

A Rubisco Mutant That Confers Growth under a Normally “Inhibitory” Oxygen Concentration[†]

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ABSTRACT: Ribulose-1,5-bisphosphate (RuBP) carboxylase/oxygenase (Rubisco) is a globally significant biocatalyst that facilitates the removal and sequestration of CO₂ from the biosphere. Rubisco-catalyzed CO₂ reduction thus provides virtually all of the organic carbon utilized by living organisms. Despite catalyzing the rate-limiting step of photosynthetic and chemoautotrophic CO₂ assimilation, Rubisco is markedly inefficient as the competition between O₂ and CO₂ for the same substrate limits the ability of aerobic organisms to obtain maximum amounts of organic carbon for CO₂-dependent growth. Random and site-directed mutagenesis procedures were coupled with genetic selection to identify an “oxygen-insensitive” mutant cyanobacterial (*Synechococcus* sp. strain PCC 6301) Rubisco that allowed for CO₂-dependent growth of a host bacterium at an oxygen concentration that inhibited growth of the host containing wild-type *Synechococcus* Rubisco. The mutant substitution, A375V, was identified as an intragenic suppressor of D103V, a negative mutant enzyme incapable of supporting autotrophic growth. Ala-375 (Ala-378 of spinach Rubisco) is a conserved residue in all form I (plant-like) Rubiscos. Structure–function analyses indicate that the A375V substitution decreased the enzyme’s oxygen sensitivity (and not CO₂/O₂ specificity), possibly by rearranging a network of interactions in a fairly conserved hydrophobic pocket near the active site. These studies point to the potential of engineering plants and other significant aerobic organisms to fix CO₂ unfettered by the presence of O₂.

The Calvin–Benson–Bassham (CBB) reductive pentose phosphate pathway provides a way for many organisms to reduce carbon dioxide to organic carbon, a process that is vital for life on earth (1). Rubisco is the rate-limiting enzyme in this pathway, catalyzing the initial steps in both autotrophic carbon assimilation (CO₂ fixation or carboxylation) and photooxidative metabolism (O₂ fixation or oxygenation) via parallel reaction mechanisms that share a common acceptor intermediate, the enediol form of RuBP (2). Thus, in aerobic organisms, the promiscuity of this enediolate for both CO₂ and O₂ limits autotrophic CO₂ assimilation (35). Severe limitations imposed on the enzyme’s efficiency, primarily due to the competition between CO₂ and O₂ at the same active site and the poor turnover rate, have prompted numerous studies directed toward improving the enzyme’s net carboxylation efficiency (2). Thus, an increase in the carboxylation catalytic efficiency (V_c/K_c) and a decrease in the oxygenation catalytic efficiency (V_o/K_o) are desirable (31). The ratio of the two catalytic efficiencies, $V_c K_o / V_o K_c$, is defined as the specificity factor (Ω). The Ω value varies greatly among Rubisco enzymes from different species (3, 4) and hence could be a potential target for engineering beneficial changes in global CO₂ fixation. Moreover, structurally superimposable proteins from different sources,

with up to 90% sequence identity, often show vastly different catalytic properties (4). Despite an excellent understanding of the details of the reaction mechanism (5), the molecular basis by which such structurally similar proteins exhibit profound differences in kinetic behavior and substrate specificity is unknown.

Naturally occurring Rubisco enzymes are highly divergent based on primary structure alignments; they have been broadly classified into four different forms (forms I, II, III, and IV) with the members of each group sharing between 37% and 85% in-group identity and an overall sequence identity of ~30% (1). However, X-ray crystal structures indicate a striking similarity among these enzymes at the level of secondary and tertiary structures (6, 7, 39). Forms I, II, and III represent enzymes with bona fide Rubisco activity, all of which contain an invariant set of active site residues, a feature that is absent in the form IV enzymes. Form I enzymes, which are present in plants, algae, and many phototrophic and chemoautotrophic proteobacteria and cyanobacteria, comprise a unique class that assembles as hexadecamers with eight ~50 kDa large subunits and eight ~15 kDa small subunits (L₈S₈). A large-subunit dimer comprises the minimum functional unit in all of the four forms, with at least two active sites per dimeric unit. Every active site is formed by a set of residues from the C-terminal α/β -barrel domain of one large subunit and a few residues from the N-terminal domain of the neighboring large subunit (1, 2, 6).

Ever since it became feasible to obtain fully active recombinant form I enzyme from *Escherichia coli* (8, 9) and perform structural analyses (10), the form I Rubisco from the cyanobacterium

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Synechococcus sp. strain PCC6301 has served as a template of choice to study the effect of structural changes on Rubisco function (4, 11). Indeed, this enzyme closely resembles plant Rubisco, and any manipulations that result in beneficial changes in the cyanobacterial enzyme might eventually be applied to plant systems. A system was recently designed for biological selection of randomly mutagenized *Synechococcus* Rubisco genes in which a Rubisco-deletion mutant of the metabolically versatile photosynthetic bacteria *Rhodobacter sphaeroides* or *Rhodobacter capsulatus* (strain SBI/II[−]) serves as a surrogate host (12–14). Using this system allows one to monitor the *in vivo* effects of changes in Rubisco function since growth of the host directly reflects the properties of the Rubisco molecule that is expressed. Subsequently, a similar approach was also used to develop a non-photosynthetic *Escherichia coli*-based selection system (15, 16). Photoautotrophic CO₂-dependent growth of *R. capsulatus* requires anaerobiosis, i.e., conditions where Rubisco function is uninhibited by the presence of oxygen. However, this organism is also capable of growth with CO₂ as sole source of carbon via the CBB pathway under dark aerobic conditions, using the oxidation of molecular H₂ to provide the energy to support growth (4). Under such chemoautotrophic growth conditions, oxygen serves as the terminal electron acceptor, thus potentially providing a means to select enzyme variants with an improved ability to support CO₂-dependent growth and interact favorably with CO₂ in the presence of normal inhibitory levels of O₂. Because of the versatile metabolic capacity of *R. capsulatus* to enable CO₂-dependent growth in both the absence or presence of O₂, under photosynthetic or nonphotosynthetic autotrophic conditions, respectively, we have undertaken studies to determine whether mutant forms of Rubisco may be isolated that support preferential growth under any of these conditions. In particular, one may easily vary the exogenous CO₂ and O₂ concentrations, so as to mimic the physiological milieu of heterologously expressed foreign Rubiscos (12–14, 17, 18).

Recently, a key region was identified in the oxygen-sensitive archaeal form III Rubisco that implicated residues of this region in specific interactions with oxygen (18). Indeed, amino acid substitutions in this region, at Ser-363 and Met-295 of the *Archaeoglobus fulgidus* enzyme, were shown to directly affect the *K_o* of this enzyme. In addition, changing Met-295 to an aspartate resulted in a significant increase in Ω . Sequence alignments indicated that Ser-363 of the *A. fulgidus* form III enzyme is equivalent to a conserved alanine in the form I enzymes (Ala-378 in spinach Rubisco) and an isoleucine in the form II enzymes (Ile-367 in *Rhodospirillum rubrum* Rubisco). Interestingly, Ser-368, which is adjacent to the Ile-367 in *R. rubrum* Rubisco (38), was previously identified as being important for oxygenase activity (19). In the current study, we have found that a substitution in the conserved alanine residue (A375V) in a hydrophobic pocket near the active site of the *Synechococcus* enzyme was able to suppress the negative phenotype caused by a D103V substitution at the enzyme's dimer–dimer interface via long-range interactions. Furthermore, the A375V substitution (equivalent to Ala-378 of spinach Rubisco and Ser-363 of the *A. fulgidus* enzyme) appeared to decrease the oxygen sensitivity of the enzyme, which is manifested as CO₂-dependent growth of the host *R. capsulatus* under aerobic conditions not supported by the wild-type enzyme. These studies suggest that residues within this conserved hydrophobic region might play a key role with respect to oxygen sensitivity by evolutionarily diverse forms of Rubisco.

MATERIALS AND METHODS

Mutagenesis, Matings, and Selection. Random mutagenesis of the *Synechococcus* wild-type or the D103V mutant *rbcLS* genes was achieved through error-prone PCR amplification with Taq DNA polymerase (Invitrogen) in the presence of 0.1 mM MnCl₂. The resulting fragment (1.8 kb) was directionally cloned into pRPS-MCS3, a broad-host range vector (12) using T4 DNA ligase (Invitrogen). Site-directed mutagenesis was accomplished using the QuikChange kit from Stratagene (Stratagene, La Jolla, CA). Wild-type and mutant genes that had been cloned into pRPS-MCS3 were expressed in *R. capsulatus* strain SBI/II[−] via triparental matings involving an *E. coli* strain that carried a helper plasmid, pRK2013 (20). The *trans*-complemented *R. capsulatus* colonies were selected under either chemoheterotrophic or photoheterotrophic conditions as described previously (12, 13, 21).

***R. capsulatus* Growth Conditions.** Aerobic chemoheterotrophic growth was accomplished on peptone yeast extract plates or in a broth containing Ormerod's basal salts, 6 g/L peptone, 5 g/L yeast extract, 10 mM NaCl, 3 mM KCl, and 0.1 μ g/mL biotin. Both media were supplemented with 1 μ g/mL nicotinic acid and 1 μ g/mL thiamin hydrochloride (13, 16, 21). The plates were incubated at 30 °C for 3 days, when using complex media, and at the same temperature for 1–2 weeks when using defined media. Growth under selective conditions, either in the presence of 0.4% DL-malate (photoheterotrophic) or in the absence of any organic carbon (photoautotrophic growth), was performed using Ormerod's minimal medium supplemented with 1 μ g/mL nicotinic acid and 1 μ g/mL thiamin hydrochloride, as described previously (12). For photoautotrophic growth, in the presence of either 5% or 1.5% CO₂, the jars were flushed for 15 min with premixed 5% CO₂/95% H₂ or 1.5% CO₂/98.5% H₂, respectively. For aerobic chemoautotrophic or mixotrophic growth in a CO₂/H₂-enriched atmosphere, plates were incubated in the dark in tightly sealed jars containing air and the CO₂/H₂-generating kits (Oxoid). To carry out biochemical analysis, cells in the stationary phase (OD₆₆₀ ~3) were harvested by centrifugation at 8000g for 10 min at 25 °C, washed with TE buffer (100 mM Tris-HCl (pH 8.0), 1 mM EDTA), and stored at −80 °C prior to cell lysis.

Preparation of Cell Extracts, Purification of Rubisco, and Biochemical Analysis. Wild-type and mutant *Synechococcus* Rubisco enzymes were purified as recombinant proteins after expressing their genes with an N-terminal hexahistidine tag using the vector pET-28a (Novagen). A two-step purification using affinity (Ni-NTA agarose; Qiagen) and ion-exchange (UnoQ; Bio-Rad) chromatographies resulted in proteins that were >95% pure, as analyzed by SDS–PAGE. The proteins were dialyzed into a Tris buffer (100 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10 mM MgSO₄, 1 mM DTT, and 2 mM NaHCO₃), mixed with 20% glycerol, and stored as aliquots at −80 °C. *R. capsulatus* cell pellets from phototrophically grown cultures that had been stored at −80 °C were thawed on ice and resuspended in TEM buffer (100 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 5 mM β -mercaptoethanol). Cells were disrupted by sonication, and low-speed supernatants were obtained by microcentrifugation at 16100g for 10 min at 4 °C. Rubisco activities and protein concentrations in both *R. capsulatus* crude extracts and purified protein samples were determined as described previously (22, 23). Rubisco purification and the levels of expression in cell extracts of *R. capsulatus* cultures were monitored via SDS–PAGE (24) and Western blot analysis, as described previously (20).

Enzyme Assays and Kinetic Measurements. RuBP carboxylase activity was measured radiometrically via the incorporation of acid-stable ^{14}C from $\text{NaH}^{14}\text{CO}_3$ (22). The Ω values of purified enzymes (20 μg) were measured from assays performed under saturating (1.23 mM) O_2 concentrations (13). $[1\text{-}^3\text{H}]\text{RuBP}$, required for these assays, was synthesized and purified by standard methods (25). The kinetic constants k_{cat} , K_c , and K_o were determined from simultaneous assays performed in vials flushed with either 100% N_2 or 100% O_2 , as per the procedure described elsewhere (26) with modifications to the substrate concentration range. The K_{RuBP} values were measured similarly in aerobic assays performed in sealed vials by varying the RuBP concentrations from 5 to 1280 μM . A constant amount of NaHCO_3 (10 mM) was used in these assays.

RESULTS

Mutagenesis, Suppressor Isolation, and Growth Phenotypes. Previous studies had indicated that surface residues involved with large subunit interactions influence the Michaelis constant for CO_2 (K_c) of the *Synechococcus* 6301 enzyme (13, 14). Unlike the wild-type enzyme, an Asp-103-to-Val mutant failed to support CO_2 -dependent growth of cultures of *R. capsulatus* strain SBI/II $^-$ in a 5% CO_2 /95% H_2 atmosphere (13). Asp-103 from one dimeric pair interacts with Ser-367 of the second dimeric pair via hydrogen bonding. In order to better understand how Asp-103, which is far from the active site, might influence catalysis and Rubisco-dependent growth, additional rounds of random mutagenesis were instituted. The rationale was that suppressor mutation(s) might be isolated that would overcome the effects of the D103V mutation and would provide further clues as to the interaction of Asp-103 with other residues that might directly or indirectly affect catalysis. This was deemed a highly feasible *modus operandi* since we could merely select for second-site suppressors of D103V that allowed for CO_2 -dependent growth in *R. capsulatus* strain SBI/II $^-$.

With the D103V construct as the starting point, several separate and parallel random mutagenesis and suppressor selection studies were performed in the *R. capsulatus* strain SBI/II $^-$ background under CO_2 -dependent growth conditions. Using SBI/II $^-$ as an indicator strain resulted in mutant enzymes which overcame the negative growth phenotype of the D103V mutant. After screening about 2000 *trans*-complemented colonies of *R. capsulatus*, isolated from five independent mutagenesis experiments, five colonies were able to reproducibly support photoautotrophic growth. Plasmid isolation from *R. capsulatus* and DNA sequencing confirmed that the suppressor mutation, i.e., a codon change from GCT to GTT, resulted in an alanine to a valine substitution at residue 375 of the large subunit. Whereas all the five plasmids had identical base changes that resulted in the A375V substitution, one of them also carried an additional mutation in the large-subunit gene (*rbcL*) that would cause phenylalanine at position 97 to be changed to leucine. Site-directed mutagenesis and subsequent screening showed that neither the F97L single mutant nor the F97L/D103V double mutant was able to complement *R. capsulatus* strain SBI/II $^-$ to photoautotrophic growth. These results were consistent with the observation that, in four of the five plasmids isolated, a single mutant substitution at Ala-375 (to a valine) was sufficient to suppress the negative phenotype caused by the D103V substitution. The A375V substitution was capable of suppressing the "negative" effects caused by both the D103V enzyme itself and

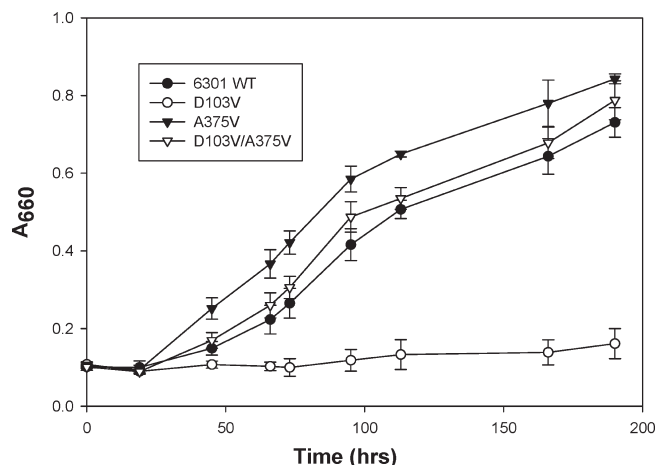


FIGURE 1: Wild-type and mutant *Synechococcus* Rubisco-dependent growth of *R. capsulatus* strain SBI/II $^-$ under CO_2 -dependent photoautotrophic growth conditions in sealed tubes under an atmosphere of 5–10% CO_2 /90–95% H_2 . Each data point represents the average \pm SD determined from four independent experiments.

D103V together with F97L. Thus, the A375V substitution on the same gene as the D103V substitution is, by definition, an intragenic suppressor that restores photoautotrophic growth when the resulting enzyme is used to complement the host *R. capsulatus* strain, SBI/II $^-$ (13). Several subsequent independent mutagenesis and selection experiments over the past 6 years by three different investigators all resulted in the isolation of the A375V enzyme as a specific suppressor of the D103V mutant enzyme. To further assess the specificity of the A375V suppressor, this alteration was combined with a previously identified G176D substitution that negatively affects enzyme activity and the ability to allow CO_2 -dependent growth in *R. capsulatus* strain SBI/II $^-$. Gly-176 is found in a completely unrelated region of the Rubisco large subunit (14). A375V was unable to suppress the negative phenotype of G176D. The apparent specificity of A375V toward mitigating the effects of D103V suggests structural correlations between Ala-375 and Asp-103 that influence significant aspects of catalysis.

The repeated isolation of the same suppressor (A375V) for D103V from several independent experiments and the strikingly conserved nature of Ala-375 among all form I enzymes, and its conservation as isoleucine in form II and serine in form III enzymes, provided a rationale for the creation of A375V, A375I, and A375S single mutant enzymes via site-directed mutagenesis. After construction and verification of the sequences of these site-directed mutants of *rbcL*, they were *trans*-complemented along with the wild-type small-subunit gene (*rbcS*) into *R. capsulatus* strain SBI/II $^-$ (21) via triparental matings and analyzed alongside wild-type, D103V, A375V, and D103V/A375V mutant enzymes. Whereas neither A375I nor A375S could support anaerobic photoautotrophic growth, both A375V and D103V/A375V mutants were fully capable of supporting anaerobic phototrophic CO_2 -dependent growth of the *R. capsulatus* SBI/II $^-$ host strain at high levels of CO_2 (Figures 1 and 2A). Unlike the wild-type *Synechococcus* enzyme, however, the A375V mutant was able to support anaerobic photoautotrophic growth at a limiting (1.5%) CO_2 concentration (Figure 2B). The inability of the wild-type *Synechococcus* enzyme to support anaerobic photoautotrophic growth at 1.5% CO_2 is most likely due to this enzyme's intrinsically high K_c value, as demonstrated and discussed in several earlier studies (4, 12, 13, 16). The results presented here

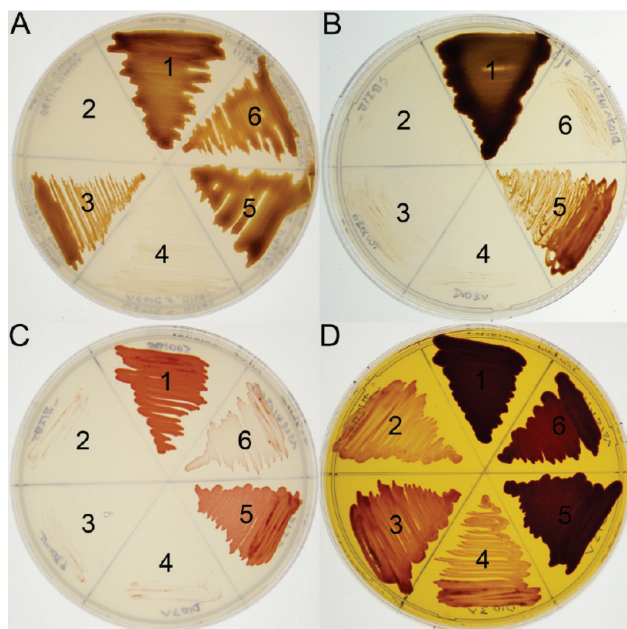


FIGURE 2: Growth phenotypes of *R. capsulatus* strain SBI/II[−] complemented with wild-type and mutant *Synechococcus* Rubisco enzymes: (A) anaerobic CO₂-dependent photoautotrophic growth under an atmosphere of 5–10% CO₂/90–95% H₂; (B) anaerobic CO₂-dependent photoautotrophic growth under an atmosphere of 1.5% CO₂/98.5% H₂; (C) aerobic CO₂-dependent chemoautotrophic growth under an atmosphere of 5–10% CO₂/40–45% H₂/50% air; (D) aerobic mixotrophic growth on a complex peptone–yeast extract medium under an atmosphere of 5–10% CO₂/40–45% H₂/50% air as previously described (1). In all four plates, sector 1 contains wild-type *R. capsulatus* strain SBI1003 with its endogenous form I and form II Rubisco genes that allow growth under all conditions (36); sector 2 contains *R. capsulatus* strain SBI/II[−] lacking endogenous functional Rubisco genes; sectors 3 to 6 contain *R. capsulatus* strain SBI/II[−] with plasmids containing the wild-type *Synechococcus rbcLrbcS* genes (sector 3), *rbcL-D103V/rbcS* (sector 4), *rbcL-A375V/rbcS* (sector 5), and *rbcL-D103V/A375V/rbcS* (sector 6).

confirm this conclusion as the wild-type *Synechococcus* enzyme was perfectly able to support anaerobic CO₂-dependent growth at high levels (5%) of CO₂ (Figure 2A). Most intriguing, however, was the unique ability of the A375V enzyme, and to a lesser extent the D103V/A375V suppressor mutant, to support aerobic CO₂-dependent chemoautotrophic growth (Figure 2C) and enhance aerobic mixotrophic growth in a complex organic growth media (Figure 2D). Inasmuch as Ala-375 resides in a hydrophobic pocket previously identified to be important for sensitivity to O₂ (18, 19), the ability of mutant cyanobacterial enzymes to specifically enable the *R. capsulatus* indicator strain to grow chemoautotrophically under aerobic conditions not supported by the wild-type *Synechococcus* enzyme (even at high levels of CO₂) suggests that this residue confers reduced sensitivity to oxygen inhibition (Figure 2C). The enhanced growth of both the A375V and D103V/A375V mutants under mixotrophic conditions in a CO₂-enriched atmosphere (Figure 2D) is also clearly attributable to the ability of these enzymes to fix and utilize CO₂ in aerobic environments. Moreover, the ability of the A375V mutant enzyme to support photoautotrophic growth under limiting CO₂ concentrations (Figure 2B) indicated that this mutation is a “positive” change relative to the wild-type enzyme.

Rubisco Expression, Activity, and in Vitro Stability. Western immunoblot analysis showed that approximate wild-type levels of holoenzyme were present in *R. capsulatus* cultures

containing D103V, A375V, and D103V/A375V mutant proteins (see Figure 1S of the Supporting Information). Furthermore, the Rubisco activities of crude extracts prepared from phototrophically grown A375V and D103V/A375V *R. capsulatus* cultures were comparable to that of the wild type, suggesting that the observed phenotypes arose from subtle changes in protein structure and were not due to major changes in holoenzyme levels *in vivo*. All of the mutant genes were subsequently expressed in *E. coli* and recombinant enzymes purified for enzymatic analysis. Both A375I and A375S had insignificant levels of Rubisco activity, perhaps because the changes led to the production of unstable enzymes or enzymes that were too severely compromised to be functional and hence were not used for further studies. However, all of the other mutant enzymes had significant amounts of Rubisco activity, ranging from 12% (for A375V) to 63% (for D103V/A375V) of the wild-type level of activity (Table 1). To further assess the effect of substitutions on the structural stability of purified enzymes *in vitro*, thermal inactivation assays were carried out. Whereas the wild-type, D103V, and D103V/A375V mutant enzymes retained comparable amounts of Rubisco activity (0–20%) after a 10 min incubation at 65 °C, the A375V single mutant enzyme consistently retained about 60% of its original activity at this temperature (Figure 3). Thus, the A375V substitution likely had a pronounced effect on both the structure and function of the enzyme, both of which could influence the behavior of the A375V mutant enzyme *in vitro* (Table 1) and *in vivo* (Figure 2).

Catalytic Properties of A375V/D103V and A375V Mutant Enzymes. As previously observed (12), the K_c value of the D103V enzyme is higher than the wild-type enzyme, accounting for its “negative” phenotype (Table 1). By contrast, the K_c value of the A375V enzyme is 23% lower than the wild-type enzyme, and this effect is partially manifested in the D103V/A375V suppressor enzyme as well. This may indicate that both Asp-103 and Ala-375 interact with a common structural or functional element that ultimately determines the Michaelis constant for CO₂. Thus, the A375V substitution likely causes changes in these structural interactions that ultimately contribute to the enhanced K_c and ability to support anaerobic CO₂-dependent phototrophic growth in the *R. capsulatus* indicator strain. An observation that is unique to the A375V substitution is its ability to cause opposing changes in the Michaelis constants for CO₂ and O₂. Indeed, the D103V/A375V and the A375V enzymes exhibit 28–36% increases in their K_o values (Table 1). As a result of these opposing changes in the Michaelis constants for CO₂ and O₂, the K_o/K_c ratio of the A375V enzyme (7.6) is considerably higher than the wild-type enzyme (4.6), while the K_o/K_c ratio of the D103V enzyme (2.2) is only about half of the wild-type value, which is restored to about 85% of the wild-type value in the D103V/A375V suppressor mutant. The inability of wild-type and D103V mutant enzymes to support aerobic CO₂-dependent chemoautotrophic growth (Figure 2C) can be attributable to their low K_o values. The beneficial changes in K_c values, relative to D103V, are likely responsible for conferring photoautotrophic growth competence to the A375V and D103V/A375V mutants (Figure 2A) and more specifically for enabling the A375V mutant to facilitate growth at lower CO₂ concentrations compared to the wild-type enzyme (Figure 2B). Much like the wild-type enzyme, the higher K_c value of the D103V/A375V mutant enzyme is likely the reason for the inability of this enzyme to support growth at 1.5% ambient CO₂ concentrations (Figure 2B). Despite the more favorable K_o/K_c ratio, the Ω ($V_c K_o/V_o K_c$) values of the A375V

Table 1: Kinetic Properties of Purified Recombinant Rubisco Enzymes

enzyme	k_{cat} (s^{-1})	Ω^a ($V_c K_o / V_o K_c$)	K_c^a ($\mu\text{M CO}_2$)	K_o^a ($\mu\text{M O}_2$)	K_o / K_c^b	V_c / V_o^b	K_{RuBP}^a (μM)
wild type	4.9 ± 0.9	39 ± 2	186 ± 13	850 ± 66	4.6	8.5	27 ± 3
D103V	4.9 ± 0.3	37 ± 3	388 ± 37	866 ± 40	2.2	16.8	25 ± 2
A375V	0.6 ± 0.1	32 ± 3	143 ± 7	1088 ± 43	7.6	4.2	34 ± 3
D103V/A375V	3.1 ± 0.3	30 ± 2	294 ± 31	1153 ± 142	3.9	7.7	269 ± 33

^aValues are the means \pm standard deviation ($n - 1$) of three separate enzyme preparations. ^bCalculated values.

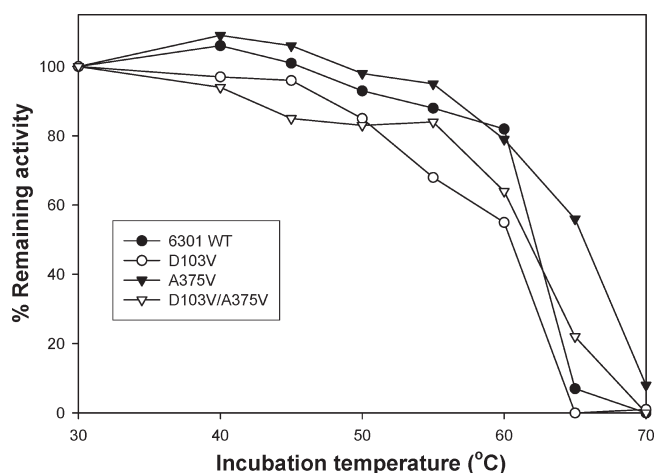


FIGURE 3: Thermal inactivation of *Synechococcus* 6301 wild-type and mutant enzymes. Aliquots of each enzyme were incubated at the respective temperatures for 10 min, cooled on ice, and assayed for the remaining amount of carboxylase activity. For each enzyme sample, the amounts of activity corresponding to the different incubation temperatures were normalized against the levels of activity calculated from the same enzyme incubated at 30 °C. Data are representative of two identical experiments that gave similar results. The A375V mutant enzyme appears to retain at least 55% of the original activity after the 65 °C incubation, indicating that the mutation likely results in major structural changes that should contribute to the catalytic properties of the enzyme assayed *in vitro*.

and D103V/A375V enzymes are slightly lower than the wild-type value (Table 1). It may also be inferred that the calculated V_c/V_o ratios of both the A375V and D103V/A375V enzymes were changed in a seemingly unfavorable direction. Moreover, mutant enzymes containing the A375V substitution showed decreases in k_{cat} , and the D103V/A375V suppressor mutant has a pronounced change in its K_{RuBP} value (Table 1). The rate-determining step of Rubisco's enzymatic mechanism is the addition of either CO_2 or O_2 to the 2,3-enediol(ate), rather than the enolization of RuBP that yields the 2,3-enediol(ate) (2). Consequently, an increase in the enzyme's K_{RuBP} value may also not limit its ability to support growth so long as the physiological levels of RuBP are in excess. Subtle changes in protein expression, not readily discernible by SDS-PAGE and Western blot analysis, or the differential structural stabilities and regulatory interactions may offset k_{cat} defects. Indeed, diverse Rubiscos or mutant forms of the same Rubisco, with lower k_{cat} values, have been shown to confer photoautotrophic growth of *Rhodobacter* Rubisco-deletion strains at rates comparable to those of the wild-type proteins (12, 13). Thus, it is likely that the opposing changes in K_o and K_c values (and consequent increase in the K_o/K_c ratio) may be the major determinants enabling enzymes containing the A375V substitution to support aerobic CO_2 -dependent (dark) chemoautotrophic growth under conditions where the wild-type or D103V negative mutant enzymes cannot (Figure 2C).

DISCUSSION

The involvement of a common set of active site residues for carboxylation and oxygenation has precluded identification of the molecular basis for Rubisco's differential interaction with its gaseous substrates CO_2 and O_2 (7). As more divergent Rubisco enzymes with differing Ω values are examined (1, 4), phylogenetic analyses, combined with structure-function studies, should increasingly contribute to delineating the molecular basis for the enzyme's ability to distinguish between the two competing substrates (27, 28). One might predict that genetic engineering of a virtually "oxygen-insensitive" Rubisco in a photosynthetic host might provide a selective growth advantage under aerobic conditions.

In several previous studies it was noted that photosynthetic bacteria expressing cyanobacterial Rubisco genes exhibit unusual sensitivity to the CO_2 levels provided in H_2/CO_2 gas mixtures used to support anaerobic photoautotrophic growth (12–14). By simply allowing CO_2 to accumulate in sealed growth vessels, or by increasing the level of CO_2 in H_2/CO_2 bubbled cultures, it was shown that the limitation in growth was related to the CO_2 concentration provided to the cultures. Providing different environmental growth conditions to select for mutant Rubisco proteins is particularly relevant to cyanobacterial Rubisco. This enzyme's normal habitat is within the carboxysomes of its natural cyanobacterial host, where the enzyme is enriched with high levels of CO_2 , negating any problem caused by this enzyme's inherently poor K_c . However, when the cyanobacterial Rubisco genes are expressed in *R. capsulatus*, where there is no apparent or substantial CO_2 -concentrating mechanism, CO_2 -dependent growth is directly tied to the properties of the Rubisco that the organism is made to utilize (4, 12–14, 17, 18). Indeed, even relatively small differences in the K_c values of cyanobacterial Rubisco appear to be well delineated by the subsequent phenotypic response of *R. capsulatus* (12–14). Such effects on growth provide convenient selection strategies to isolate mutations that affect cyanobacterial Rubisco function. Particularly intriguing for the selection purposes is the inability of the wild-type cyanobacterial Rubisco to support aerobic CO_2 -dependent chemoautotrophic growth in *R. capsulatus* strain SBI/II[−] (21), whereas Rubisco genes from other sources that encode proteins with more favorable properties are able to support aerobic chemoautotrophic growth.

Because Asp-103 of the *Synechococcus* large subunit is involved in a pair of reciprocal hydrogen bond interactions with Ser-367 at the interface of large-subunit dimers (Figure 4A), this hydrogen bond disruption was thought to be responsible for bringing about conformational changes in the holoenzyme that resulted in an increased K_c . Being far away from the active site, it was intriguing that the D103V mutation had a selective effect on the K_c value, with little or no changes in the other kinetic constants (13) (Table 1). A more detailed analysis of the X-ray crystal structure of *Synechococcus* Rubisco indicates that the

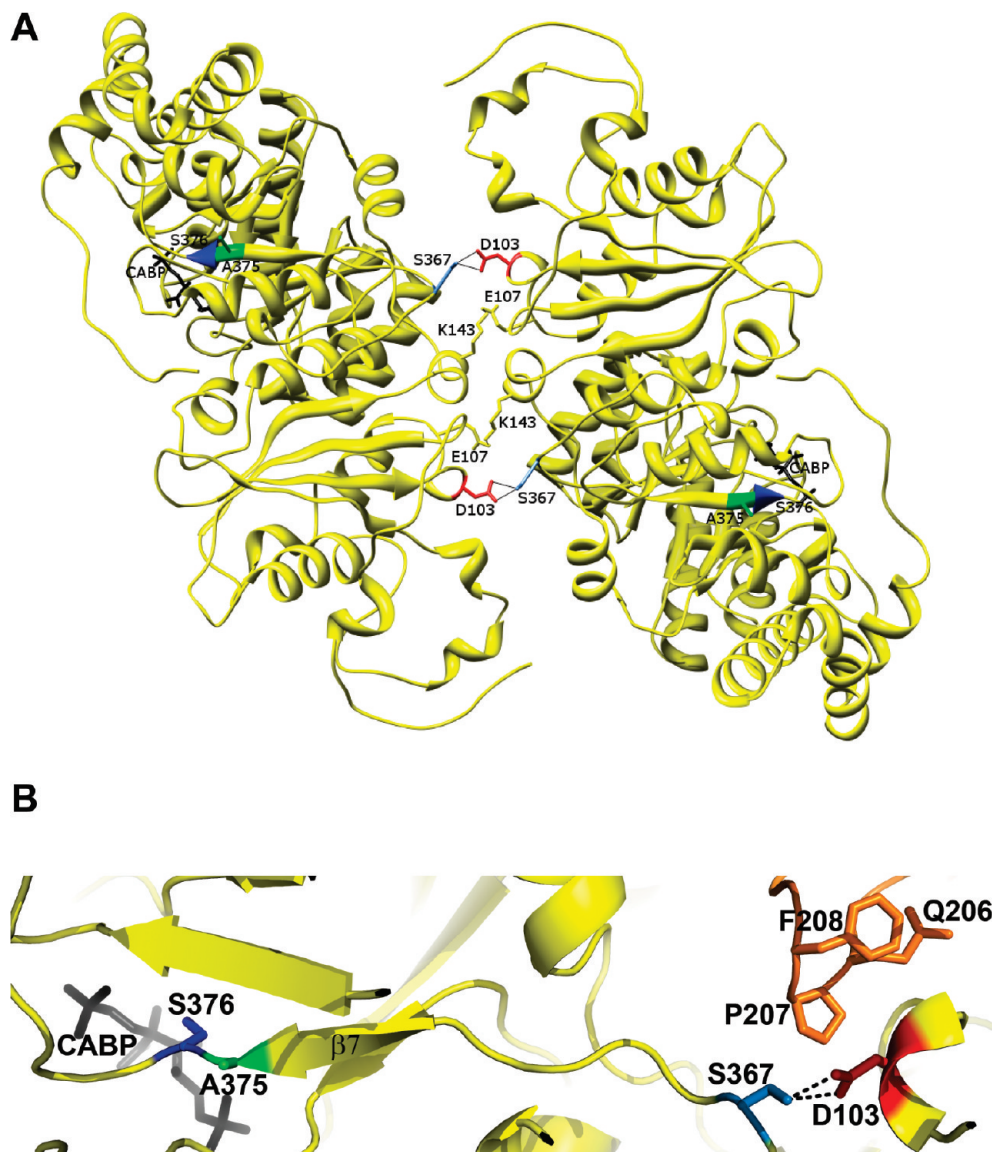


FIGURE 4: (A) Interaction of Asp-103 (red) from one monomer of a dimer of *Synechococcus* Rubisco large subunits with Ser-367 (light blue) of a monomer from a different dimer (1RBL) (37). There are two such interactions for each pair of interacting monomers (yellow). (B) Close-up view of the surface interactions of Asp-103 from one subunit with Ser-367 of another subunit and residues Gln-206, Pro-207, and Phe-208 of yet another large subunit (orange). Ser-367 is on the same strand as Ala-375 (green), which contacts active site Ser-376 (blue). Residues Gln-206 to Phe-208 are only a few residues away from the active site residues Lys-198, Asp-200, and Asp-201.

D103V substitution at the dimer–dimer interface could very well be affecting the active site in the adjacent large subunit, because its hydrogen bond partner, Ser-367, is adjacent to the β -strand (β -7) that leads into the active site. Asp-103 is also within 4 Å from residues Gln-206, Pro-207, and Phe-208 of yet another large subunit, which are present in the same strand as the active site residues Lys-198, Asp-200, and Asp-201 (Lys-201, Asp-203, and Asp-204 in spinach Rubisco) (Figure 4B). Thus, suppressor selection might have been expected to result in the identification of one or more residues in these regions. The identification of A375V as the specific suppressor for D103V is in support of this hypothesis, although it is curious that none of the other residues were selected after random mutagenesis. Perhaps this can be attributed to limitations imposed by the methodology employed, i.e., the requirement for absolutely favorable substitutions (structurally and functionally viable) to occur with an appropriate frequency and among all possible base changes sampled by error-prone PCR. Statistically, single-base changes best suit a scenario like this, which is the case with suppressor A375V identified in this study.

Ala-375 of the *Synechococcus* enzyme is adjacent to conserved active site Ser-376 (Ser-379 in spinach form I Rubisco and Ser-368 of *R. rubrum* form II Rubisco) (Figure 4B). Ser-376, and its equivalent residue in other Rubiscos, forms a hydrogen bond with the C3-hydroxyl group of the substrate RuBP (5). It is thus not surprising that the A375V substitution causes alterations in the K_{RuBP} (Table 1). The proximity to the active site Ser-376 also explains why all of the three substitutions at Ala-375 result in substantial loss of carboxylation activity. Ser-376 is only nine residues away from Ser-367 and could very well be the link to the active site that ultimately determines the changes in K_c caused by the loss of the D103–S367 hydrogen bond (Figure 4B). In previous studies where various substitutions were made in Ser-376 of the cyanobacterial enzyme, or the equivalent residues in the *R. rubrum* form II and *Chlamydomonas* form I enzymes, they all resulted in substantial loss of carboxylase activity and elevated K_{RuBP} values. In each instance the enzymes became more oxygen insensitive (19, 29, 30). Thus, the decreased oxygen sensitivities and k_{cat} values and the increased K_{RuBP} values that accompany

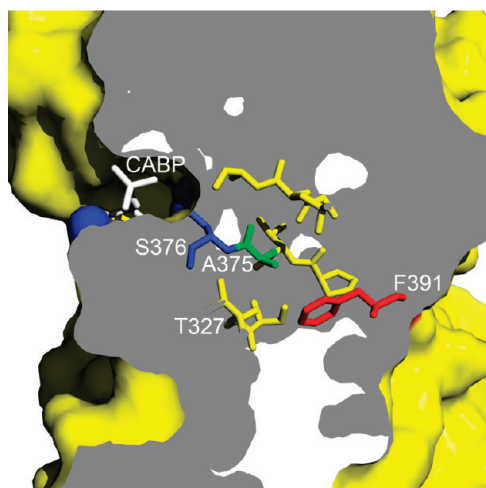


FIGURE 5: A hypothetical model for oxygen diffusion into the RubisCO active site via the hydrophobic pocket of residues. The figure shows the cross-sectional view of a surface rendering of the α/β -barrel motif of a single large subunit from the *Synechococcus* 6301 RubisCO (1RBL) (37). The active site, marked by the presence of CABP (black), is indicated. The active site residues, loop 6 Lys-331 and Ser-376 (blue), are on either side of the carbon that is attacked by CO_2 or O_2 in the rate-limiting step of the reaction mechanism. Ser-376 is adjacent to the hydrophobic pocket of residues that have been proposed to play a role in oxygen sensitivity. Residue Phe-391 (red), one of the hydrophobic pocket residues, appears to be positioned at the surface opposite to the active site.

the A375V mutant substitution observed here in either the wild type or the D103V mutants (Table 1) could very well be caused by similar changes in the interactions between active site Ser-376 and the substrate and/or Rubisco reaction intermediates. A manifestation of these changes in structural interactions could be seen in the form of the increased thermal stability of the A375V mutant enzyme (Figure 3). Notably, the S368A substitution in the *R. rubrum* (form II) enzyme caused an increase in the Ω value (19), whereas the equivalent substitution in the *Chlamydomonas* Rubisco (30) or the A375V substitution in the *Synechococcus* Rubisco (Table 1) results in lower Ω values relative to the wild type. This may be due to subtle differences in the interactions involving these residues in the form I and form II enzymes.

It is apparent that Ala-375 or its equivalent residue in other Rubisco enzymes (Ile or Ser in form II and III, respectively) is surrounded by a hydrophobic pocket of residues, a feature that appears to be common to all Rubisco enzymes. Substitutions (Ile and Val) at the equivalent Ser-363 in the *A. fulgidus* form III enzyme (18) and an S368A substitution in the *R. rubrum* form II enzyme (19) also resulted in enzymes that were less sensitive to O_2 . Because divergent Rubisco enzymes also have dissimilar catalytic properties (2–4), it is thus quite likely that the hydrophobic pockets surrounding Ala-375 (*Synechococcus*) or the equivalent residues in other Rubiscos might play a role in determining the enzyme's sensitivity to O_2 . Further analysis of residues in this region should provide additional insights as to why diverse Rubiscos exhibit differences in their interactions with the substrates CO_2 and O_2 . Certainly, with other proteins that require or use O_2 as a substrate, it has been observed that hydrophobic areas or pockets facilitate O_2 diffusion to the protein interior (32, 33). With respect to Rubisco, the aforementioned hydrophobic region may influence or facilitate the diffusion of oxygen to the active site (Figure 5). It can be seen in this

cross-sectional view of the large-subunit monomer that the substrate analogue, CABP, is present on one side of the surface, in a cleft juxtaposed by the active site residues Lys-331 and Ser-376 (blue). Lys-331 coordinates with the carboxyl group of CABP that would be analogous to the incoming CO_2 . The side chain of residue Ser-376, which seems to be involved in differential O_2 sensitivity, is on the other side of the CABP. The hydrophobic pocket of residues extending from Ala-375 leads to residue Phe-391 (red) on the other side of the surface of the protein. One might thus envision that the hydrophobic pocket shown here (Figure 5) might provide a facile diffusion channel to the active site from the opposite surface of the large-subunit monomer.

In summary, we have used the surrogate *R. capsulatus* system to isolate mutant forms of cyanobacterial Rubisco that greatly improve our perception of residues of the protein that influence oxygen sensitivity and mitigate important kinetic properties. This study clearly illustrates that changing Ala-375 to a more bulky valine residue influences both K_o and K_c of cyanobacterial Rubisco such that the A375V enzyme possesses a relatively high K_o/K_c ratio and this mutant enzyme appears inherently more stable than the wild-type enzyme. These properties, either singly or in combination, or with the mediation of other presently unknown factors, contribute to conferring the A375V mutant enzyme with the unique ability to support aerobic CO_2 -dependent growth in *R. capsulatus*. Moreover, it is apparent from the results of these studies that changes in the k_{cat} and/or any perceived trade-offs between k_{cat} and substrate specificity (34) may not be inimical for the construction or selection of Rubisco enzymes capable of substantial CO_2 fixation in the presence of high levels of oxygen. The current study represents a further effort to understand aspects of the bifunctionality of Rubisco and could very well form the basis for future genetic engineering of an "oxygen-insensitive" Rubisco.

SUPPORTING INFORMATION AVAILABLE

Western immunoblots (Figure S1A,B) depicting the levels of wild-type and mutant *Synechococcus* Rubisco proteins in photoheterotrophically and photoautotrophically grown *R. capsulatus* strain SBI/II[−]. This material is available free of charge via the Internet at <http://pubs.acs.org>.

REFERENCES

1. Tabita, F. R., Hanson, T. E., Li, H., Satagopan, S., Singh, J., and Chan, S. (2007) Function, structure, and evolution of the RubisCO-like proteins and their RubisCO homologs. *Microbiol. Mol. Biol. Rev.* 71, 576–599.
2. Spreitzer, R. J., and Salvucci, M. E. (2002) RubisCO: structure, regulatory interactions, and possibilities for a better enzyme. *Annu. Rev. Plant Biol.* 53, 449–475.
3. Jordan, D. B., and Ogren, W. L. (1981) Species variation in the specificity of ribulose biphosphate carboxylase/oxygenase. *Nature* 291, 513–515.
4. Tabita, F. R. (1999) Microbial ribulose 1,5-bisphosphate carboxylase/oxygenase: A different perspective. *Photosynth. Res.* 60, 1–28.
5. Cleland, W. W., Andrews, T. J., Gutteridge, S., Hartman, F. C., and Lorimer, G. H. (1998) Mechanism of Rubisco: the carbamate as general base. *Chem. Rev.* 98, 549–561.
6. Andersson, I., and Taylor, T. C. (2003) Structural framework for catalysis and regulation in ribulose-1,5-bisphosphate carboxylase/oxygenase. *Arch. Biochem. Biophys.* 414, 130–140.
7. Tabita, F. R., Satagopan, S., Hanson, T. E., Kreel, N. E., and Scott, S. S. (2008) Distinct form I, II, III, and IV Rubisco proteins from the three kingdoms of life provide clues about Rubisco evolution and structure/function relationships. *J. Exp. Bot.* 59, 1515–1524.
8. Gatenby, A. A., van der Vies, S. M., and Bradley, D. (1985) Assembly in *E. coli* of a functional multi-subunit ribulose biphosphate carboxylase from a blue-green alga. *Nature* 314, 617–620.

9. Tabita, F. R., and Small, C. S. (1985) Expression and assembly of active cyanobacterial ribulose 1,5-bisphosphate carboxylase/oxygenase in *Escherichia coli* containing stoichiometric amounts of large and small subunits. *Proc. Natl. Acad. Sci. U.S.A.* 82, 6100–6103.
10. Newman, J., and Gutteridge, S. (1993) The X-ray structure of *Synechococcus* ribulose-bisphosphate carboxylase/oxygenase-activated quaternary complex at 2.2 Å resolution. *J. Biol. Chem.* 268, 25876–25886.
11. Parry, M. A. J., Andralojc, P. J., Mitchell, R. A. C., Madgwick, P. J., and Keys, A. J. (2003) Manipulation of Rubisco: its amount, activity, function and regulation. *J. Exp. Bot.* 54, 1321–1333.
12. Falcone, D. L., and Tabita, F. R. (1991) Expression of endogenous and foreign ribulose 1,5-bisphosphate carboxylase-oxygenase (RubisCO) genes in a RubisCO deletion mutant of *Rhodobacter sphaeroides*. *J. Bacteriol.* 173, 2099–2108.
13. Smith, S. A., and Tabita, F. R. (2003) Positive and negative bioselection of mutant forms of prokaryotic (cyanobacterial) ribulose-1,5-bisphosphate carboxylase/oxygenase. *J. Mol. Biol.* 331, 557–569.
14. Smith, S. A., and Tabita, F. R. (2004) Glycine 176 affects catalytic properties and stability of the *Synechococcus* sp. strain PCC 6301 ribulose 1,5-bisphosphate carboxylase/oxygenase. *J. Biol. Chem.* 279, 25632–25637.
15. Parikh, M. R., Greene, D. H., Woods, K. K., and Matsumura, I. (2006) Directed evolution of Rubisco hypermorphs through genetic selection in engineered *E. coli*. *Protein Eng. Des. Sel.* 19, 113–119.
16. Green, D. M., Whitney, S. M., and Matsumura, I. (2007) Artificially evolved PCC6301 Rubisco variants exhibit improvements in folding and catalytic efficiency. *Biochem. J.* 404, 517–524.
17. Finn, M. W., and Tabita, F. R. (2003) Synthesis of catalytically active form III ribulose 1,5-bisphosphate carboxylase/oxygenase in archaea. *J. Bacteriol.* 185, 3049–3059.
18. Kreel, N. E., and Tabita, F. R. (2007) Substitutions at methionine 295 of *Archaeoglobus fulgidus* ribulose-1,5-bisphosphate carboxylase/oxygenase affect oxygen binding and CO₂/O₂ specificity. *J. Biol. Chem.* 282, 1341–1351.
19. Harpel, M. R., and Hartman, F. C. (1992) Enhanced CO₂/O₂ specificity of a site-directed mutant of ribulose-bisphosphate carboxylase/oxygenase. *J. Biol. Chem.* 267, 6475–6478.
20. Figurski, D. H., and Helinski, D. R. (1979) Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. *Proc. Natl. Acad. Sci. U.S.A.* 76, 1648–1652.
21. Paoli, G. C., Vichivanives, P., and Tabita, F. R. (1998) Physiological control and *cbh* gene regulation in *Rhodobacter capsulatus*. *J. Bacteriol.* 180, 4258–4269.
22. Whitman, W., and Tabita, F. R. (1976) Inhibition of D-ribulose 1,5-bisphosphate carboxylase by pyridoxal 5'-phosphate. *Biochem. Biophys. Res. Commun.* 71, 1034–1039.
23. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254.
24. Laemmli, U. K. (1971) Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
25. Kuehn, G. D., and Hsu, T. C. (1978) Preparative-scale enzymic synthesis of D-[¹⁴C]ribulose 1,5-bisphosphate. *Biochem. J.* 175, 909–912.
26. Chen, Z., Chastain, C. J., Al-Abed, S. R., Chollet, R., and Spreitzer, R. J. (1988) Reduced CO₂/O₂ specificity of ribulose-1,5-bisphosphate carboxylase/oxygenase in a temperature-sensitive chloroplast mutant of *Chlamydomonas reinhardtii*. *Proc. Natl. Acad. Sci. U.S.A.* 85, 4696–4699.
27. Du, Y. C., Peddi, S. R., and Spreitzer, R. J. (2003) Assessment of structural and functional divergence far from the large subunit active site of ribulose-1,5-bisphosphate carboxylase/oxygenase. *J. Biol. Chem.* 278, 49401–49405.
28. Spreitzer, R. J., Peddi, S. R., and Satagopan, S. (2005) Phylogenetic engineering at the interface between large and small subunits impart land-plant kinetic properties to algal Rubisco. *Proc. Natl. Acad. Sci. U.S.A.* 102, 17225–17230.
29. Lee, G. J., and McFadden, B. A. (1992) Serine-376 contributes to the binding of substrate by ribulose-bisphosphate carboxylase/oxygenase from *Anacystis nidulans*. *Biochemistry* 31, 2304–2308.
30. Zhu, G., and Spreitzer, R. J. (1994) Directed mutagenesis of chloroplast ribulosebisphosphate carboxylase/oxygenase: substitutions at large subunit asparagine 123 and serine 379 decrease CO₂/O₂ specificity. *J. Biol. Chem.* 269, 3952–3956.
31. Karkehabadi, S., Satagopan, S., Taylor, T. C., Spreitzer, R. J., and Andersson, I. (2007) Structural analysis of altered large-subunit loop-6/carboxy-terminus interactions that influence catalytic efficiency and CO₂/O₂ specificity of ribulose-1,5-bisphosphate carboxylase/oxygenase. *Biochemistry* 46, 11080–11089.
32. Fiorucci, S., Golebiowski, J., Cabrol-Bass, D., and Antonczak, S. (2006) Molecular simulations reveal a new entry site in quercetin 2,3-dioxygenase. A pathway for dioxygen? *Proteins* 64, 845–850.
33. Luna, V. M., Chen, Y., Fee, J. A., and Stout, C. D. (2008) Crystallographic studies of Xe and Kr binding within the large internal cavity of cytochrome *ba*₃ from *Thermus thermophilus*: Structural analysis and role of oxygen transport channels in the heme-Cu oxidases. *Biochemistry* 47, 4657–4665.
34. Tcherkez, G. G. B., Farquhar, G., and Andrews, T. J. (2006) Despite slow catalysis and confused substrate specificity, all ribulose bisphosphate carboxylases may be perfectly optimized. *Proc. Natl. Acad. Sci. U.S.A.* 103, 7246–7251.
35. Ogren, W. L. (1984) Photorespiration: pathways, regulation, and modification. *Annu. Rev. Plant Physiol.* 35, 415–442.
36. Paoli, G. C., and Tabita, F. R. (1998) Aerobic chemolithoautotrophic growth and RubisCO function in *Rhodobacter capsulatus* and a spontaneous gain of function mutant of *Rhodobacter sphaeroides*. *Arch. Microbiol.* 170, 8–17.
37. Newman, J., Branden, C. I., and Jones, T. A. (1993) Structure determination and refinement of ribulose 1,5-bisphosphate carboxylase/oxygenase from *Synechococcus* PCC6301. *Acta Crystallogr., Sect. D* 49, 548–560.
38. Lundqvist, T., and Schneider, G. (1991) Crystal structure of activated ribulose-1,5-bisphosphate carboxylase complexed with its substrate, ribulose-1,5-bisphosphate. *J. Biol. Chem.* 266, 12604–12611.
39. Kitano, K., Maeda, N., Fukui, T., Atomi, H., Imanaka, T., and Miki, K. (2001) Crystal structure of a novel-type archaeal Rubisco with pentagonal symmetry. *Structure* 9, 473–481.