was gassed with 100% O₂ at 7 PSI for 15 min before the addition of the NaH¹⁴CO₃ and
Rubisco. The reaction was allowed to proceed for 30 min in a 25°C water bath, and then stopped with 0.1 ml of 50 mM ZnSO₄ in 0.5 M HCl. The reaction mix was adjusted to pH 6.3 with 0.1 mL of 1.5 M sodium cacodylate, added with 0.1 mL of 0.2 M 3-PGA/0.5 mM 2-PG, 0.1 mL (0.25 units) of phosphoglycolate phosphatase, and incubated at 30°C for 30 min in a water bath to convert the [1-³H]phosphoglycolate into [1-³H]glycolate. The phosphatase reaction was stopped with the addition of 0.9 mL of 1 M formic acid, which brings the total reaction volume to 1.8 mL. Of the 1.8 mL reaction-mix, 0.75 mL was mixed with 0.1 mL of 1 M HCl, dried in a force-draft oven at 65°C overnight, redissolved in 350 µL H₂O, and the acid-stable ¹⁴CO₂ was measured by liquid scintillation spectroscopy. Another 0.75 mL of the 1.8-mL reaction mix was loaded onto a Dowex AG 1-X8 formate column (Biorad) that was pre-equilibrated with H₂O, and the flow-through was collected. The column was further eluted with 3 mL of 1 M formic acid, collecting a total of 3.75 mL of flow-through, which contained the uncharged non-phosphorylated [1-³H]glycolate. Two separate 1.5-mL aliquots of flow-through was lyophilized at -40°C overnight, re-dissolved in 250 µL of H₂O, and the ³H measured by liquid scintillation spectroscopy. Ω is simply calculated as the rate of carboxylation (v_c) per the rate of oxygenation (v_o) times the concentration of O₂ per CO₂ (Laing et al., 1974):

Ω = v_c/v_o x [O₂]/[CO₂]

where v_c/v_o is equivalent to the moles of CO₂ fixed per moles of phosphoglycolate formed, [O₂] is 1.15 mM, and [CO₂] is 30 µM (Jordan and Ogren, 1981a). However, even before the specificity-factor assay can be carried out, the [1-³H]RuBP had to be
synthesized and the phosphoglycolate phosphatase enzyme had to be partially purified (Christeller and Tolbert, 1978; Kuehn and Hsu, 1978; Jordan and Ogren, 1981a).

\[^{1-3}\text{H}]\text{RuBP}\) was previously synthesized by Dr. Todor N. Genkov from D-[2-\(^{3}\text{H}\)]glucose according to an established method (Kuehn and Hsu, 1978; Jordan and Ogren, 1981a). An 8-mL reaction mixture comprised of 40 mM Hepes-NaOH, pH 7.6, 5 mM MgCl\(_2\), 1 mM KCl, 2 mM ATP, 15 mM phosphoenol pyruvate, 15 mM NADP, 2 mM DTT, 0.2 mM EDTA, and 6 mM D-glucose was prepared at 0°C and adjusted to pH 7.6 with 1 M NaOH. Then, 1 mg of BSA, 100 U of pyruvate kinase, 150 U of glucose-6-phosphate dehydrogenase, 25 U of 6-phosphogluconate dehydrogenase, 25 U of phosphoribulokinase, and 1 mCi of D-[2-\(^{3}\text{H}\)]glucose were added. When the reaction mix returned to room temperature, the pH was adjusted to 7.6 with 1 M NaOH, and RuBP-synthesis was initiated by the addition of 325 U of hexokinase. During RuBP synthesis, which took about 45 min, the mixture was continuously stirred, and the pH was monitored and maintained at 7.6 with drop-wise additions of 1 M NaOH until there was no further change in pH, indicating the completion of the reaction. The reaction mix was adjusted to pH 3.5 using 3 M HCl, loaded onto a Dowex AG 1-X8 Cl\(^{-}\) column (200-400 mesh) that was pre-equilibrated with H\(_2\)O, and sequentially washed with 300 mL of H\(_2\)O, 300 mL of 10 mM LiCl/1 mM HCl, and 200 mL of 10 mM to 100 mM LiCl/1 mM HCl as a linear gradient. \[^{1-3}\text{H}]\text{RuBP}\) fractions, which were eluted with 300 mL of 100 mM to 350 mM LiCl/1 mM HCl as a linear gradient, were identified by liquid scintillation spectroscopy, concentrated to 20 mL by lyophilization at -40°C, and then adjusted to pH 6.5 with Ba(OH)\(_2\). To precipitate out the \[^{1-3}\text{H}]\text{RuBP}\), 1 mL of 1 M barium acetate and 20 mL of 100% ethanol were added. The \[^{1-3}\text{H}]\text{RuBP}\) was pelleted, resuspended in 1 mL
H₂O and mixed with 0.75 g AG 50W-X4 H⁺- resin, which had been prewashed with H₂O. The flow-through from the resin mix was collected, and the resin mix was further eluted with 5 mL H₂O, collecting the flow-through as well. The total flow-through, which contained [1⁻³H]RuBP, was adjusted to pH 6.5 with 1 M NaOH. The mole-amount of [1⁻³H]RuBP was determined by measuring the [¹⁴C]carboxylation product in a Rubisco reaction with unknown but limited amount of substrate [1⁻³H]RuBP, but excess amounts of Rubisco and NaH¹⁴CO₃. [1⁻³H]RuBP was stored at -20ºC.

The phosphoglycolate phosphatase enzyme was partially purified from tobacco leaves (Christeller and Tolbert, 1978; Jordan and Ogren, 1981a). Homogenate from 300 g of leaves ground in 1 L of Buffer A (20 mM sodium cacodylate pH 6.3, 2 mM ZnSO₄, 2 mM citrate), with 2% w/v polyvinylpolypyrrolidone added, was filtered through cheesecloth and centrifuged at 12,000 g for 20 min at 4ºC to pellet leaf debris. The supernatant containing the leaf extract was acetone-precipitated, and the precipitate between 25% to 40% v/v acetone was pelleted, air-dried, dissolved in 50 mL of Buffer A and loaded on a Bufffer A-equilibrated DEAE-cellulose column (2.5 X 30 cm). The column was then washed with 150 mL of Buffer A and the phosphoglycolate phosphatase was eluted with a linear gradient of 0 M to 0.4 M KCl in Buffer A. Eluate-fractions were assayed for phosphoglycolate phosphatase activity by adding 50 µL of eluate to 0.5 mL of Buffer A containing 2 µmol of MgCl₂ and 1 µmol of 2-phosphoglycolate, incubated at 30ºC for 5 min, and stopped with 0.2 mL of 10% w/v trichloroacetic acid. To determine the phosphatase activity, absorbance at 820 nm by free inorganic-phosphate released from 2-phosphoglycolate was measured and compared to a phosphate standard curve. Eluate fractions containing peak phosphatase activities were pooled and subjected to
ammonium-sulfate fractionation. The precipitate between 50% and 80% ammonium sulfate, which contained phosphoglycolate phosphatase, was pelleted, resuspended in 10 mL of Buffer A, and stored at -80°C. When needed for the Rubisco Ω assay, the required amount of phosphoglycolate phosphatase was adjusted to 2.5 U/mL with Buffer A.

**Determination of Rubisco kinetic-properties**

The kinetic properties of Rubisco were determined from measurements of Rubisco carboxylase activity at six different CO₂ concentrations with or without O₂ (Chen et al., 1988). V₀ was calculated from the Ω value (V_cK_o/V_oK_c) and the V_c, K_c, and K_o values (Laing et al., 1974; Chen et al., 1988). Purified Rubisco (10 µg) in 20 µL of buffer (1 mM DTT, 50 mM Bicine-NaOH, pH 8.0, 10 mM MgCl₂, 10 mM NaHCO₃) was injected with a Hamilton syringe into a rubber-stopper-sealed 7-mL scintillation vial containing reaction buffer for a total of 1 mL of reaction mix (10 µg Rubisco, 0.4 mM RuBP, 50 mM Bicine-NaOH, pH 8.0, 10 mM MgCl₂, and NaHCO₃ concentrations of 0.6 mM, 1.6 mM, 2.6 mM, 4.6 mM, 8.6 mM, or 16.6 mM). The reaction buffers had been gassed at 7 PSI for 15 min with either 100% N₂ (to create an environment without O₂) or 100% O₂ before the addition of the NaHCO₃, which included 25 µL of radiolabeled NaH¹⁴CO₃ (0.4 mCi/mL, 60 Ci/mol). The reaction was allowed to proceed for 1 min in a 25°C water bath, and then stopped with 1 mL of 3 M formic acid in methanol. The rubber stopper was removed from the scintillation vial, the reaction mix was dried in a force-draft oven at 65°C overnight, 350 µL of 0.25 M HCl was added to the dried product, and the remaining fixed, acid-stable ¹⁴CO₂ was measured by liquid scintillation spectroscopy. The V_c and K_c values were determined from the double-reciprocal plot of
1/[CO₂] (x-axis) to 1/vₐ (y-axis) for the 100% N₂-gassed O₂-free carboxylase reactions. Vₐ is the 1/x-intercept and Kₐ is the Vₐ times the slope. Kₒ is determined from the relation between [CO₂] and the ratio (R) of the carboxylase activity in the absence and presence of O₂ where R = 1 + Kₐ[O₂]/(KₐKₒ[KO₂]), which can be rearranged as 1/(R-1) = Kₒ[CO₂]/Kₐ[O₂] + Kₒ/[O₂]. (Chen et al., 1988). By plotting [CO₂] (x-axis) to 1/(R-1) (y-axis), Kₒ is simply the x-intercept times 1.15 mM, which is [O₂] for the 100% O₂-gassed reactions (Jordan and Ogren, 1981a; Chen et al., 1988).

**Determination of Rubisco thermostability**

Rubisco stability was determined with a thermostability assay (Chen et al., 1993). Purified Rubisco (5 µg) in 50 µL of buffer (1 mM DTT, 50 mM Bicine-NaOH, pH 8.0, 10 mM MgCl₂, 10 mM NaHCO₃) was incubated at 35°C, 45°C, 50°C, 55°C, 60°C, 65°C, or 70°C for 10 min, cooled on ice for 5 min, and then injected with a Hamilton syringe into a rubber-stopper-sealed 7-mL scintillation vial containing reaction buffer for a total of 0.5 mL of reaction-mix (5 µg of purified Rubisco, 0.4 mM RuBP, 50 mM Bicine-NaOH, pH 8.0, 10 mM MgCl₂, 10 mM of 2 Ci/mol NaH¹⁴CO₃). The reaction was allowed to proceed for 1 min in a 25°C water bath, and then stopped with 0.5 mL of 3 M formic acid in methanol. The rubber stopper was removed from the scintillation vial, the reaction mix was dried in a force-draft oven at 65°C overnight, 350 µL of 0.25 M HCl was added to the dried product, and the remaining fixed, acid-stable ¹⁴CO₂ measured by liquid scintillation spectroscopy.
SDS-PAGE of *Chlamydomonas* total soluble proteins

Total soluble proteins from sonicated *Chlamydomonas* cells were separated with a linear-gradient SDS-PAGE method that gives high resolution of chloroplast stromal proteins (Chua, 1980). A 14 x 16 x 0.15-cm gel was poured consisting of 28 mL of resolving linear-gradient gel of 7.5% to 15% w/v polyacrylamide/N,N'-methylene-bis-acrylamide (37.5:1), 5% to 17% w/v sucrose, 85 mM Tris-HCl, pH 9.18, and 0.1% w/v SDS. The resolving gel was then overlaid with 6 mL of stacking gel of 6% w/v acrylamide/N,N'-methylene-bis-acrylamide (37.5:1), 13.5 mM Tris-H$_2$SO$_4$, pH 6.1, and 0.1% w/v SDS. The lower reservoir buffer was 85 mM Tris-HCl, pH 9.18, and 0.1% w/v SDS whereas the upper reservoir buffer was 2 mM Tris-borate, pH 8.64. Each well of the gel was loaded with 60 µg of *Chlamydomonas* protein, which had been mixed with sample loading buffer (5% w/v SDS, 30% w/v sucrose, 0.05% w/v bromophenol blue, 100 mM DTT) at a 3:2 ratio and boiled for 3 min. Electrophoresis was carried out at 25°C, and initially set to 15 mA per gel, but turned up to 30 mA per gel after the dye front had passed the stacking/resolving gel interface. Electrophoresis was stopped as soon as the dye front moved off the end of the gel. The gel was stained overnight with 0.25% w/v Coomassie Brilliant Blue R, 50% v/v methanol, and 7% v/v acetic acid (Chua, 1980). Destaining was then carried out with a solution of 40% v/v methanol and 7% v/v acetic acid until bands were visible. The gel was then further destained and stored in 10% acetic acid.

Western blotting of Rubisco

SDS-PAGE gels were run in duplicate, and one was used for western blotting
(Towbin et al., 1979). Proteins from the gel were transferred to a nitrocellulose membrane at 15 V for 12 hr at 4°C in blotting buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol). Then, the membrane was blocked with 3% w/v gelatin in TBS buffer (500 mM NaCl, 20 mM Tris-HCl, pH 7.4) for 1 hr at room temperature. The membrane was probed with rabbit anti-Chlamydomonas Rubisco large (0.76 µg) and small-subunit IgGs (0.68 µg) (Karkehabadi et al., 2005) in 50 mL of 1% w/v gelatin in TBS buffer for 7 hr. It was then washed with TTBS buffer (0.05% v/v Tween 20 in TBS buffer), incubated with 12.5 µL of goat anti-rabbit IgG/horseradish peroxidase conjugate (Biorad) in 1% w/v gelatin in 50 mL TBS buffer for 2 hr, washed with TTBS buffer, washed with TBS buffer, and visualized on an x-ray film using horseradish peroxidase chemiluminescence (National Diagnostics).
RESULTS AND DISCUSSION

PHYLOGENETIC GROUPS

Defining phylogenetic groups

Protein-sequence alignment of the Rubisco large subunit of *Chlamydomonas* with 500 flowering-plant large subunits showed that there are 34 "phylogenetic residues" that differ between *Chlamydomonas* and plants (Du *et al.*, 2003). Phylogenetic residues are those that differ between *Chlamydomonas* Rubisco and over 95% of the 500 flowering-plant sequences (Du *et al.*, 2003). These phylogenetic residues may account for the differences in kinetic properties between algae and plants, and are potential targets for engineering. A direct approach to determine the phylogenetic-residue combinations that can influence Rubisco catalysis would be to change the *Chlamydomonas* residues to plant residues, and then assay the mutant enzymes for changes in kinetic properties. However, site-directed mutagenesis of all possible combinations of the 34 phylogenetic residues would involve creating $2^{34}$ mutant enzymes, which is over 17 billion mutant strains (Du *et al.*, 2003).

Instead, to simplify the problem, phylogenetic residues can be first clustered into "phylogenetic groups" based on the closeness of the residues in the highest-resolution, 1.4-Å x-ray crystal structure of *Chlamydomonas* Rubisco (Table 2 and Fig. 4) (Taylor *et al.*, 2001; Du *et al.*, 2003). Specifically, a phylogenetic group consists of phylogenetic residues within 5 Å of each other (Du *et al.*, 2003). Each phylogenetic group defines a structural region, and directed mutagenesis of each group as a whole would determine whether a structural region influences catalysis. Single-residue changes are often not
Table 2: Phylogenetic groups in the catalytic large subunit of *Chlamydomonas* Rubisco (Du et al., 2003).

<table>
<thead>
<tr>
<th>Group number $^a$</th>
<th>Group notation $^b$</th>
<th>Residue substitutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11</td>
<td>A11V</td>
</tr>
<tr>
<td>2</td>
<td>30-32</td>
<td>V30E, V31T, R32K</td>
</tr>
<tr>
<td>--</td>
<td>42-53</td>
<td>M42V, C53A</td>
</tr>
<tr>
<td>3</td>
<td>86</td>
<td>D86H</td>
</tr>
<tr>
<td>4</td>
<td>105-369 $^c$</td>
<td>I105L, C369V $^c$</td>
</tr>
<tr>
<td>5</td>
<td>149-282</td>
<td>V149Q, I282H</td>
</tr>
<tr>
<td>6</td>
<td>168-399</td>
<td>G168P, L326I, M349L, M375L, A398S, C399V</td>
</tr>
<tr>
<td>7</td>
<td>221</td>
<td>V221C</td>
</tr>
<tr>
<td>--</td>
<td>235</td>
<td>V235I</td>
</tr>
<tr>
<td>--</td>
<td>256-258</td>
<td>C256F, K258R</td>
</tr>
<tr>
<td>--</td>
<td>265</td>
<td>I265V</td>
</tr>
<tr>
<td>8</td>
<td>305-474</td>
<td>R305K, D470E, T471A, I472M, K474T</td>
</tr>
<tr>
<td>--</td>
<td>341</td>
<td>V341I</td>
</tr>
<tr>
<td>9</td>
<td>391-428</td>
<td>V391T, T428V</td>
</tr>
<tr>
<td>10</td>
<td>442-447</td>
<td>G442N, D443E, V444I, S447E</td>
</tr>
</tbody>
</table>

$^a$ Group number, which is based on the order of the phylogenetic groups, will be used for labeling of western-blot and phenotype-data figures where space is limited. Mutant phylogenetic groups that were previously created and analyzed are designated "--" (Zhu and Spreitzer, 1996; Du et al., 2003; Spreitzer et al., 2005).

$^b$ Group notation, which is based on the first and last residue substituted in a phylogenetic group, is the favored method for referring to specific phylogenetic groups throughout the text.

$^c$ The phylogenetic-residues Ile-105 and Cys-369 are from neighboring large-subunits.
Figure 4: Distribution of the 34 phylogenetic residues, which cluster into 15 groups, in the large subunit of *Chlamydomonas* Rubisco (PDB 1GK8) (Taylor et al., 2001).

Each color represents a phylogenetic group. Only one large subunit is shown with the backbone represented as white ribbon. At the left is the N-terminal domain. At the right is the C-terminal α/β-barrel active-site domain with the bound transition-state analog CABP shown as black sticks. Residues Ile-105 and Cys-369 are in contact between two large subunits. The βA-βB loops from two different small subunits are shown as yellow and orange ribbons at the bottom right of the figure. These loops vary in size among species, and interact with phylogenetic groups 256-258 and 235 at the bottom of the α/β barrel (Spreitzer et al., 2005).
sufficient to cause a noticeable change in catalysis, but residue-group changes have
greater effects on structure (Zhu and Spreitzer, 1996; Du et al., 2003; Spreitzer et al.,
2005; Satagopan and Spreitzer, 2008).

The objective of the first phase of this study was to determine which phylogenetic
groups contribute to the differences in kinetic properties between *Chlamydomonas* and
plant Rubisco enzymes, and possibly determine their other roles in Rubisco structure and
function. Some of the groups have only one residue (i.e., groups 11, 86, 221, 235, 265
and 341) (Table 2 and Fig. 4). Five of the phylogenetic groups (i.e., groups 42-53, 235,
256-258, 265 and 341) were studied previously, but did not result in any significant
changes in catalysis (Zhu and Spreitzer, 1996; Du et al., 2003; Spreitzer et al., 2005).
Thus, only ten remaining phylogenetic-group mutants needed to be created and analyzed
in this study.

Recovery of phylogenetic-group mutants and their phenotypes

*Chlamydomonas* phylogenetic-group mutants were recovered by chloroplast
transformation of the MX3312 *rbcL*-knockout strain (Satagopan and Spreitzer, 2004,
2008; Zhu et al., 2005) followed by photosynthetic selection. All ten phylogenetic-group
mutant strains could grow photoautotrophically, and the transformation frequencies with
the mutant genes were not significantly different from that of wild type *rbcL* (1 X 10⁻⁶ per
cells shot). Thus, none of the mutations affected essential functions of Rubisco (Fig. 5).
Moreover, similar to wild-type *rbcL* transformation, all the phylogenetic-group mutant
transformants started appearing as photosynthetic colonies on minimal medium within 2
to 4 weeks.
Figure 5: Growth phenotypes of *Chlamydomonas* wild type (WT) and phylogenetic-group mutants. About $2 \times 10^5$ cells were spotted at each position, and grown for one week with the conditions indicated (Spreitzer and Mets, 1981). Spot numbering is based on Table 2.
Comparison of the growth phenotypes of the phylogenetic-group mutants (Spreitzer and Mets, 1981) indicated that only the 442-447 phylogenetic-group mutant (G442N/D443E/V444I/S447E) had reduced photosynthetic growth on minimal medium in the light (spot 10, Fig. 5). When grown heterotrophically on acetate medium in the dark, the 442-447 mutant-Rubisco strain was indistinguishable from wild type indicating that the reduction in growth on minimal medium must result from a decrease in photosynthesis.

**Rubisco holoenzyme level**

To determine the effects of the mutations on the amount of Rubisco *in vivo*, which would depend on holoenzyme expression, stability, or assembly, cell extracts were subjected to SDS-PAGE and western blotting (Fig. 6). The amount of assembled Rubisco *in vivo* is directly related to the observed subunit levels *in vitro* because free small subunits are rapidly degraded *in vivo*, and expression of large subunits is blocked at translation in the absence of small subunits (Spreitzer *et al.*, 1985; Khrebtukova and Spreitzer, 1996). Almost all the phylogenetic-group mutant strains expressed equal amounts of Rubisco when compared with wild type except for the 442-447 mutant strain, which had reduced *in vivo* holoenzyme level as evidenced by the western blot (lane 10, Fig. 6). Thus, the decreased holoenzyme level of the 442-447 mutant strain could account for its decreased photosynthetic growth (spot 10, Fig. 5).

A thermal-inactivation experiment (Chen *et al.*, 1993) was performed with the 442-447 mutant Rubisco to determine whether the enzyme was unstable. As shown in Fig. 7, the wild-type and 442-447 mutant enzymes have similar thermal inactivation.
Figure 6: SDS-PAGE (left) and western-blot (right) analysis of total soluble protein from *Chlamydomonas* wild type (WT) and phylogenetic-group mutants (lanes 1 through 10). Sixty micrograms of total soluble protein from sonicated dark-grown cells were run in each lane. Mutant numbering is based on Table 2. LS denotes the Rubisco large subunit, and SS denotes the small subunit.
Figure 7: Thermal inactivation of Rubisco purified from *Chlamydomonas* wild type (WT) and the 442-447 phylogenetic-group mutant. Five micrograms of Rubisco were pre-incubated at the indicated temperatures for 10 min, and then cooled on ice for 5 min, before assaying carboxylase activity at 25°C for 1 min. The measured activity was normalized to that of the 35°C pre-incubation. The specific activity after the 35°C pre-incubation was 1.1 µmol CO₂ fixed/min/mg protein for wild-type Rubisco, and 0.9 µmol/min/mg for the mutant enzyme.
Therefore, the phylogenetic substitutions G442N/D443E/V444I/S447E in the 442-447 mutant do not affect stability in vitro, but may affect structure leading to degradation in vivo.

**Kinetic properties**

Despite the reduced holoenzyme level of the 442-447 phylogenetic-group mutant, sufficient amounts of Rubisco could be purified from all the mutants for kinetic analysis. An initial N$_2$/O$_2$-ratio assay (Spreitzer and Chastain, 1987) was performed with all the phylogenetic-group mutant enzymes to determine whether the phylogenetic substitutions affected the O$_2$ sensitivity of Rubisco (Ratio A/B, Table 3). Carboxylase specific activities were measured at saturating CO$_2$ (12.4 mM NaHCO$_3$, Table 3), and the Ω values were also measured (Table 3).

Only the 168-399, 305-474 and 442-447 phylogenetic-group mutant enzymes have altered O$_2$-sensitivity in comparison with wild type Rubisco, which has an N$_2$/O$_2$ ratio of 2.4 (Table 3). The rest of the phylogenetic mutants have ratios within ±10% of the wild-type value. The 168-399 and 442-447 mutants have increases in the N$_2$/O$_2$ ratio to 3.2 and 2.9, respectively, which indicates that the phylogenetic substitution G168P/L326I/M349L/M375L/A398S/C399V in phylogenetic-group 168-399, close to catalytic loop-6, increased the sensitivity of Rubisco to O$_2$, causing the carboxylase reaction to be more prone to O$_2$ inhibition (Table 3). The phylogenetic substitution G442N/D443E/V444I/S447E in phylogenetic-group 442-447 also caused a similar but less pronounced increase in O$_2$ inhibition. On the other hand, phylogenetic-group 305-474, consisting of R305K/D470E/T471A/I472M/K474T substitutions at the carboxy
Table 3: Ω values, specific activities, and oxygen inhibition of Rubisco purified from *Chlamydomonas* wild type and phylogenetic-group mutants. Values are the means ±S.D. (n - 1) of three separate enzyme preparations.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Ω</th>
<th>V&lt;sub&gt;c&lt;/sub&gt;</th>
<th>V&lt;sub&gt;c&lt;/sub&gt;</th>
<th>100% N&lt;sub&gt;2&lt;/sub&gt;</th>
<th>100% N&lt;sub&gt;2&lt;/sub&gt;</th>
<th>100% O&lt;sub&gt;2&lt;/sub&gt;</th>
<th>Ratio (A/B)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>V&lt;sub&gt;c&lt;/sub&gt;/V&lt;sub&gt;c&lt;/sub&gt;</td>
<td>NaHCO&lt;sub&gt;3&lt;/sub&gt;</td>
<td>0.98 mM NaHCO&lt;sub&gt;3&lt;/sub&gt;</td>
<td>0.98 mM NaHCO&lt;sub&gt;3&lt;/sub&gt;</td>
<td>0.98 mM NaHCO&lt;sub&gt;3&lt;/sub&gt;</td>
<td>A/B</td>
</tr>
<tr>
<td>Wild type</td>
<td></td>
<td>59 ± 1</td>
<td>101 ± 2</td>
<td>28.6 ± 0.4</td>
<td>11.8 ± 1.0</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td></td>
<td>62 ± 3</td>
<td>115 ± 5</td>
<td>33.6 ± 0.4</td>
<td>13.7 ± 0.8</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>30-32</td>
<td></td>
<td>61 ± 3</td>
<td>107 ± 9</td>
<td>31.1 ± 0.4</td>
<td>13.0 ± 0.3</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td>86</td>
<td></td>
<td>58 ± 2</td>
<td>92 ± 5</td>
<td>22.8 ± 1.3</td>
<td>8.7 ± 1.2</td>
<td>2.6</td>
<td></td>
</tr>
<tr>
<td>105-369</td>
<td></td>
<td>63 ± 2</td>
<td>115 ± 16</td>
<td>35.5 ± 1.0</td>
<td>13.8 ± 0.3</td>
<td>2.6</td>
<td></td>
</tr>
<tr>
<td>149-282</td>
<td></td>
<td>61 ± 1</td>
<td>122 ± 7</td>
<td>33.7 ± 2.9</td>
<td>13.3 ± 1.6</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>168-399</td>
<td></td>
<td>56 ± 2</td>
<td>46 ± 4</td>
<td>10.7 ± 1.1</td>
<td>3.3 ± 0.3</td>
<td>3.2</td>
<td></td>
</tr>
<tr>
<td>221</td>
<td></td>
<td>59 ± 1</td>
<td>116 ± 10</td>
<td>32.3 ± 2.0</td>
<td>14.6 ± 0.8</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td>305-474</td>
<td></td>
<td>61 ± 2</td>
<td>99 ± 16</td>
<td>21.7 ± 0.8</td>
<td>11.5 ± 0.4</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td>391-428</td>
<td></td>
<td>62 ± 3</td>
<td>57 ± 9</td>
<td>18.1 ± 1.1</td>
<td>8.3 ± 0.2</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td>442-447</td>
<td></td>
<td>61 ± 4</td>
<td>98 ± 11</td>
<td>28.5 ± 2.9</td>
<td>9.9 ± 1.2</td>
<td>2.9</td>
<td></td>
</tr>
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</table>
terminus of the Rubisco large subunit, decreased the sensitivity of Rubisco to O$_2$, evidenced by the reduction of the N$_2$/O$_2$ ratio to 1.9 (Table 3). Of the three phylogenetic mutants 168-399, 305-474 and 442-447, only the 168-399 mutant has a decrease in specific carboxylase activity, which is over 50% (Table 3).

Because of the decrease in specific activity and increase in oxygen sensitivity, the 168-399 mutant enzyme has the lowest Ω value among the phylogenetic-group mutants, with Ω = 59 for the wild-type enzyme, and Ω = 56 for the 168-399 mutant enzyme (Table 3). On the other hand, it was surprising that none of the phylogenetic substitutions increases Ω by at least 10% considering that plant Rubisco enzymes have Ω values at least 15% higher than that of *Chlamydomonas* Rubisco (Jordan and Ogren, 1981b; Genkov *et al.*, 2010). Perhaps further combinations of phylogenetic-group substitutions are required to achieve the shift in catalytic properties observed between *Chlamydomonas* and plant Rubisco enzymes.

Detailed kinetic analysis was performed on the 168-399, 305-474 and 442-447 phylogenetic-group mutant Rubisco enzymes to determine the specific changes in kinetic constants (Table 4). For the 168-399 and 442-447 mutants, which have increases in O$_2$ inhibition, the $K_o$ values are decreased to 386 and 352 µM O$_2$, respectively, compared with 458 µM O$_2$ for the wild-type enzyme (Table 4).

For the 305-474 mutant, which has a decrease in O$_2$-inhibition, $K_o$ is increased to 637 µM O$_2$ (Table 4). However, the beneficial decrease in O$_2$ sensitivity of the 305-474 mutant is offset by an increase in $K_c$ to 58 µM CO$_2$, compared to 35 µM CO$_2$ for the wild-type enzyme (Table 4). In addition, for the 168-399 mutant, $V_c$ is reduced to 65 µmol CO$_2$ fixed/hr/mg protein, from 105 µmol/hr/mg for the wild-type enzyme, which,
Table 4: Kinetic properties of Rubisco purified from *Chlamydomonas* wild type and three phylogenetic-group mutants with altered oxygen inhibition.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$\Omega^{a,b}$</th>
<th>$V_c^{b}$</th>
<th>$K_c^{b}$</th>
<th>$K_o^{b}$</th>
<th>$V_c/K_c^{c}$</th>
<th>$K_o/K_c^{c}$</th>
<th>$V_c/V_o^{c}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\mu$mol/hr/mg</td>
<td>$\mu$M CO$_2$</td>
<td>$\mu$M O$_2$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>59 ± 1</td>
<td>105 ± 2</td>
<td>35 ± 1</td>
<td>458 ± 3</td>
<td>3.0</td>
<td>13</td>
<td>4.5</td>
</tr>
<tr>
<td>168-399</td>
<td>56 ± 2</td>
<td>65 ± 11</td>
<td>36 ± 3</td>
<td>386 ± 29</td>
<td>1.8</td>
<td>11</td>
<td>5.1</td>
</tr>
<tr>
<td>305-474</td>
<td>61 ± 2</td>
<td>96 ± 10</td>
<td>58 ± 7</td>
<td>637 ± 7</td>
<td>1.7</td>
<td>11</td>
<td>5.5</td>
</tr>
<tr>
<td>442-447</td>
<td>61 ± 4</td>
<td>82 ± 8</td>
<td>34 ± 4</td>
<td>352 ± 32</td>
<td>2.4</td>
<td>10</td>
<td>6.1</td>
</tr>
</tbody>
</table>

$a$ Values are from Table 3.

$b$ Values are the means ± S.D. ($n$ - 1) of three separate enzyme preparations.

$c$ Calculated values.
together with the reduced $K_0$, contributes to a lower $\Omega$ (Table 4).

**Structural analysis of the phylogenetic-group mutant enzymes**

The decrease in $\Omega$ caused by the 168-399 phylogenetic-group substitutions (G168P/L326I/M349L/M375L/A398S/C399V) (Table 4), at the base and flank of catalytic loop 6 (Fig. 8), is reminiscent of the decreased $\Omega$ previously observed for an L326I/M349L mutant enzyme (Zhu and Spreitzer, 1996). The side chains of Leu-326 and Met-349 are in van der Waals contact, and, because the two residues are located in $\beta$-strand 6 and $\alpha$-helix 6, respectively (Fig. 8), substituting the two residues could directly affect the flexibility of the loop, and affect the stability of the interactions between Lys-334 and the gaseous substrates (Gutteridge *et al.*, 1993; Zhu and Spreitzer, 1996). Besides that, changes of Leu-326 could also affect the adjacent active-site residue His-327 (Fig. 8). Mutations of His-327 have been previously found to weaken the binding of the CABP carboxylation transition-state analog to Rubisco, which would decrease $\Omega$ (Harpel *et al.*, 1991).

Because the base-of-loop-6 phylogenetic-group 168-399 mutant enzyme, which has four substitutions G168P/M375L/A398S/C399V additional to the previous L326I/M349L, does not have $\Omega$ restored to the wild-type value or increased to plant values (Table 4), it must be concluded that these additional phylogenetic-residue changes are not sufficient to complement the L326I/M349L substitutions in terms of $\Omega$ (Zhu and Spreitzer, 1996). The four additional phylogenetic-residue substitutions are at the amino-terminal ends of the $\beta$ strands forming the $\beta$-sheet wall of the active-site barrel (Fig. 8). In fact, as part of the network of hydrogen bonds keeping the $\beta$-sheet intact, there is a
Figure 8: Structural comparison of residues in the 168-399 phylogenetic group (red) at the base of catalytic loop 6 between *Chlamydomonas* (left, PDB 1GK8) and spinach Rubisco (right, PDB 8RUC). Except for Leu-326 and Met-349, which flank loop 6, the other phylogenetic residues are at the N-terminal ends of β-strands β1, β8, and β7, which are also part of the α/β-barrel active-site domain. The CABP carboxylation transition-state analog denotes the active site with the side chain of Lys-334 stabilizing the partial negative charge on the CO₂ (or O₂) moiety that is represented by a carboxylate side group of CABP. His-327 at the C-terminal end of β6, adjacent to Leu-326, is another active-site residue that can influence Ω (Harpel *et al.*, 1991).
hydrogen bond between the backbone carbonyl oxygen of Met-375 and the amide nitrogen of Leu-326, suggesting that there could be some other complementary effects between the phylogenetic residues unrelated to $\Omega$.

As for the phylogenetic-group 305-474 substitutions (R305K/D470E/T471A/I472M/K474T) at the carboxy terminus, the increase in $K_o$ (Table 4) is reminiscent of the increased $K_o$ observed in a previous mutant Chlamydomonas Rubisco that had the carboxy terminus changed to that of spinach (D470P/T471A/I472M/K474T) (Satagopan and Spreitzer, 2008). Whereas the previous D470P/T471A/I472M/K474T mutant enzyme had a 10% increase in $\Omega$ (Satagopan and Spreitzer, 2008), the 305-474 phylogenetic-group mutant enzyme has no change in $\Omega$ despite an increase in $K_o$ because of a concomitant increase in $K_c$ (Table 4).

The 305-474 mutant enzyme (R305K/D470E/T471A/I472M/K474T) is different from the previous D470P/T471A/I472M/K474T enzyme in that Asp-470 was replaced with Glu-470, found in 41% of plant-Rubisco sequences, as opposed to being replaced with Pro-470, found in only 18% of the plant sequences including spinach (Du et al., 2003). Also, an additional Arg-305-to-Lys substitution was included in the 305-474 mutant because Arg-305 in Chlamydomonas Rubisco is in van der Waals contact with Lys-474 (Fig. 9). It was previously suggested that the increase in $\Omega$ for D470P/T471A/I472M/K474T might be attributed to an increase in carboxy-terminal flexibility caused by the elimination of the salt bridge between Asp-470 and Lys-474 (Satagopan and Spreitzer, 2008). For the 305-474 mutant enzyme, even though the salt bridge between residue 470 and 474 is disrupted, the increased flexibility of the carboxy terminus could be offset by the formation of an ionic interaction between Thr-474 and
Figure 9: Structural comparison of residues in phylogenetic group 305-474 (green) at the carboxy-terminal tail between *Chlamydomonas* (top) and spinach Rubisco (bottom). The Asp-470-Lys-474 salt bridge present in *Chlamydomonas* is absent among plants. Instead, Lys-305 and Thr-474 form an ionic interaction in plants. Phe-467 packs against catalytic Lys-334, which interacts with the carboxylation transition-state analog CABP. Residues Glu-336 and Glu-338 in loop 6 interact with the 305-474 phylogenetic residues, and could be responsible for transmitting changes in 305-474 directly to the loop, especially to Glu-338, which has a different conformation between *Chlamydomonas* and spinach Rubisco.
Lys-305, which was an additional phylogenetic substitution in the 305-474 mutant (Fig. 9). Nonetheless, the conformational rigidity of the carboxy terminus could affect Phe-467, which packs against active-site Lys-334, thus transmitting changes in the carboxy terminus to the active-site (Fig. 9). Also, interactions between the 305-474 residues and Glu-336 and 338 directly affects catalytic loop 6 (Fig. 9).

As for 442-447 mutant Rubisco, the phylogenetic substitutions are mainly in α-helix G, on the surface of the large-subunit, over 20Å away from the active-site. Thus, it is difficult to map the network of interactions between the 442-447 phylogenetic group and the active site to account for the decreased $K_o$ of the mutant enzyme (Table 4). Nevertheless, the 442-447 phylogenetic group could play a greater role in holoenzyme assembly, which would account for the decreased growth and in vivo Rubisco level of the mutant strain (Figs. 5 and 6).

Even though phylogenetic groups 168-399, 305-474 and 442-447 do play a role in Rubisco catalysis, more extensive combinations of phylogenetic groups could be responsible for the shift in kinetic properties between *Chlamydomonas* and plant Rubisco.
ASSOCIATED GROUPS

Defining associated groups

Because individual phylogenetic-group substitutions in *Chlamydomonas* Rubisco do not produce plant Rubisco kinetics, combinations of the phylogenetic groups might be required (Tables 3 and 4). However, to create all possible combinations of the 15 phylogenetic groups, which is $2^{15}$ or over 32,000 combinations, would still be overwhelming. Instead, phylogenetic groups can be combined into "associated groups," which are each defined as a combination of a phylogenetic group and all other surrounding phylogenetic groups (Table 5). To be included in an associated-group, the surrounding phylogenetic groups have to be within interacting distance (5 Å) of a non-phylogenetic residue that also interacts with the center phylogenetic group (Table 5). It is not possible for two separate phylogenetic groups to interact with a common phylogenetic residue directly, because the two groups, including the bridging phylogenetic residue, would have been clustered as one phylogenetic group.

Because the previous penta/ABSO mutant had plant-like kinetic properties, the small-subunit βA-βB loop also influences catalysis (Spreitzer *et al*., 2005). Therefore, this loop was also considered in the associated-group analysis. The βA-βB loop is variable between *Chlamydomonas* and plant Rubisco enzymes (Spreitzer, 2003). The penta/ABSO mutant was created by combining five phylogenetic-residue substitutions and a chimeric Rubisco small subunit that has the βA-βB loop from spinach (Spreitzer *et al*., 2005). To test the possible role of the βA-βB loop in the associated groups, any associated-group mutants that have the center phylogenetic group in direct contact with the βA-βB loop was combined with the chimeric Rubisco small subunit that has the
Table 5: Associated groups in *Chlamydomonas* Rubisco. Phylogenetic groups are based on Table 2. Residue substitutions are listed in Table 6.

<table>
<thead>
<tr>
<th>Center phylogenetic group</th>
<th>Surrounding phylogenetic groups</th>
<th>Shared non-phylogenetic residues between groups&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Associated groups</th>
<th>Name</th>
<th>Letter</th>
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<tr>
<td>11</td>
<td>None</td>
<td>None</td>
<td></td>
<td></td>
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<tr>
<td>30-32</td>
<td>86</td>
<td>Y85, T34</td>
<td>30-32Assoc</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>42-53</td>
<td>86</td>
<td>A99, R41</td>
<td>42-53Assoc</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td>86</td>
<td>30-32, 42-53</td>
<td>Y85, A99</td>
<td>86Assoc</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td>105-369</td>
<td>30-32, 149-282</td>
<td>T34, M371</td>
<td>105-369Assoc</td>
<td>D</td>
<td></td>
</tr>
<tr>
<td>149-282</td>
<td>105-369, 256-258</td>
<td>Y283, L280, C284</td>
<td>149-282Assoc</td>
<td>E</td>
<td></td>
</tr>
<tr>
<td>168-399</td>
<td>265, 341, 391-428</td>
<td>Y239, F345, F394, L424</td>
<td>168-399Assoc</td>
<td>F</td>
<td></td>
</tr>
<tr>
<td>221</td>
<td>256-258, 265</td>
<td>F218, L240</td>
<td>221Assoc</td>
<td>G</td>
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<tr>
<td>235</td>
<td>149-282</td>
<td>Y283</td>
<td>235Assoc</td>
<td>H</td>
<td></td>
</tr>
<tr>
<td>256-258</td>
<td>221, 265</td>
<td>F218, A257</td>
<td>256-258Assoc</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>265</td>
<td>168-399, 221, 256-258</td>
<td>Y239, L240, A257</td>
<td>265Assoc</td>
<td>J&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>305-474</td>
<td>42-53, 341</td>
<td>R41, L475</td>
<td>305-474Assoc</td>
<td>K</td>
<td></td>
</tr>
<tr>
<td>341</td>
<td>168-399, 305-474</td>
<td>F345, L475</td>
<td>341Assoc</td>
<td>L</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Shared non-phylogenetic residues are within 5 Å from both the center and surrounding phylogenetic groups.

<sup>b</sup> Because the βA-βB loop from the Rubisco small subunit also influences catalysis, phylogenetic-groups that interact with residues in the βA-βB loop are combined with the spinach βA-βB loop when substituted as associated groups.

<sup>c</sup> The 265Assoc associated group alone cannot be recovered by photosynthetic selection, but, when combined with the Rubisco small subunit from *Arabidopsis*, can support photosynthesis. Thus, this strain is noted as "J" in further studies.

<sup>d</sup> The 391-428Assoc and 442-447Assoc associated groups cannot be recovered by photosynthetic selection, and thus, cannot be analyzed.
spinach βA-βB loop. Therefore, the βA-βB loop was also considered a phylogenetic group with regard to associated groups, but it is included in an associated group only if residues of the βA-βB loop are in contact with a phylogenetic group (Table 5).

Different regions of Rubisco can interact. Thus, directed mutagenesis of the associated groups as a whole, coupled with data from separately substituting each phylogenetic group, may provide information about complementation between regions of the holoenzyme. Perhaps combinations of structurally-related phylogenetic groups complement to produce catalytic changes, which might not be obvious from separate phylogenetic-group substitution.

Because each phylogenetic group and the other surrounding phylogenetic groups form each associated group, like the number of phylogenetic groups, there are 15 associated group, for which mutant enzymes can be created and analyzed. However, for phylogenetic group 11, Ala-11 is too distant from any other phylogenetic groups to form an associated group (Table 5). Thus, only 14 associated-group mutants need to be created.

**Recovery of associated-group mutants and their phenotypes**

*Chlamydomonas* associated-group mutants were recovered by chloroplast transformation of the MX3312 (Satagopan and Spreitzer, 2004, 2008; Zhu et al., 2005) and rbcLΔ/ABSO rbcL-knockout strains followed by photosynthetic selection. The rbcLΔ/ABSO strain, which has the spinach-substituted Rubisco small-subunit βA-βB loop (Spreitzer et al., 2005), was used to create associated-group mutants that involve the βA-βB loop (235Assoc and 256-258Assoc) (Table 5). Eleven of the fourteen associated-
group mutant strains could grow photoautotrophically. The 265Assoc, 442-447Assoc, and 391-428Assoc mutants could not be recovered (Table 5). For the mutants that could be recovered, the transformation frequencies were 1-3 × 10^{-6} per cells transformed, and the transformants started appearing as photosynthetic colonies on minimal medium within 2 to 4 weeks, which was similarly observed for the wild-type rbcL transformants.

Comparison of the growth phenotypes between the associated-group mutants and wild-type Chlamydomonas by spot tests (Spreitzer and Mets, 1981) indicated that the 30-32Assoc, 86Assoc, 149-282Assoc, and 221Assoc mutants have reduced photosynthetic growth on minimal medium in the light at the restrictive temperature of 35°C (spots A, C, E, and G, respectively, Fig. 10). Because these "temperature-conditional phenotypes" are limited to photosynthetic growth, but not heterotrophic growth at 35°C (Fig. 10), the temperature sensitivity must be specific to a defect in Rubisco. Considering that the 30-32Assoc mutant (V30E/V31T/R32K/D86H/I105L/C369V) and the 86Assoc mutant (D86H/V30E/V31T/R32K/M42V/C53A) have substitutions V30E/V31T/R32K/D86H in common, which is also a combination of phylogenetic groups 30-32 and 86 (Tables 2 and 5), the temperature-conditional phenotypes might be caused by combining the 30-32 and 86 phylogenetic-group substitutions. Similarly, considering that the 149-282Assoc mutant (V149Q/I282H/I105L/C369V/C256F/K258R/I265V) and the 221Assoc mutant (V221C/C256F/K258R/I265V) have substitutions C256F/K258R/I265V in common, which is a combination of phylogenetic groups 256-258 and 265 (Tables 2 and 5), the temperature-conditional phenotypes might be caused by combining the 256-258 and 265 phylogenetic-group substitutions. Moreover, a previous study has shown that combining
Figure 10: Growth phenotypes of *Chlamydomonas* wild type (WT) and associated-group mutants (spots A through L). About $2 \times 10^5$ cells were spotted at each position at the conditions indicated (Spreitzer and Mets, 1981). Panel A (top) cells were grown for a week. Panel B (bottom) cells were supplemented with 5% CO$_2$ in air and scanned after one week (bottom-left) and after two weeks (bottom-right). Spot letter is based on Table 5.
the 256-258 and 265 phylogenetic-group substitutions does produce a temperature-
conditional *Chlamydomonas* mutant (Spreitzer *et al.*, 2005). On the other hand, the
temperature-conditional phenotypes of the 30-32Assoc and 86Assoc mutant strains could
be attributed to phylogenetic substitutions in a region that affects the interaction between
Rubisco and Rubisco activase (Larson *et al.*, 1997; Ott *et al.*, 2000).

For the associated-group mutants that could not be recovered, attempts were also
made to transform into the *rbcLΔ/ABSO* and *rbcLΔ/SSAT* strains (Dent *et al.*, 2005;
Genkov *et al.*, 2010; Genkov and Spreitzer, unpublished). Interestingly, the 265Assoc
mutant produced photosynthetic colonies when transformed in the cell-walled
*rbcLΔ/SSAT* strain, which has the Rubisco small subunit substituted with that of
However, this 265Assoc/SSAT transformant required 5% v/v CO₂ in air for growth, and
colonies were visible only after three weeks compared to six days for the wild-type *rbcL*
transformants. In fact, spot tests indicated that the 265Assoc/SSAT strain has less
photosynthetic growth than wild type even after two weeks on minimal medium with 5%
CO₂ (spot J, Fig. 10). The other two associated-group mutants, 391-428Assoc and 442-
447Assoc, could not be recovered in any of the transformation hosts.

**Rubisco holoenzyme level**

To determine the effects of the mutations on the amount of Rubisco *in vivo*, SDS-
PAGE and western blotting were performed. Almost all the associated-group mutant
strains expressed equal amounts of Rubisco when compared with wild type except for the
265Assoc/SSAT mutant strain, which had reduced *in vivo* holoenzyme level as evidenced on the western blots (lane J, Fig. 11). The decreased Rubisco holoenzyme level of the 265Assoc/SSAT mutant strain could account for the high-CO$_2$-requiring phenotype (spot J, Fig. 10).

A thermal-inactivation experiment (Chen *et al.*, 1993) was performed to see whether the 265Assoc/SSAT mutant enzyme was unstable (Fig. 12). Whereas the wild-type enzyme was unaffected by a 55°C incubation, the mutant enzyme lost 80% of its activity at this temperature. Thus, the phylogenetic substitutions I265V/V149Q/I282H/G168P/L326I/M349L/M375L/A398S/C399V/V221C/C256F/K258R, together with the *Arabidopsis* small subunit, caused the 265Assoc/SSAT mutant enzyme to be more unstable than the wild-type enzyme (Fig. 12).

Reductions in holoenzyme levels for 35°C-grown *Chlamydomonas* cells were observed in the western blots for the 149-282Assoc and 221Assoc mutant strains (lanes E and G, respectively, Fig. 11), which would explain their temperature-conditional phenotypes (spots E and G, respectively, Fig. 10). A previous study has shown that the C256F/K258R/I265V substitutions, which are present in the 149-282Assoc and 221Assoc associated-group mutants, caused a similar reduction in holoenzyme level in *Chlamydomonas* cells grown at 35°C (Spreitzer *et al.*, 2005). However, the reduction in holoenzyme level was not caused by increased holoenzyme instability, but likely due to increased susceptibility to proteolysis (Du *et al.*, 2003; Spreitzer *et al.*, 2005). Thus, the 149-282Assoc and 221Assoc associated-group substitutions might also alter Rubisco structure sufficiently to increase the susceptibility of the enzymes to proteolysis *in vivo*, especially at elevated 35°C.
Figure 11: SDS-PAGE (left) and western-blot analysis (right) of total soluble proteins from *Chlamydomonas* wild type (WT) and associated-group mutants (A to L). Sixty micrograms of total soluble protein from cells grown in the dark at 25°C (top) or 35°C (bottom) were run in each lane. Mutant letter is based on Table 5. LS denotes the Rubisco large subunit, and SS denotes the small subunit.
Figure 12: Thermal inactivation of Rubisco purified from *Chlamydomonas* wild type (WT) and the 265Assoc/SSAT mutant. Five micrograms of Rubisco were pre-incubated at the indicated temperatures for 10 min, and then cooled on ice for 5 min before assaying for carboxylase activity at 25°C for 1 min. The measured activity after each pre-incubation temperature was normalized to that of 35°C-pre-incubation. The specific activity after the 35°C pre-incubation was 1.1 µmol CO₂ fixed/min/mg protein for wild-type Rubisco, and 0.1 µmol/min/mg for the 265Assoc/SSAT enzyme.
Kinetic properties

Because $\Omega$ defines the rate-limiting step of the carboxylation and oxygenation reactions of Rubisco, and differs between land-plant and Chlamydomonas enzymes, $\Omega$ was measured for all the associated-group mutant enzymes. Similar to the phylogenetic-group mutant enzymes, none of the associated-group mutant enzymes have increased $\Omega$ values (Table 6). The 265Assoc/SSAT mutant enzyme has an increased $\Omega$ (Table 6), but this 10% increase can be attributed to substituting the entire small subunit from Arabidopsis (Genkov et al., 2010).

Phylogenetic-group mutant 168-399 has a decrease in $\Omega$ (Table 3), but when combined with phylogenetic groups 305-474 and 341 to produce the 341Assoc associated-group mutant, the resulting mutant enzyme has $\Omega$ restored to the wild-type value (Table 6). Therefore, the 168-399 group substitutions are complemented by the 305-474 and 341 groups. More likely, the 305-474 substitutions are responsible for restoring the $\Omega$ value of the 168-399 mutant enzyme because the 305-474 mutant enzyme has an increased $K_o$ (Tables 3 and 4) whereas the 341 mutant enzyme (V341I) has kinetic properties indistinguishable from wild-type Rubisco (Zhu and Spreitzer, 1996).

Structural analysis of the associated-group mutants

The $\Omega$-reducing 168-399 phylogenetic-group (G168P/L326I/M349L/M375L/A398S/C399V) is at the base of loop 6 (Figs. 8 and 13). The other complementary phylogenetic groups 305-474 (R305K/D470E/T471A/I472M/K474T) and 341 (V341I), which restore the $\Omega$ value as demonstrated by the 341Assoc associated-group mutant
Table 6: $\Omega$ values of Rubisco purified from *Chlamydomonas* wild type and associated-group mutants.

<table>
<thead>
<tr>
<th>Enzymes (Letter)</th>
<th>Residue substitutions</th>
<th>$\Omega\ a$ $(V_c K_d/V_o K_c)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>None</td>
<td>61 ± 2</td>
</tr>
<tr>
<td>30-32Assoc (A)</td>
<td>V30E/V31T/R32K/D86H/I105L/C369V</td>
<td>61 ± 2</td>
</tr>
<tr>
<td>42-53Assoc (B)</td>
<td>M42V/C53A/D86H/R305K/D470E/T471A/I472M/K474T</td>
<td>60 ± 2</td>
</tr>
<tr>
<td>86Assoc (C)</td>
<td>D86H/V30E/V31T/R32K/M42V/C53A</td>
<td>55 ± 2</td>
</tr>
<tr>
<td>105-369Assoc (D)</td>
<td>I105L/C369V/V30E/V31T/R32K/V149Q/I282H</td>
<td>63 ± 2</td>
</tr>
<tr>
<td>149-282Assoc (E)</td>
<td>V149Q/I282H/I105L/C369V/C256F/K258R/I265V</td>
<td>61 ± 2</td>
</tr>
<tr>
<td>168-399Assoc (F)</td>
<td>G168P/L326I/M349L/M375L/A398S/C399V/I265V/V341I/V391T/T428V</td>
<td>57 ± 4</td>
</tr>
<tr>
<td>221Assoc (G)</td>
<td>V221C/C256F/K258R/I265V</td>
<td>54 ± 2</td>
</tr>
<tr>
<td>235Assoc (H)</td>
<td>V235I/ABSO (spinach small-subunit βA-βB loop)</td>
<td>56 ± 1</td>
</tr>
<tr>
<td>256-258Assoc (I)</td>
<td>C256F/K258R/V149Q/I282H/V221C/I265V/ABSO (spinach small-subunit βA-βB loop)</td>
<td>60 ± 2</td>
</tr>
<tr>
<td>265Assoc/SSAT (J)</td>
<td>I265V/V221C/V149Q/I282H/G168P/L326I/M349L/M375L/A398S/C399V/C256F/K258R/SSAT (Arabidopsis small-subunit)</td>
<td>66 ± 2</td>
</tr>
<tr>
<td>305-474Assoc (K)</td>
<td>R305K/D470E/T471A/I472M/K474T/M42V/C53A/V341I</td>
<td>62 ± 3</td>
</tr>
<tr>
<td>341Assoc (L)</td>
<td>V341I/G168P/L326I/M349L/M375L/A398S/C399V/R305K/D470E/T471A/I472M/K474T</td>
<td>64 ± 2</td>
</tr>
</tbody>
</table>

$^a$ Values are the means ± S.D. ($n$ - 1) of three separate enzyme preparations.
Figure 13: Structural comparison of residues in associated group 341Assoc consisting of phylogenetic groups 168-399 (red) at the base of catalytic loop 6, 305-474 (green) at the carboxy-terminal tail, and 341 (blue) in α-helix 6. Glu-338 in α-helix 6 is in van der Waals contact with Ala-471 in Chlamydomonas Rubisco, but caps the N-terminal end of α-helix 6 in spinach Rubisco. The CABP transition-state analog denotes the active site.
enzyme, are in the carboxy terminus (Figs. 9 and 13) and α-helix 6 of the Rubisco large subunit, respectively (Fig. 13). Comparison of the x-ray crystal structures of *Chlamydomonas* and spinach Rubisco indicates that the T471A substitution could change the conformation of the carboxylate side chain of Glu-338 at the amino-terminal end of α-helix 6 (Fig. 13). In spinach Rubisco, Ala-471 is in van der Waals contact with Glu-338, but in *Chlamydomonas*, the bulkier Thr-471 prevents the carboxylate side chain of Glu-338 from occupying the same space. Instead, the negatively-charged side chain is turned towards the amino-terminal end of α-helix 6, and may neutralize the partial positive charge from the α-helix dipole (Fig. 13).

In addition, the phylogenetic V341I substitution enables residue 341 in α-helix 6 to form van der Waal contact with residue 474 in the carboxy terminus through the longer isoleucine side chain as seen in spinach Rubisco (Fig. 13). This may strengthen the interaction between α-helix 6 and the carboxy terminus. Perhaps the increased interaction between α-helix 6 and the carboxy terminus, namely between Glu-338 and Ala-471, and between Ile-341 and Thr-474, complements some subtle structural perturbations caused by the 168-399 phylogenetic substitutions at the base of loop 6 (Fig. 13).

In the 265Assoc associated-group mutant, a combination of the 168-399 phylogenetic-group substitutions (G168P/L326I/M349L/M375L/A398S/C399V) at the base of loop 6 with phylogenetic groups 149-282, 221, 256-258, and 265 (V149Q/I282H, V221C, C256F/K258R and I265V) near the interface between large and small subunits cannot be recovered through photosynthetic selection. However, transforming the 265Assoc mutant gene into a *Chlamydomonas* strain that expresses the *Arabidopsis* small
subunit (Genkov and Spreitzer, unpublished) produces a mutant strain that can grow photosynthetically, albeit at only 5% CO₂ in air (Fig. 10). In other words, the land-plant small subunit complements the large-subunit phylogenetic changes in the 265Assoc associated group in *Chlamydomonas*. Because replacement of the small-subunit βA-βB loop with that of spinach complements the large-subunit changes in the penta/ABSO enzyme to produce plant-like kinetic properties (Spreitzer *et al.*, 2005), an attempt was made to transform the 265Assoc mutant gene into the *rbcLΔ*/ABSChlamydomonas strain to determine whether replacement of the βA-βB loop with that of spinach was sufficient to complement the 265Assoc large-subunit substitutions. Moreover, among the residue changes in 265Assoc are V221C/C256F/K258R/I265V, which are four of the five phylogenetic substitutions in the large subunit of the penta/ABSO mutant (Spreitzer *et al.*, 2005). However, no photosynthetic transformants were recovered in this experiment. Therefore, some other regions of the Rubisco small subunit may complement the 265Assoc substitutions. Because the loop between β-strands C and D of the small subunit, the βC-βD loop, is also variable among species, with photosynthetic bacteria and non-green algae having two additional residues in the loop (Spreitzer, 2003), changes in the βC-βD loop could be responsible for complementing the 265Assoc substitutions (Fig. 14). The *Chlamydomonas* small subunit has Lys-114 in the βC-βD loop, and the corresponding plant residue is Arg-108. Both of these residues form a salt bridge with Asp-397 in the large subunit.

For the 391-428Assoc and 442-447Assoc associated-group substitutions, which cannot be recovered by photosynthetic selection, the substitutions common to both associated groups are V391T/T428V/G442N/D443E/V444I/S447E (Table 6). These are
Figure 14: Structural comparison of residues in the associated group 265Assoc consisting of phylogenetic groups 149-282 (pale green), 168-399 (red), 221 (bright green), 256-258 (pink), and 265 (purple). The entire small subunit is colored yellow. Non-phylogenetic residues at the interface between large and small subunits are shown as faded sticks. Residues of the βC-βD loop of the small subunit are labeled (112 to 115 in Chlamydomonas; 106 to 109 in spinach). The CABP transition-state analog denotes the active site.
a combination of the 391-428 and 442-447 phylogenetic-group substitutions (Tables 2 and 5). The 442-447 residues are in α-helix G of the Rubisco large subunit whereas the 391 and 428 residues are in α-helices 7 and 8, respectively (Fig. 15).

Of the 391-428 and 442-447 phylogenetic groups, only phylogenetic residues Gly-442, Asp-443, and Ser-447, which are all in α-helix G, are on the surface of the holoenzyme (Fig. 13). Phylogenetic residues Gly-442 and Asp-443 are substituted with Asn-442 and Glu-443 in most land plants, effectively replacing the residue side chains with longer polar functional groups, thus allowing for better interaction with Arg-446 (Fig. 13). Similarly, phylogenetic residue Ser-447 is more commonly Asp-447 in land plants, which forms a salt bridge with Arg-435 (Fig. 13). Another residue in α-helix G, Trp-451, also has a different conformation between *Chlamydomonas* and Spinach Rubisco (Fig. 13). Because the 442-447 phylogenetic-group substitutions, G442N/D443E/V444I/S447E, caused a decrease in Rubisco holoenzyme level (Fig. 4), and the mutant strain has reduced photosynthetic growth (Fig. 3), changes in electrostatic interactions on the surface of α-helix G involving phylogenetic residues 442, 443 and 447 might affect interactions with chaperones or other protein molecules that assist in the assembly of the hexadecameric Rubisco.

Perhaps the addition of other phylogenetic-residue substitutions in the 391-428Assoc and 442-447Assoc associated-group mutants exacerbate the reduction in Rubisco holoenzyme assembly, which would explain the inability to recover the associated-group mutants in *Chlamydomonas* through photosynthetic selection. The other non-surface phylogenetic residues common to the 391-428Assoc and 442-447Assoc phylogenetic-groups are Val-391, Thr-428, and Val-444. Of these residues, Thr-428 is in
Figure 15: Structural comparison of phylogenetic residues common to both the 391-428Assoc and 442-447Assoc associated groups consisting of phylogenetic groups 391-428 (brown) in α-helices 7 and 8, and 442-447 (blue) in α-helix G. Non-phylogenetic residues that might be altered in their interactions because of the phylogenetic changes are shown as white sticks for the large-subunit or yellow sticks for the small subunit. The backbone of large-subunit α-helices 7, 8, and G, and small-subunit N-terminal loop (residues 1-22) are represented as ribbons.
van der Waals contact with Phe-15 from the Rubisco small subunit (Fig. 13). In land plants, large-subunit phylogenetic-residue Thr-428 is substituted with Val-428, small-subunit residue Phe-15 is substituted with Leu-15, and the distance between the two residues is greater in plant Rubisco (Fig. 13). A previous study showed that a Rubisco mutant with a small-subunit L18A substitution, which might affect the interaction with residue 15, lacked holoenzyme even though the subunits were expressed (Genkov and Spreitzer, 2009).

A switch in species-specific intermolecular interactions between Rubisco and Rubisco activase was also observed when the surface residues 89 and 94 of the Rubisco large subunit were changed from those of Chlamydomonas (non-Solanaceae) to those of tobacco (Solanaceae) (Larson et al., 1997; Ott et al., 2000). Similarly, the surface residues in the 442-447 phylogenetic-group on α-helix G could define a switch region for species-specific interactions between Rubisco and chaperones. Flaveria and sunflower Rubisco large subunits can be expressed and assembled in tobacco, and all three species are identical for the surface phylogenetic-residues 442, 443, and 447 in the 442-447 phylogenetic group (Kanevski et al., 1999; Whitney et al., 2011). On the other hand, the Rubisco large subunits from the rhodophyte Galdieria sulphuraria and the diatom Phaeodactylum tricornutum can be expressed but not assembled in tobacco, and the identities of the residues 442, 443 and 447 from the foreign Rubiscos are different from tobacco (Whitney et al., 2001). Rubisco chaperone RbcX binds specifically to a stretch of seven amino acids in the carboxy terminus of the Synechococcus large subunit (Saschenbrecker et al., 2007), which corresponds to residues 464-470 in Chlamydomonas. However, a subsequent study indicated that α-helices 8, G, and H
could be involved in chaperone release by undergoing a conformational change of as much as 8 Å (Saschenbrecker et al., 2007; Liu et al., 2010).

It is surprising that none of the associated-group substitutions have significant increases in Ω characteristic of plant Rubisco even though the holoenzyme is being structurally altered to mimic the plant enzyme. The phylogenetic substitutions in the penta/ABSO mutant, and the large/small-subunit region defined by the substitutions, must be truly unique because penta/ABSO is the only phylogenetic mutant enzyme with kinetic properties similar to those of plants (Spreitzer et al., 2005).
PENTA/ABSO DISSECTION

Penta/ABSO

The success of the phylogenetic approach was demonstrated by a previous study in which a Rubisco \textit{Chlamydomonas}-to-plant substitution of five large-subunit phylogenetic residues (V221C/V235I/C256F/K258R/I265V), together with the spinach small-subunit βA-βB loop, produced a mutant enzyme with kinetic properties similar to those of plants (Spreitzer \textit{et al.}, 2005). This penta/ABSO enzyme had an increase in Ω by 17% and a decrease in $V_c$ by 50% (Spreitzer \textit{et al.}, 2005). To determine the minimal number of phylogenetic changes that actually plays a role in the catalytic shift of the penta/ABSO enzyme, further dissection of the interactions within the phylogenetic residues and βA-βB loop is warranted. Because five large-subunit phylogenetic residues and the βA-βB loop are changed in the penta/ABSO mutant enzyme, to dissect the structural interactions by creating all possible combinations would require the creation and analysis of $2^5$ (= 64) mutant enzymes. Instead, the five large-subunit phylogenetic residues can be grouped into four phylogenetic groups, and the βA-βB loop can be considered an additional group. To be specific, the residue substitutions in penta/ABSO, V221C/V235I/C256F/ K258R/I265V/ABSO, can be separated into substitutions of phylogenetic groups 221, 235, 256-258, 265, and ABSO (Table 2). Previous studies have shown that just substituting the five separate groups alone do not produce plant-Rubisco kinetic-properties (Du \textit{et al.}, 2003; Karkehabadi \textit{et al.}, 2005; Spreitzer \textit{et al.}, 2005). At a next level, pairwise combinations of the groups could be substituted, but that would still require creating and analyzing ten mutant enzymes. Instead, if only pairs of associated phylogenetic groups are considered, then only five mutant enzymes need to be created...
One of these mutant enzymes, C256F/K258R/I265V, was created previously, but it had a 10% decrease in Ω rather than the >30% increase characteristic of plant Rubisco (Du et al., 2003; Spreitzer et al., 2005). The V235I/ABSO mutant enzyme was created as the 235Assoc enzyme (Tables 5 and 6). Thus, only three additional mutant enzymes remained to be created and analyzed.

**Recovery of penta/ABSO-dissection mutants and their phenotypes**

*Chlamydomonas* penta/ABSO-dissection mutants were recovered by chloroplast transformation of the MX3312 (Zhu et al., 2005; Satagopan and Spreitzer, 2004, 2008) and rbcLΔ/ABSO rbcL-knockout strains followed by photosynthetic selection. The rbcLΔ/ABSO strain, which has the small-subunit βA-βB loop from spinach (Spreitzer et al., 2005), was used to create the V235I/ABSO and C256F/K258R/ABSO mutants (Table 7). All the penta/ABSO-dissection mutant strains could grow photoautotrophically, and transformation frequencies were 1-3 X 10^-6 per cells shot. Transformants started to appear on minimal medium within 2 to 4 weeks, which was also observed for wild-type rbcL transformants.

Comparison of the growth phenotype between the penta/ABSO-dissection mutants and wild-type *Chlamydomonas* by spot tests (Spreitzer and Mets, 1981) indicated that the C256F/K258R/I265V mutant had reduced photosynthetic growth (spot 4, Fig. 16) as observed previously (Du et al., 2003; Spreitzer et al., 2005). The rest of the mutant strains had somewhat reduced growth on minimal medium at 35°C in comparison to wild type (Fig. 16). When grown on acetate medium in the dark, the mutant strains were indistinguishable from wild type (Fig. 16). Therefore, the reduction in growth must be
Table 7: Pairwise combinations of associated phylogenetic groups created to dissect the penta/ABSO mutant. Phylogenetic groups are defined in Table 2.

<table>
<thead>
<tr>
<th>First phylogenetic group</th>
<th>Second Phylogenetic group</th>
<th>Shared non-phylogenetic residues between groups</th>
<th>Necessary residue substitutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>221</td>
<td>256-258</td>
<td>F218</td>
<td>V221C/C256F/K258R</td>
</tr>
<tr>
<td>221</td>
<td>265</td>
<td>L240</td>
<td>V221C/I265V</td>
</tr>
<tr>
<td>235</td>
<td>βA-βB loop</td>
<td>Direct contact</td>
<td>V235I/ABSO</td>
</tr>
<tr>
<td>256-258</td>
<td>265</td>
<td>A257</td>
<td>C256F/K258R/I265V</td>
</tr>
<tr>
<td>256-258</td>
<td>βA-βB loop</td>
<td>Direct contact</td>
<td>C256F/K258R/ABSO</td>
</tr>
</tbody>
</table>

* Shared non-phylogenetic residues are within 5 Å from both phylogenetic groups.

* Because the small-subunit βA-βB loop also influences catalysis, it is included as a phylogenetic group.

* V235I/ABSO was created and analyzed as the 235Assoc mutant enzyme (Tables 5 and Table 6).

* The C256F/K258R/I265V mutant strain was created and analyzed previously (Du et al., 2003; Spreitzer et al., 2005).
Figure 16: Growth phenotypes of *Chlamydomonas* wild type (WT), penta/ABSO-dissection mutants V221C/C256F/K258R (spot 1), V221C/I265V (spot 2), V235I/ABSO (spot 3), C256F/K258R/I265V (spot 4) and C256F/K258R/ABSO (spot 5), and penta/ABSO (V221C/V235I/C256F/K258R/I265V/ABSO) (spot 6). About 2 X 10^5 cells were spotted at each position, and grown for one week on minimal medium at 4000 Lux or acetate medium in darkness at either 25 or 35°C.
specific to a defect in Rubisco. Previous studies showed that the reduced growth of the C256F/K258R/I265V mutant strain is caused by a decrease in the amount and activity of Rubisco in vivo (Du et al., 2003; Spreitzer et al., 2005).

**Kinetic properties**

All the penta/ABSO-dissection mutant enzymes have reduced carboxylase specific activities when measured with saturating CO$_2$ (12.4 mM NaHCO$_3$) (Table 8). The V221C/C256F/K258R, V221C/I265V, V235I/ABSO, and C256F/K258R/ABSO mutant enzymes have carboxylase specific activities of 30%, 64%, 56%, and 44%, respectively, in comparison with the wild-type enzyme. In fact, even the C256F/K258R/I265V enzyme that was analyzed previously had a $V_c$ decreased to 46% of the wild-type value (Du et al., 2003; Spreitzer et al., 2005). Considering that the penta/ABSO enzyme has only 57% of the $V_c$ value of the wild-type enzyme (Spreitzer et al., 2005), the reduction in carboxylase activity seems to be a common feature of changing several phylogenetic residues at a time. However, whereas the penta/ABSO mutant has an $\Omega$ value increased by 17% (Spreitzer et al., 2005), none of the dissection mutants has an increase in $\Omega$. Instead, $\Omega$ is either unchanged, or reduced by 12% and 7% for the V221C/C256F/K258R and V235I/ABSO mutant enzymes, respectively. There are no significant changes in O$_2$ inhibition among the dissection-mutant enzymes when compared to the wild-type enzyme. However, for the penta/ABSO enzyme, because of a beneficial increase in $K_o$ and decrease in $K_c$, a decrease in O$_2$ inhibition would be expected (Spreitzer et al., 2005). Perhaps all the phylogenetic-residue substitutions in the penta/ABSO mutant are required for the 17% increase in $\Omega$, 54% decrease in $V_c$, 30%
Table 8: Ω, specific activity, and oxygen inhibition of Rubisco purified from *Chlamydomonas* wild type and penta/ABSO-dissection mutants.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>RuBP carboxylase activity</th>
<th>Ratio (A/B)</th>
<th>Ω&lt;sup&gt;a&lt;/sup&gt; V&lt;sub&gt;c&lt;/sub&gt;K&lt;sub&gt;c&lt;/sub&gt;/V&lt;sub&gt;e&lt;/sub&gt;K&lt;sub&gt;c&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100% N&lt;sub&gt;2&lt;/sub&gt;</td>
<td>100% N&lt;sub&gt;2&lt;/sub&gt;</td>
<td>100% O&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td>12.4 mM NaHCO&lt;sub&gt;3&lt;/sub&gt;</td>
<td>0.98 mM NaHCO&lt;sub&gt;3&lt;/sub&gt;</td>
<td>0.98 mM NaHCO&lt;sub&gt;3&lt;/sub&gt;</td>
</tr>
<tr>
<td>Wild type</td>
<td>103.7</td>
<td>28.2</td>
<td>11.2</td>
</tr>
<tr>
<td>V221C/V235I/C256F/K258R/I265V/ABSO</td>
<td>29.8</td>
<td>5.7</td>
<td>2.7</td>
</tr>
<tr>
<td>V221C/C256F/K258R</td>
<td>64.9</td>
<td>17.0</td>
<td>7.2</td>
</tr>
<tr>
<td>V235I/ABSO</td>
<td>56.4</td>
<td>6.8</td>
<td>2.8</td>
</tr>
<tr>
<td>C256F/K258R/I265V</td>
<td>44.0</td>
<td>9.9</td>
<td>4.4</td>
</tr>
<tr>
<td>C256F/K258R/ABSO</td>
<td>56 ± 1</td>
<td>58 ± 1</td>
<td>56 ± 1</td>
</tr>
<tr>
<td></td>
<td>60 ± 1</td>
<td>70 ± 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>53 ± 1</td>
</tr>
<tr>
<td></td>
<td>54 ± 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>58 ± 1</td>
<td>54 ± 2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values are the means ± S.D. (n - 1) of three separate enzyme preparations.

<sup>b</sup> Values are from a previous study (Spreitzer *et al.*, 2005).
decrease in $K_c$, 6% increase in $K_o$, and 37% decrease in $V_o$ (Spreitzer et al., 2005), which represent a shift to the kinetic properties of plant Rubisco. In conclusion, future genetic engineering aimed at improving Rubisco should target all the penta/ABSO phylogenetic residues and the small-subunit $\beta$A-$\beta$B loop.

It is interesting that Cys-256, which is one of the penta/ABSO residues in van der Waals contact with the small-subunit $\beta$A-$\beta$B loop, is S-methylated (Taylor et al., 2001). Because plant Rubisco has Phe-256 in place of Cys-256, perhaps S-methylation, which also occurs at phylogenetic residue Cys-369 in Chlamydomonas Rubisco (Taylor et al., 2001), is another cause for the differences in kinetic properties between algal and plant Rubisco enzymes. Another posttranslational modification observed in Chlamydomonas Rubisco but not in plant Rubisco is hydroxylation of Pro-104 and Pro-151, which are conserved among algal and plant Rubisco enzymes (Taylor et al., 2001).
POSTTRANSLATIONAL MODIFICATIONS

Posttranslational modification residues

Four new posttranslational modifications of the Rubisco large subunit were observed in the highest-resolution 1.4-Å x-ray crystal structure of *Chlamydomonas* Rubisco (Taylor *et al.*, 2001). The Cγ atoms of Pro-104 and Pro-151 are hydroxylated, and the Sγ atoms of Cys-256 and Cys-369 are methylated (Taylor *et al.*, 2001; Mizohata *et al.*, 2002). The function of these posttranslational modifications is unknown.

Even though the Pro-104 and Pro-151 residues are conserved among species, hydroxylation of the prolines has not been observed in Rubisco crystal structures from other species (Knight *et al.*, 1990; Taylor *et al.*, 2001; Andersson and Backlund, 2008). Though unlikely, it is also possible that the other Rubisco structures are less well-defined, thus obscuring the hydroxylation modification. Proline hydroxylation has a history of serving as a cellular mechanism for sensing O₂ such as for tagging the hypoxia-inducible factor transcription complex for degradation (Jaakkola *et al.*, 2001). In addition, because hydroxyprolines stabilize the triple-helix conformation of collagen (Motoooka *et al.*, 2012), the hydroxylation of Pro-104 and Pro-151 in Rubisco could also have a structural effect on the holoenzyme in response to O₂ levels. The additional hydroxyl groups might provide extra hydrogen-bonding partners or increase the hydrophilicity of the residues. A chloroplast-localized prolyl 4-hydroxylase has been identified in *Chlamydomonas* that is expressed under anaerobic conditions (Terashima *et al.*, 2010). However, ten other prolyl 4-hydroxylases were also identified in *Chlamydomonas* (Keskiaho *et al.*, 2007), and a crystal-structure is available for one of them (Koski *et al.*, 2007). One of these prolyl 4-hydroxylases plays a role in cell-wall assembly (Keskiaho *et al.*, 2007).
The methylated Cys-256 and Cys-369 residues are phylogenetic residues that are replaced by Phe-256 and Val-369 in plants (Taylor et al., 2001; Du et al., 2003). Perhaps these residues might account for the kinetic differences between algal and plant Rubisco (Jordan and Ogren, 1981b). Methylation of the cysteine residues could prevent formation of disulfide bridges. Methyltransferases have been identified in some plant species, but these chloroplast-localized methyltransferases only methylate Lys-14 of the Rubisco large subunit in plants (Houtz et al., 2008).

Pro-104 and Cys-369 are solvent exposed at the large-subunit interdimeric interface (Knight et al., 1990; Taylor et al., 2001) (Fig. 17). The modification of these residues could occur after formation of the Rubisco holoenzyme. However, because Pro-151 and Cys-256 are inaccessible to solvent, modifications of these residues must occur before assembly. Pro-151 is buried in the large subunit whereas Cys-256, which is in van der Waals contact with small-subunit Val-63, is only slightly exposed to the interior cavity of the holoenzyme at the large/small-subunit interface (Taylor et al., 2001; Spreitzer et al., 2005) (Fig. 17).

To test the importance of the posttranslationally-modified residues, each was separately changed to alanine in Chlamydomonas Rubisco. P104A/P151A and C256A/C369A double mutants were also created to assess the essentiality of hydroxylation and methylation. Because Cys-256 and Cys-369 are phylogenetic residues, they were also changed to the common plant residues Phe-256 and Val-369 either singly or as a pair to determine the effect of the plant residues.
Figure 17: Distribution of posttranslationally modified residues in *Chlamydomonas* Rubisco (PDB 1GK8). Large subunits are represented in gray and white whereas small subunits are represented in orange and yellow. Surface residues hydroxy-Pro-104 (red) and methyl-Cys-369 (cyan) are shown in the holoenzyme (top). Residues hydroxy-Pro-151 (light green) and methyl-Cys-256 (pink) in the interior are shown by removing four of the front large subunits (bottom).
Recovery of modified-residue mutants and their phenotypes

The *Chlamydomonas* mutants P104A, P151A, P104A/P151A, C256A, C369A, C256A/C369A, C256F, and C256F/C369V were created previously (Spreitzer et al., unpublished), but only the C256F mutant had been analyzed (Du et al., 2003). The C369V Rubisco mutant was created in the current study by chloroplast transformation of the MX3312 *rbcL*-knockout strain followed by photosynthetic selection.

Comparison of the growth phenotypes between the directed mutants and wild type indicated that only the C256A and C256A/C369A mutants had reduced photosynthetic growth, which was even more pronounced at 35°C (spots 4 and 6 respectively, Fig. 18). The P104A and P104A/P151A mutants also had reduced growth, but only at 35°C (spots 1 and 3 respectively, Fig. 18).

Rubisco holoenzyme level

To determine the effects of the mutant substitutions on the amount of Rubisco *in vivo*, which would depend on holoenzyme expression, stability, or assembly, SDS-PAGE and western analysis were performed. Only the C256A and C256A/C369A mutant strains had reduced holoenzyme levels when grown at both 25 and 35°C (lanes 4 and 6 respectively, Fig. 19). The P104A/P151A mutant had some decrease in holoenzyme at 35°C (lane 3, Fig. 19). The decreased Rubisco holoenzyme levels of the C256A and C256A/C369A mutant strains could account for their decreased photosynthetic growth at 25 and 35°C (spots 4 and 6, Fig. 18). The slight decrease in holoenzyme level of the 35°C-grown P104A/P151A mutant (lane 3, Fig. 19) may account for its decreased growth at 35°C (spot 3, Fig. 18).
Figure 18: Growth phenotypes of *Chlamydomonas* wild type (WT) and posttranslational-residue mutants P104A (spot 1), P151A (spot 2), P104A/P151A (spot 3), C256A (spot 4), C369A (spot 5), C256A/C369A (spot 6), C256F (spot 7), C369V (spot 8) and C256F/C369V (spot 9). About 2 X 10^5 cells were spotted on each position, and grown for one week at the conditions indicated.
Figure 19: SDS-PAGE (left) and western-blot analysis (right) of total soluble protein from *Chlamydomonas* wild type (WT) and posttranslational-residue mutants P104A (lane 1), P151A (lane 2), P104A/P151A (lane 3), C256A (lane 4), C369A (lane 5), C256A/C369A (lane 6), C256F (lane 7), C369V (lane 8) and C256F/C369V (lane 9). Sixty micrograms of total soluble protein from dark-grown cells were run in each lane. LS denotes the Rubisco large subunit, and SS denotes the small subunit.
Thermal inactivation experiments (Chen et al., 1993) were performed to determine whether the mutant enzymes are unstable (Fig. 20). All the mutant enzymes had similar temperature inactivation profiles (Fig. 20), indicating that they are not inherently unstable. Perhaps the P104A/P151, C256A, and C256A/C369A mutants have reduced holoenzyme levels because the mutations increase the susceptibility of the holoenzyme to proteolysis \textit{in vivo}.

**Kinetic properties**

Among the hydroxy-Pro-to-Ala mutant enzymes, only P104A and P104A/P151A have reduced $V_c$ of over 50%, and increased $K_c$ of 18% and 36%, respectively, which are compensated by increased $K_o$ of about 80% (Table 9). Because the P104A/P151A enzyme has kinetic properties similar to those of the P104A enzyme, the changes in kinetic properties may be caused by the P104A mutation alone. The kinetic properties of the P151A enzyme are similar to those of the wild-type enzyme. The P151A and P104A/P151A enzymes have significant reductions in $\Omega$ of 5% and 8%, respectively (Table 9).

More pronounced changes in kinetic properties were observed for the methyl-Cys-to-Ala mutant enzymes. C256A and C256A/C369A enzymes have reduced $V_c$ of over 79%, and increased $K_c$ of 29% and 39%, respectively. These negative effects are compensated by increased $K_o$ values of 36% and 70%, respectively (Table 9). Because the kinetic properties of the C256A and C256A/C369A enzymes are similar, changes in the kinetic properties of the C256A/C369A enzyme may be caused by the C256A substitution alone. Methyl-Cys-256 is one of the phylogenetically-substituted residue in
Figure 20: Thermal inactivation of Rubisco purified from *Chlamydomonas* wild type (WT) and posttranslational-residue mutants P104A, P151A, P104A/P151A, C256A, C369A, C256A/C369A, C256F, C369V, and C256F/C369V. Five micrograms of Rubisco were pre-incubated at the indicated temperatures for 10 min, cooled on ice for 5 min, and assayed for carboxylase activity at 25°C for 1 min. The measured activity after each pre-incubation temperature was normalized to that of the 35°C pre-incubation. The specific activity after the 35°C pre-incubation was 1.1 µmol CO₂/min/mg protein for wild-type Rubisco, 0.8 µmol CO₂/min/mg for mutant P104A, 1.3 µmol CO₂/min/mg for mutant P151A, 0.9 µmol CO₂/min/mg for mutant P104A/P151A, 0.5 µmol CO₂/min/mg for mutant C256A, 1.4 µmol CO₂/min/mg for mutant C369A, 0.3 µmol CO₂/min/mg for mutant C256A/C369A, 0.9 µmol CO₂/min/mg for mutant C256F, 1.0 µmol CO₂/min/mg for mutant C369V, and 1.1 µmol CO₂/min/mg for mutant C256F/C369V.
Table 9: Kinetic properties of Rubisco purified from *Chlamydomonas* wild type and posttranslational mutants.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$\Omega^a$</th>
<th>$V_c^a$</th>
<th>$K_c^a$</th>
<th>$K_o^a$</th>
<th>$V_o/K_c^b$</th>
<th>$K_o/K_c^b$</th>
<th>$V_c/V_o^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$V_cK_o/V_oK_c$</td>
<td>$\mu\text{mol/hr/mg}$</td>
<td>$\mu\text{M CO}_2$</td>
<td>$\mu\text{M O}_2$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>60 ± 1</td>
<td>134 ± 12</td>
<td>28 ± 2</td>
<td>569 ± 51</td>
<td>4.9</td>
<td>21</td>
<td>2.9</td>
</tr>
<tr>
<td>P104A</td>
<td>58 ± 2</td>
<td>50 ± 2</td>
<td>33 ± 5</td>
<td>1062 ± 62</td>
<td>1.5</td>
<td>32</td>
<td>1.8</td>
</tr>
<tr>
<td>P151A</td>
<td>57 ± 1</td>
<td>98 ± 14</td>
<td>24 ± 4</td>
<td>574 ± 42</td>
<td>4.1</td>
<td>24</td>
<td>2.3</td>
</tr>
<tr>
<td>P104A/P151A</td>
<td>55 ± 3</td>
<td>55 ± 8</td>
<td>38 ± 6</td>
<td>1031 ± 306</td>
<td>1.5</td>
<td>27</td>
<td>2.0</td>
</tr>
<tr>
<td>C256A</td>
<td>50 ± 3</td>
<td>28 ± 7</td>
<td>36 ± 6</td>
<td>771 ± 174</td>
<td>0.8</td>
<td>22</td>
<td>2.3</td>
</tr>
<tr>
<td>C369A</td>
<td>51 ± 2</td>
<td>113 ± 15</td>
<td>28 ± 2</td>
<td>581 ± 61</td>
<td>4.1</td>
<td>21</td>
<td>2.5</td>
</tr>
<tr>
<td>C256A/C369A</td>
<td>54 ± 3</td>
<td>24 ± 6</td>
<td>39 ± 8</td>
<td>965 ± 71</td>
<td>0.6</td>
<td>25</td>
<td>2.2</td>
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<tr>
<td>C256F$^c$</td>
<td>59</td>
<td>91</td>
<td>34</td>
<td>602</td>
<td>2.7</td>
<td>18</td>
<td>3.3</td>
</tr>
<tr>
<td>C369V</td>
<td>65 ± 3</td>
<td>124 ± 22</td>
<td>21 ± 4</td>
<td>582 ± 30</td>
<td>5.9</td>
<td>28</td>
<td>2.3</td>
</tr>
<tr>
<td>C256F/C369V</td>
<td>58 ± 4</td>
<td>86 ± 16</td>
<td>31 ± 2</td>
<td>645 ± 69</td>
<td>2.8</td>
<td>21</td>
<td>2.8</td>
</tr>
</tbody>
</table>

$^a$ Values are the means ± S.D. ($n$ - 1) of three separate enzyme preparations.

$^b$ Calculated values.

$^c$ Values are from Du *et al.* (2003).
the penta/ABSO mutant (Spreitzer et al., 2005). In terms of $\Omega$, the C256A, C369A and C256A/C369A enzymes have decreases by 17%, 15%, and 10%, respectively (Table 9). In contrast, the phylogenetic substitutions of the methyl-Cys residues (C256F, C369V and C256F/C369V) do not cause any significant changes to the kinetic properties of the enzymes except for a small increase in $\Omega$ for the C369V mutant.

**Structural analysis of posttranslationally-modified mutant enzymes**

Of the two hydroxylated proline residues in *Chlamydomonas* Rubisco, only Pro-104 seems to play a role in catalysis based on the measured kinetic properties of the Pro-to-Ala mutants (Table 9). Moreover, unlike Pro-151, Pro-104 is solvent exposed, which would allow the modification to take place after the assembly of the holoenzyme, and might provide a means for modulating enzyme activity in response to environmental conditions such as CO$_2$ and O$_2$ concentrations. The hydroxyl group of hydroxy-Pro-104 is in van der Waals contact with the backbone carbonyl oxygens of large-subunit residues Arg-79 and Lys-81 (Fig. 21), which are both in a stretch of loop region known as the 60s loop in the N-terminal domain of the large subunit (Knight et al., 1990; Duff et al., 2000; Spreitzer and Salvucci, 2002). Active-site residues Glu-60, Thr-65, and Trp-66 are also located in the 60s loop (Knight et al., 1990; Duff et al., 2000; Spreitzer and Salvucci, 2002). Perhaps hydroxy-Pro-104 influences catalysis through the interaction with the 60s loop. In addition to being located in the 60s loop, Arg-79 forms a hydrogen bond with the carbonyl oxygen of Gln-113 in the small-subunit $\beta$C-$\beta$D loop (Fig. 21). Therefore, the modification state of Pro-104 might also influence small-subunit structure.

Of the two methylated cysteine residues in *Chlamydomonas* Rubisco, only
Figure 21: Structural interactions from hydroxy-Pro-104 (red) to the catalytic 60s loop and the small subunit. The large-subunit 60s loop is represented in white ribbon. Residues that interact with hydroxy-Pro-104 (Arg-79, Lys-81 and Ile/Leu-105) or active-site residues (Glu-60, Thr-65 and Trp-66) are represented as sticks. The small subunit is represented in yellow. Small-subunit residue Gln-113/Asn-107 that interacts with Arg-79 is represented as sticks. The CABP transition-state analog (black) denotes the active site.
Cys-256 influences catalysis (Table 9). The methylation of Cys-256 enables contact with small-subunit residue Val-63, which is absent in the shorter βA-βB loop of plants (Fig. 22). Changes in the interaction between the small-subunit βA-βB loop and the large subunit could partially account for the shift in kinetic properties of the penta/ABSO mutant enzyme (Spreitzer et al., 2005). Therefore, Cys-256 could be one of the large-subunit residues mediating the interaction between the large and small subunits in a methylation-dependent manner. Moreover, when Cys-256 is replaced with the phylogenetically-related Phe-256, which has a longer and bulkier side chain, the changes in kinetic properties are less drastic than that of replacing with Ala-256, which has a shorter side chain. Perhaps the loss of interaction between residue 256 and small-subunit residue 63 is the reason for this change in catalysis.

In conclusion, among the four posttranslational modifications observed in the large subunit of *Chlamydomonas* Rubisco, only hydroxylation of Pro-104 and methylation of Cys-256 provide potential mechanisms for controlling Rubisco catalysis. Hydroxy-Pro-104 and methyl-Cys-256 can form networks of interactions to the small-subunit βC-βD and βA-βB loops, respectively, whereas the unmodified Pro-104 and Cys-256 are unable to participate in the interactions. Based on a previous study (Spreitzer et al., 2005) and also the analysis of the 265Assoc/SSAT associated-group mutant enzyme (Fig. 14), interactions between the large subunit and small-subunit βA-βB and βC-βD loops could affect the function of Rubisco.
Figure 22: Comparison of structural interactions of methyl-Cys-256/Phe-256 (red) and the small subunit. The large subunit is represented in white. Residues within 4 Å of methyl-Cys/Phe-256 are represented as sticks. The small subunit is represented in yellow. The CABP transition-state analog (black) that denotes the active site is 20Å away from this region.
Ever since the discovery of the role that Rubisco plays in photosynthesis and photorespiration (Bowes et al., 1971), engineering the enzyme for increased photosynthesis or decreased photorespiration has been touted as a means to increase crop productivity (Spreitzer and Salvucci, 2002). Considering that the kinetic properties of Rubisco are variable among species (Jordan and Ogren, 1981b), modifying the enzyme to produce the desired kinetic properties seems possible. However, the active-site residues, which at first glance would seem like obvious targets for engineering, are conserved among species, and any changes in the active-site residues are detrimental to Rubisco function (Gutteridge et al., 1993; Hartman and Harpel, 1994; Zhu and Spreitzer, 1994). Therefore, the variable non-active-site residues, known as phylogenetic residues, and the corresponding structural regions of the holoenzyme must define targets for engineering. Because engineering of eukaryotic Rubisco is most amenable in Chlamydomonas, this alga is a suitable host for testing out engineered Rubisco enzymes, and also provides a system for genetic selection. In the present study, phylogenetic residues, grouped according to their distribution in the x-ray crystal structure of Chlamydomonas Rubisco (Taylor et al., 2001) were changed to those most common in land plants. Phylogenetic substitutions that altered the kinetic properties of Rubisco defined non-active-site regions of the holoenzyme that influence catalysis. Complementary phylogenetic substitutions between separate regions of the holoenzyme were also observed.

Phylogenetic substitutions (G168P/L326I/M349L/M375L/A398S/C399V) at the base of loop 6 reduced Ω by 5%, which was consistent with a previous study in the same region of the holoenzyme (Zhu and Spreitzer, 1996). Further substitutions
(R305K/D470E/T471A/I472M/K474T) at the carboxy terminus of the large subunit complement the base-of-loop-6 substitutions, and restore the Ω value of the enzyme back to normal. This indicates that the phylogenetic residues at the base of loop 6 and at the carboxy terminus form complementary interactions, possibly affecting catalytic loop 6, which is located between the two structural regions. However, because land plant Rubisco has a greater Ω value, other regions of the holoenzyme must also affect catalysis.

When the base-of-loop-6 substitutions were combined with several substitutions at the large/small-subunit interface region at the bottom of the α/β barrel, a mutant enzyme was created that requires the presence of the land-plant Arabidopsis small subunit to enable viable photosynthetic growth in Chlamydomonas. Perhaps the large-subunit residues that were changed in the mutant enzyme define a network of interactions between the small subunit and the active site. Though the βA-βB and βC-βD loops of the small subunit could be involved in the large/small-subunit interaction, defining the exact small-subunit residues that are responsible for complementing the large-subunit changes would be an avenue for future research.

A couple of associated-group mutants with phylogenetic substitutions in α-helices 7, 8, and G cannot be recovered as photosynthetic transformants in any of the available Chlamydomonas rbcL-knockout hosts. Therefore, phylogenetic differences in the three α helices, especially surface α-helix G, might be important for species-specific assembly of the holoenzyme. In fact, creating all the other phylogenetic substitutions did not complement the changes in the three α helices, and failed to produce a functional Rubisco enzyme in Chlamydomonas. If the species-specific assembly region of Rubisco could be defined in the future, it might be possible to express the high-Ω non-green algal Rubisco
in crop plants, which might increase net CO$_2$ fixation, and it might also be possible to express eukaryotic Rubisco in *E. coli*, which would expedite structure-function studies of the enzyme.

Because none of the phylogenetic substitutions in the current study produced a corresponding shift in the kinetic properties of Rubisco, the focus was turned to the previously created penta/ABSO phylogenetic mutant that has plant-like kinetic properties. Further dissection of the substitutions within the penta/ABSO mutant indicated that all of the residue substitutions are required for the shift in catalysis, which means that the five large-subunit phylogenetic residues at the bottom of the $\alpha/\beta$ barrel, and the small-subunit $\beta$A-$\beta$B loop in the same region, are important targets for attempts at engineering an improved Rubisco enzyme.

Some of the posttranslationally-modified residues also play a role in Rubisco catalysis. Alanine substitutions of hydroxy-Pro-104 and methyl-Cys-256 affect the kinetic properties of Rubisco negatively by reducing $V_c$ and $\Omega$ values. Therefore, targeting the modified residues or the modification states of the residues might be another avenue for Rubisco engineering. A variety of nuclear non-*RbcS* mutations that affect Rubisco properties were previously recovered in *Chlamydomonas* (Spreitzer *et al.*, 1992; Gotor *et al.*, 1994), and may have lesions in the posttranslational modification pathway of Rubisco. If the genetic loci of the catalysis-influencing nuclear mutations could be identified in the future, other players that are involved in the modification of Rubisco might be defined, and serve as additional targets for engineering.

In conclusion, the present study narrowed down the primary region for Rubisco engineering to only the interface area between the large-subunit bottom-of-the-$\alpha/\beta$-barrel
region and the small-subunit βA-βB loop. Other structural interactions, such as those between the large-subunit regions at the base of loop 6 and the carboxy terminus, and between the base of loop 6, the bottom of the α/β barrel and the small-subunit βC-βD loop, also influence Rubisco function. These regions may also be worthy targets for molecular-evolution experiments.
LITERATURE CITED


