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ROLE OF THE RUBISCO SMALL SUBUNIT

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INTRODUCTION

CO_2 and O_2 are mutually competitive at the active site of ribulose-1,5-bisphosphate (RuBP) carboxylase/oxygenase (Rubisco). Whereas carboxylation of RuBP is the rate limiting step of photosynthetic CO_2 fixation, oxygenation of RuBP ultimately leads to the loss of fixed carbon via the photorespiratory pathway. The CO_2/O_2 specificity (Ω) of Rubisco is determined by the V_{max} of carboxylation and oxygenation (V_c and V_o, respectively) and the K_m for CO_2 and O_2 (K_c and K_o, respectively) such that $\Omega = V_cK_o/V_oK_c$ (Laing et al. 1974). Directed mutagenesis of the active-site large subunit has deepened our understanding of the Rubisco catalytic mechanisms (reviewed by Hartman and Harpel 1994; Cleland et al. 1998; Spreitzer 1998), and classical genetic methods have identified large-subunit regions that control CO_2/O_2 specificity (reviewed by Spreitzer 1993, 1998). We now have a much better idea of the prospects and limitations for improving the enzyme in favor of carboxylation (Chen and Spreitzer 1992; reviewed by Spreitzer 1999). Because of its pivotal role in both plant productivity and the balance of our planet's atmospheric CO_2 concentration (reviewed by Tolbert 1997), further study of this enzyme is warranted. However, Rubisco contains two subunits, each present in eight copies. In plants and green algae, the well-studied 55-kD large subunit is coded by the chloroplast *rbcL* gene, but the 15-kD small subunit is coded by a family of nuclear *RbcS* genes. The role of the small subunit in Rubisco structure or catalytic efficiency is not known. Only recently have we succeeded in eliminating the two *RbcS* genes in the green alga *Chlamydomonas reinhardtii* (Khrebtukova and Spreitzer 1996). The *RbcS* deletion mutant lacks photosynthesis, but it can be maintained with acetate-supplemented medium in darkness. This mutant can also be rescued on minimal medium in the light by transformation with either the *RbcS1* or *RbcS2* gene (Khrebtukova and Spreitzer 1996). It is now possible to address questions about the structure-function relationships of the eukaryotic small subunit.

SPECIFIC AIMS OF THE CURRENT PROJECT

There are three specific aims in the current project: (1) Alanine scanning mutagenesis is being used to dissect the importance of the βA/βB loop, a feature unique to the eukaryotic small subunit. (2) Random mutagenesis is being used to identify additional residues or regions of the small subunit that are important for holoenzyme assembly and function. (3) Attempts are being made to express foreign small subunits in *Chlamydomonas* to examine the contribution of small subunits to holoenzyme assembly, catalytic efficiency, and CO_2/O_2 specificity.

ACCOMPLISHMENTS AND WORK IN PROGRESS

Directed Mutagenesis

It was previously reported that differential expression of *RbcS* family members in fern might be correlated with differences in Rubisco carboxylase specific activity (Eilenberg et al. 1991), but detailed kinetic analysis of fern Rubisco has not been done (Eilenberg et al. 1998). Because the two small subunits of *Chlamydomonas* differ by four residues (T22S, A47S, T128S, and F132W), and may be differentially expressed in darkness (Goldschmidt-Clermont and Rahire 1986), we constructed and analyzed an *RbcS1/RbcS2* chimera (Khrebtukova and Spreitzer 1996). Small subunits expressed by the *RbcS1*, *RbcS2*, or chimeric gene (following transformation into the *RbcS* deletion mutant) produced functional holoenzymes in light or darkness, but *RbcS1* appeared to produce a slightly higher transformation frequency and level of holoenzyme (Khrebtukova and Spreitzer 1996). More recently, when we compared holoenzymes purified from wild type, an *RbcS1* transformant, an *RbcS2* transformant, and the chimeric *RbcS* transformant, no significant differences were found in V_c, K_c/K_o, or holoenzyme thermal stability. Thus, both small subunits appear to be equivalent, further indicating that the differences in small-subunit sequence play no

essential role in Rubisco function. We have used *RbcS1* for directed mutagenesis studies due to its higher transformation frequency and presumed level of expression.

ALANINE SCANNING MUTAGENESIS

Several directed mutagenesis studies of the small subunit have been performed with cyanobacterial Rubisco expressed in *Escherichia coli* (reviewed by Spreitzer 1993, 1999). Changes in conserved residues dispersed within primary structure either have little effect, decrease holoenzyme stability, or decrease carboxylation catalytic efficiency. No clear picture of the role of the small subunit has emerged from these studies, and it has been difficult to define functionally-significant interactions between large and small subunits.

The small subunit of cyanobacterial Rubisco lacks 12 residues of a 22-residue loop (between β-strands A and B) that is characteristic of plant small subunits (Fig. 1) (Newman and Gutteridge 1993; Schreuder et al. 1993; Andersson 1996). This βA/βB loop extends between and over the ends of two large subunits from the bottom side of the α/β-barrel active site, and also interacts with the same loop of a neighboring small subunit (Andersson 1996). Wasmann et al. (1989) inserted the pea *RbcS* loop region (residues 45 through 65) into cyanobacterial *RbcS* (replacing residues 45 through 53), performed *in vitro* transcription and translation of the chimeric gene, and checked for holoenzyme assembly after import of the small subunit into pea chloroplasts. The chimeric small subunit was now able to assemble with pea large subunits within the chloroplast. Although it would appear that the loop is essential for assembly, subsequent directed mutagenesis and chloroplast import studies identified only one residue (Arg-53) that was critical to this process (Flachmann and Bohnert 1992; Adam 1995). The other substitutions (E54R, H55A, P59A, D63G, D63L, and Y66A; Flachmann and Bohnert 1992) may have affected Rubisco function, but not enough holoenzyme can be assembled by transport into isolated chloroplasts to permit biochemical analysis.

Spinach	39-	TDHGFV YREHHNSPGYYDGRYW	-74
Tobacco	39-	·E···· ···NNK·····	-74
Pea	39-	LEK··· ···NK·····	-74
Maize	39-	SKV··· ···NST··C·····	-73
Chlamydomonas	39-	AEADKAYVSNESAI·FGSV·CL···N·	-80
Synechococcus	39-	NEHSNPE EF·····L	-62

Figure 1. Comparison of small-subunit sequences in the βA/βB loop region. Assignment of secondary structure (β-strands A and B in *gray*) is based on the X-ray crystal structure of spinach Rubisco (Andersson 1996). Residues identical with those of the spinach small-subunit sequence are marked (·). Residues investigated via directed mutagenesis of pea, *Chlamydomonas*, and cyanobacterial small subunits are underlined in the respective sequences (Voordouw et al. 1987; Fitchen et al. 1990; Lee et al. 1991; Flachmann and Bohnert 1992).

Our primary focus has been placed on examining the significance of the βA/βB-loop residues that are conserved between *Chlamydomonas* and land plants, but that differ or are absent from the loop of the cyanobacterial small-subunit (i.e., *Chlamydomonas* Arg-59, Ser-64, Tyr-67, Tyr-68, Asp-69, and Arg-71) (Fig. 1). Although most of these residues and others have now been replaced via directed mutagenesis and transformation of *Chlamydomonas*, only two (F60A and R71A) fail to restore photosynthesis to the *RbcS* deletion mutant (Table I). In particular, Tyr-67, Tyr-68, and Asp-69 are highly conserved residues (Fig. 1), but they are clearly not essential for holoenzyme function or assembly (Table I). We doubt that the βA/βB loop is an "assembly domain" that actively facilitates holoenzyme assembly (Flachmann and Bohnert 1992). Further biochemical analysis of the mutant enzymes may reveal the true significance of this region with respect to Rubisco function. Preliminary results indicate that βA/βB-loop substitutions can influence carboxylation efficiency, CO_2/O_2 specificity, and holoenzyme stability (Table I).

Table I. Directed mutations in the Rubisco small subunit of *Chlamydomonas*. Mutations were made in the *RbcS1* gene, which was then transformed into a strain that lacks both *RbcS* genes (Khrebtukova and Spreitzer 1996). The N_2/O_2 ratio is the ratio of RuBP carboxylase activities determined at limiting CO_2 under 100% N_2 and 100% O_2. The N_2/O_2 ratio is comparable to K_c/K_o (Chen et al. 1988). ND, not determined.

Amino-acid substitution	Base change	Transformation frequency	Biochemical result
R59A	CGC → GCC	1.4×10^{-6}	↓thermal stability
R59E	CGC → GAG	1.7×10^{-6}	↓k_{cat}, ↓holoenzyme, ↓N_2/O_2 ratio
F60A	TTC → GCC	0 (per 8×10^{7})	no photosynthesis
S62A	AGC → GCC	1.0×10^{-5}	↓k_{cat}
S64A	TCT → GCC	ND	ND
L66F	CTG → TTC	5.0×10^{-7}	↓k_{cat}, ↓thermal stability, ↓N_2/O_2 ratio
L66G	CTG → GGC	5.0×10^{-6}	wild-type characteristics
Y67A	TAC → GCC	5.0×10^{-7}	↓k_{cat}
Y68A	TAC → GCC	1.3×10^{-5}	ND
D69A	GAC → GCC	6.5×10^{-6}	ND
R71A	CGC → GCC	0 (per 8×10^{7})	no photosynthesis
F132UAA	TTC → TAA	2.5×10^{-8}	↓k_{cat}, ↓thermal stability

ARG-59 IS NOT ESSENTIAL

In previous studies of pea small subunits transported into isolated chloroplasts (Flachmann and Bohnert 1992; Adam 1995), an R53E substitution was found to block holoenzyme assembly. This observation led Flachmann and Bohnert (1992) to conclude that the highly conserved Arg residue was essential for assembly. When the homologous residue in the *Chlamydomonas* small subunit (Fig. 1, Arg-59) was changed to Ala in the current project, we found that this mutant small subunit restored photosynthesis in our *RbcS* deletion strain (Table I). Although the mutant Rubisco has some associated thermal instability *in vitro* (Fig. 2), Arg-59 is clearly not essential for assembly.

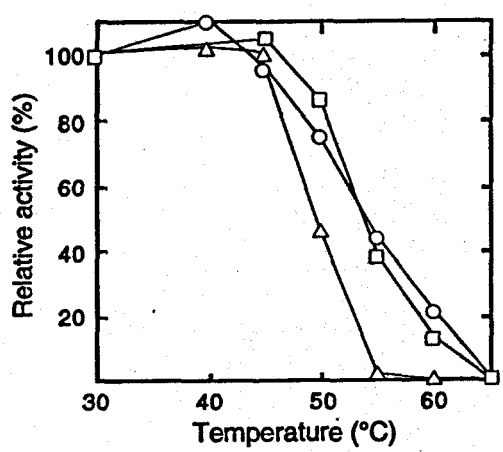

Figure 2. Thermal inactivation of purified Rubisco from wild type (○), *RbcS1*-wt (□), and mutant *RbcS1*-R59A (△). Purified Rubisco (0.1–0.4 mg ml^{-1}) in 10 mM $NaHCO_3$, 10 mM $MgCl_2$, 1 mM DTT, and 50 mM Bicine, pH 8.0, was incubated at each temperature for 10 min. The samples were then cooled on ice, and RuBP carboxylase activity was assayed at 25°C. Activities for each enzyme were normalized against the level of activity measured after the 30°C incubation. The *RbcS1*-wt strain was obtained by transforming the *RbcS* deletion mutant with the wild-type *RbcS1* gene (i. e., This strain lacks *RbcS2*, as does the *RbcS1*-R59A strain).

Because one could argue that Arg-59 has a different function in *Chlamydomonas* Rubisco than in pea Rubisco (Fig. 1), we also created an R59E substitution. Once again, photosynthesis-

competent transformants were recovered (Table I). However, in this case, the R59E mutant cells have a greatly-reduced level of Rubisco holoenzyme and grow exceptionally slowly, indicating that the presence of Glu at residue 59 is, in fact, detrimental to Rubisco assembly or stability. In other words, Arg-59 is not essential for assembly, but a negatively charged residue at this position appears to disrupt holoenzyme stability. Whereas R59A Rubisco has apparently normal kinetic constants, the R59E enzyme has a reduced k_{cat} and reduced N_2/O_2 ratio (Table II). Thus, the small-subunit βA/βB loop can contribute to the catalytic efficiency of the distant large-subunit active site. Further study via directed mutagenesis and transformation in *Chlamydomonas* will allow this region to be dissected in detail.

Table II. RuBP carboxylase activity of Rubisco purified from wild type and *RbcS1* directed mutants. Wild-type Rubisco contains two different small subunits (encoded by *RbcS1* and *RbcS2* genes), but transformants contain only the *RbcS1* gene.

Strain	Carboxylase activity at 25°C (μmol CO_2 h^{-1} mg Rubisco^{-1})			N_2/O_2 ratio
	100% N_2 9.86 mM $NaHCO_3$	(A) 100% N_2 0.53 mM $NaHCO_3$	(B) 100% O_2 0.53 mM $NaHCO_3$	(A/B)
Wild type	83.2	18.8	6.2	3.0
Transformant *RbcS1*-wt	89.5	16.7	6.0	2.8
Transformant *RbcS1*-R59A	81.8	16.3	5.5	3.0
Transformant *RbcS1*-R59E	6.8	1.5	0.6	2.5

LEU-66 SPECIFIC INTERACTIONS

We have also been investigating the βA/βB loop in a different way. Some years ago, a *Chlamydomonas* chloroplast mutant was recovered by genetic screening, named 68-4PP, that has a wild-type phenotype at 25°C, but lacks Rubisco holoenzyme and requires acetate for growth at 35°C (Chen et al. 1988). When Rubisco was purified from 25°C-grown cells, the mutant enzyme was found to have a decreased V_c, increased K_c/K_c, and a 13% decrease in Ω. Further studies showed that the mutant Rubisco is unstable at 35°C *in vivo* or *in vitro* (Chen et al. 1988, 1993). All of these altered enzyme properties arise from an *rbcL* mutation that causes an L290F substitution in large-subunit β-strand 5, far from the active site at the bottom of the α/β-barrel (Chen et al. 1988). More recently, photosynthesis-competent revertants were recovered from mutant 68-4PP (L290F) that arise from second-site mutations in *rbcL* (Hong and Spreitzer 1997). Either an A222T substitution in α-helix 2 or a V262L substitution below β-strand 4 (Hong and Spreitzer 1997) improves the thermal stability of the original mutant Rubisco and increases its Ω to the wild-type value (Hong and Spreitzer 1997). The "long distance" interactions between residue 290 and residues 222 and 262 are particularly interesting because all three residues are in Van der Waals contact with small-subunit residues in the βA/βB loop (residues Pro-59, Gly-60, and Tyr-61 of spinach) (Fig. 3) (Andersson 1996). Now that it is possible to perform directed mutagenesis and transformation of the *Chlamydomonas* small subunit, the significance of these comparable residues can be studied (residues Cys-65, Leu-66, and Tyr-67 in *Chlamydomonas*, see Fig. 1). We have created an L66F substitution in the small subunit that should mimic the effect of the L290F substitution in the large subunit (Fig. 3). This L66F small-subunit mutant has the same temperature-conditional phenotype as the L290F large-subunit mutant (Chen et al. 1988), and both mutant enzymes display similar thermal instability *in vitro* (Chen et al. 1993). Preliminary biochemical analysis further indicates that the L66F and L290F enzymes have catalytic properties

ered in similar ways (Chen et al. 1988, 199[illegible]993). Because land plants and some green algae y at residue 66 (e. g., Fig. 1), we also crea[illegible]reated an L66G substitution. The L66G mutant similar to the wild-type enzyme, indicating [illegible]ng that it is the increased size of residue 66, not identity, that is responsible for the modi[illegible]odified properties of the L66F mutant enzyme. nteraction at the large/small-subunit interf[illegible]rface is specific. It may be possible to select esis-competent revertants of mutant L66F [illegible]F at 35°C to further explore this site.

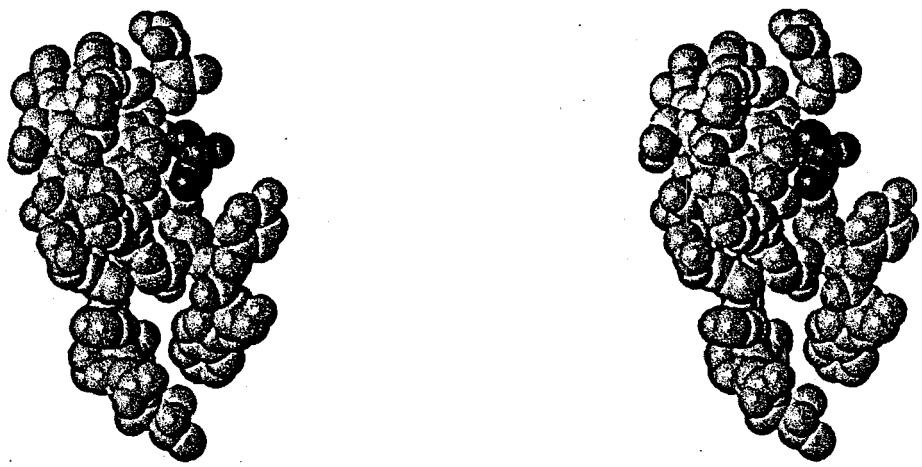

Figure 3. Stereo image of the spinach βA/[illegible]βA/βB loop (residues 46–67 in *green*) (Andersson 1996). Interactions between th[illegible]n the Rubisco large and small subunits of *Chlamydomonas* that may influence ca[illegible]catalysis are illustrated. An L290F substitution (*pink*) at the bottom of the lar[illegible]large-subunit α/β barrel (Chen et al. 1988) is complemented by a V262L substitu[illegible]stitution (*light blue*) in the same large subunit or by an A222T substitution (*dark b[illegible]rk blue*) in a neighboring large subunit (Hong and Spreitzer 1997). Although[illegible]n these large-subunit residues are relatively far from each other, all are in [illegible] in close contact with small-subunit residues. Large-subunit residues 222[illegible]2, 262, and 290 are identical in *Chlamydomonas* and spinach Rubisco. T[illegible] The spinach small subunit contains Gly at residue 60 (*red*), but *Chlam*[illegible]*amydomonas* contains Leu. The *Chlamydomonas* small subunit also conta[illegible]ntains six additional residues (Fig. 1) that would reside between Val-51 and Tyr-[illegible]Tyr-52 (both in *yellow*) of the spinach small subunit.

C-TERMINAL TR[illegible]TRUNCATION

ibisco enzymes from eukaryotic red and b[illegible]n brown algae have been found to have Ω values ier than those of land-plant enzymes (e. g[illegible]. g., *Chlamydomonas* Ω = 60, spinach Ω = 80, ieca Ω = 110, *Galdieria partita* Ω = 230[illegible]30) (Read and Tabita 1992, 1994; Uemura et al. n an attempt to understand the structural [illegible]al basis for these differences, the X-ray crystal nas recently been solved for Rubisco fro[illegible]rom the thermophilic red alga *Galdieria partita* n et al. 1999). This structure shows that the[illegible]ne C-terminus of the small subunit, which is 32 onger than that of spinach, folds back on [illegible]n itself and interacts with the N-terminal side of loop (Fig. 3). The *Galdieria* βA/βB loop, [illegible]n, like that of the cyanobacterium *Synechococcus* acks the extra residues that are characteris[illegible]ristic of green plants and algae. Sugawara et al. oposed that the small-subunit C-termina[illegible]nal extension may play a role in an enhanced ability and elevated Ω value of *Galdieria* [illegible]*a* Rubisco. The *Chlamydomonas* small subunit n extended C-terminus, 11 residues longe[illegible]ger than that of the spinach small subunit. By ; directed mutagenesis and transformation[illegible]on, we have introduced a stop codon that would : length of the small subunit by 9 residues[illegible]ues. Surprisingly, this F132UAA mutant strain otosynthetic competence (Table I). Althou[illegible]nough its V_c is reduced by 56% relative to the V_c ld-type enzyme, it has a normal N_2/O_2 r[illegible]ratio. Thus, this C-terminal extension is not

essential for holoenzyme function or assembly. However, the F132UAA mutant enzyme displays significant thermal instability *in vitro* (Fig. 4), supporting the idea that the C-terminus plays a role in holoenzyme stability. Based on the *Galdieria* structure, we suspect that the C-terminus of the *Chlamydomonas* small subunit interacts with the extra six residues of its βA/βB loop (Fig. 3).

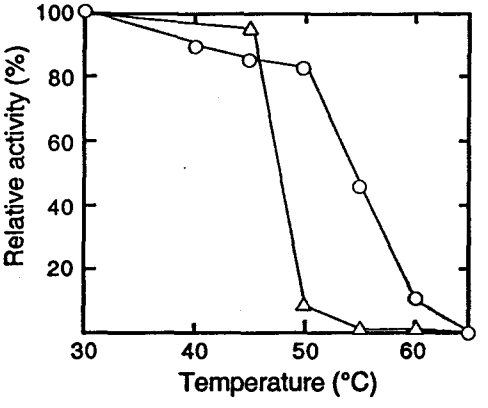

Figure 4. Thermal inactivation of purified Rubisco from wild type (○) and mutant *RbcS1*-F132UAA (△). Purified Rubisco (0.1–0.4 mg ml^{-1}) in 10 mM $NaHCO_3$, 10 mM $MgCl_2$, 1 mM DTT, and 50 mM Bicine, pH 8.0, was incubated at each temperature for 10 min. The samples were then cooled on ice, and RuBP carboxylase activity was assayed at 25°C. Activities for each enzyme were normalized against the level of activity measured after the 30°C incubation.

Random Mutagenesis

Random genetic screening and selection in *Chlamydomonas* have proven to be quite useful for identifying functionally-significant regions of the Rubisco large subunit (reviewed by Spreitzer 1993, 1998). The importance of such regions could not have been predicted by simply examining the existing crystal structures. Although *rbcL* mutants were recovered at random, the identified amino-acid substitutions are not distributed randomly. Only the most deleterious (and important) substitutions are recovered, and, by selecting for second-site suppressors, specific interactions within tertiary and quaternary structure are identified (e. g., Thow et al. 1994; Spreitzer et al. 1995; Hong and Spreitzer 1997). *RbcS* mutants have not been recovered in *Chlamydomonas* by screening for photosynthesis-deficient, acetate-requiring mutants. Because there are two *RbcS* genes, and either one is sufficient for photosynthetic growth (Khrebtukova and Spreitzer 1996), the probability of getting a deleterious point mutation in both genes simultaneously is vanishingly small (reviewed by Spreitzer 1993). Now that we have a *Chlamydomonas* strain that lacks both *RbcS* genes (Khrebtukova and Spreitzer 1996), the application of *in vivo* genetic methods is possible.

XL-1 RED AND TEMPERATURE-CONDITIONAL MUTANTS

With respect to the second specific aim of the current project, random mutagenesis of *RbcS* in the DNA-repair-deficient XL-1 Red *E. coli* strain has been found to give rise to temperature-conditional, photosynthesis-deficient strains when transformed into the *Chlamydomonas RbcS* deletion strain. However, none of these mutants has yet been found to arise from a mutation in *RbcS*. When grown with acetate at the nonpermissive temperature of 35°C, all of the mutant strains retain substantial levels of RuBP carboxylase activity. Because DNA integrates into the nucleus by nonhomologous recombination (Kindle 1990), we anticipated that the transforming DNA might produce nonspecific temperature-conditional mutants by disrupting other genes. We also expect that temperature-conditional *RbcS* mutants will be rare (reviewed by Spreitzer 1993). Thus, it is still necessary to screen more colonies to assess the utility of this method. Even one temperature-conditional *RbcS* mutant could be extremely valuable for identifying important regions of the small subunit (e. g., Spreitzer 1998).

SMALL-SUBUNIT SUPPRESSION

We have made an interesting discovery via genetic selection that has become a focus for further study. As apparent from the discussion above (see LEU-66 SPECIFIC INTERACTIONS, page 5),

it took a long time to recover second-site *rbcL* suppressors (A222T and V262L) of the 68-4PP (L290F) *rbcL* mutant (Chen et al. 1988; Hong and Spreitzer 1997). The reason for this is that most photosynthesis-competent revertants of the temperature-conditional 68-4PP mutant arise from mutations in nuclear genes (Chen et al. 1990, 1993; Hong and Spreitzer 1997). Detailed genetic and biochemical analysis of one of these suppressors indicated that it acted posttranslationally to restore the low Ω value and thermal instability of 68-4PP (L290F) mutant Rubisco, but this nuclear suppressor mutation did not reside in either of the two *RbcS* genes (Chen et al. 1990, 1993; Gotor et al. 1994). Recent genetic analysis has indicated that there are two distinct nuclear genes that can suppress the 68-4PP (L290F) *rbcL* mutation. Now that we have a better understanding of the structural interactions near the small-subunit βA/βB loop (Hong and Spreitzer 1997; see LEU-66 SPECIFIC INTERACTIONS, page 5), we decided to screen alleles of the second nuclear-suppressor gene for mutations in *RbcS*. By PCR amplifying and sequencing DNA in the small-subunit βA/βB-loop region (Fig. 1), we found that either an N54S or A57V substitution in the *RbcS2* small subunit can complement the L290F substitution. Of the three revertant strains analyzed, one contained the *RbcS2*-N54S mutation and two contained the *RbcS2*-A57V mutation. These small-subunit substitutions increase the amount of Rubisco in 35°C-grown cells to the wild-type level. A comparison of Rubisco enzymes purified from 25°C-grown cells also indicated that the *RbcS* mutations improve catalysis of the original 68-4PP (L290F) mutant enzyme by increasing its Ω value to that of wild-type Rubisco (Table III). This is an exciting finding because we now have evidence that the small-subunit βA/βB-loop region can, in fact, influence Rubisco Ω.

Table III. RuBP carboxylase activity and CO_2/O_2 specificity (Ω) of Rubisco purified from the temperature-conditional 68-4PP mutant and its photosynthesis-competent revertants R116-1B and R116-10C. The N_2/O_2 ratio is comparable to K_c/K_o (Chen et al. 1988). Ω values are the average of three separate enzyme preparations ±SD as determined with the [^{3}H]RuBP/NaH$^{14}CO_3$ dual-labeling assay (Jordan and Ogren 1981a; Spreitzer et al. 1982).

Strain	Carboxylase activity at 25°C (μmol CO_2 h^{-1} mg Rubisco^{-1})			N_2/O_2 ratio	Ω
	100% N_2 9.86 mM $NaHCO_3$	(A) 100% N_2 0.53 mM $NaHCO_3$	(B) 100% O_2 0.53 mM $NaHCO_3$	(A/B)	(V_cK_o/V_oK_c)
Wild type	65.2	11.2	3.7	3.0	60 ±1
Mutant 68-4PP (*rbcL*-L290F)	19.9	2.9	1.2	2.4	54 ±1
Revertant R116-1B (*rbcL*-L290F, *RbcS2*-N54S)	44.9	6.1	2.2	2.8	60 ±3
Revertant R116-10C (*rbcL*-L290F, *RbcS2*-A57V)	41.6	11.6	4.7	2.5	58 ±1

Genetic analysis of the R116-1B and R116-10C revertants, which contain the *RbcS2*-N54S and *RbcS2*-A57V suppressor mutations, respectively, confirmed that the suppressors were inherited in a Mendelian pattern and have similar map distances (Table IV). After recovering *mt*$^-$ revertants from these crosses, backcrosses were performed to obtain progeny that lacked the original 68-4PP *rbcL* mutation but retained the *RbcS2* suppressor mutation. In the absence of the *rbcL* mutation, both suppressors were found to have wild-type (photosynthesis-competent) phenotypes. Strains that contain an *RbcS2* suppressor mutation are, in fact, heterogeneous for wild-type *RbcS1* and mutant *RbcS2* small subunits. It is also interesting that the small-subunit N54S and A57V

substitutions occur in the region of the βA/βB loop that is six residues longer than the land-plant small-subunit βA/βB loop (Figs. 1 and 3). We suspect that this inserted region is relatively far from both the large-subunit L290F substitution and the large-subunit residues that suppress it (A222T and V262L) (Hong and Spreitzer 1997) (Fig. 3).

Table IV. Genetic analysis of photosynthesis-competent R116-1B and R116-10C revertants of the temperature-conditional, photosynthesis-deficient 68-4PP mutant (*rbcL*-L290F).

Test cross	Tetrads (score *ac-pf*)[a]			Map distance[b] (X 100)
	PD	NPD	T	
rbcL-L290F, *Rbc*S2-N54S, +, *mt*$^+$ X +, +, *pf-2*, *mt*$^-$ (revertant R116-1B) (wild type)	10	10	13	20
rbcL-L290F, *Rbc*S2-A57V, +, *mt*$^+$ X +, +, *pf-2*, *mt*$^-$ (revertant R116-10C) (wild type)	5	5	4	14

[a]All progeny receive the temperature-conditional chloroplast *rbcL*-L290F mutation, but only two in a tetrad receive the nuclear *RbcS2* suppressor mutation. In the absence of the suppressor, progeny lack photosynthesis at 35°C and require acetate (*ac*) for growth. Segregation was scored relative to the centromere-linked paralyzed flagella (*pf*) marker, which allows parental-ditype (PD), nonparental-ditype (NPD), and tetratype (T) tetrads to be defined. [b]Distances (% recombination) between the *RbcS2* suppressors and their centromeres were calculated as 0.5T / (PD + NPD + T).

DNA sequencing and restriction-enzyme analysis have been used to verify that *RbcS1* has a wild-type sequence and that the *RbcS2* mutations are genetically linked with the suppressor phenotypes (e. g., see Fig. V). This work has also allowed the identification and recovery of strains that contain the *RbcS2* suppressor mutations but lack the original *rbcL*-L290F mutation (e. g., backcross progeny in Fig. V, lanes 9 and 10).

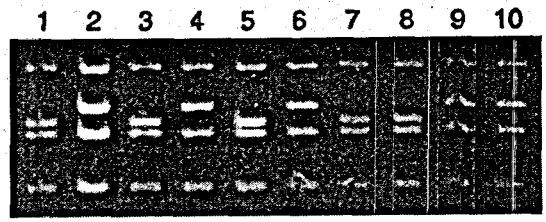

Figure V. Segregation of the *RbcS2*-A57V suppressor mutation in reciprocal crosses. DNA was purified, and a 818-bp *RbcS2* sequence was amplified by PCR. The PCR product was digested with *Hae*III and separated on a 3.5% agarose gel. The mutation eliminates a *Hae*III site, increasing the size of a 189-bp fragment to 210 bp. Lane 1, wild type; lane 2, revertant R116-10C (*RbcS2*-A57V, *rbcL*-L290F); lanes 3–6, progeny from the cross R116-10C *mt*$^+$ X wild-type *mt*$^-$ (progeny 3 and 5 had temperature-conditional, acetate-requiring phenotypes); lanes 7–10, progeny from the cross wild-type *mt*$^+$ X R116-10C *mt*$^-$ (progeny 7–10 had wild-type phenotypes).

Because we have a strain that lacks both *RbcS* genes (Khrebtukova and Spreitzer 1996), we have recently succeeded in transforming each of the *RbcS2* suppressor genes into *Chlamydomonas* in the absence of *RbcS1*. If the suppressors improve the V_c and Ω of mutant *rbcL*-L290F Rubisco, perhaps they will improve the wild-type enzyme. However, the *RbcS2*-N54S and *RbcS2*-A57V

strains were recovered at a substantially lower transformation frequency (2.5×10^{-7}) than that for the wild-type *RbcS2* gene (3.4×10^{-6}), indicating that the homogeneous *RbcS2*-N54S and *RbcS2*-A57V enzymes may be compromised in function or stability. By employing the [^{3}H]RuBP/ $NaH^{14}CO_3$ dual-labeling assay (Jordan and Ogren 1981a), we have recently found that the *RbcS2* mutant enzymes have wild-type Ω values. Further kinetic analysis of the mutant enzymes is in progress. Regardless of the outcome, the discovery of these small-subunit substitutions will help guide future directed-mutagenesis studies of the βA/βB loop.

X-Ray Crystallography

In a separate but related project, we are also making progress towards solving the X-ray crystal structure of *Chlamydomonas* Rubisco (Yen et al. 1998). Once this structure is available, it will become easier to ask questions about the large/small-subunit interface.

Expression of Foreign Small Subunits

The kinetic constants and CO_2/O_2 specificities differ among Rubisco enzymes from phylogenetically diverse species (Jordan and Ogren 1981b). To determine whether these differences may arise from differences in the small subunit, we have tried to complement the photosynthesis deficiency of our *RbcS* deletion mutant with *RbcS* genes from land plants. A few attempts with genomic clones proved unsuccessful, and we assumed that differences in introns and transit peptides may prevent expression of mature plant small subunits in *Chlamydomonas*. As a first step for eliminating these potential problems, we used directed mutagenesis to construct a gene in which the mature small-subunit coding region of the *Chlamydomonas RbcS1* gene is replaced exactly by the mature small-subunit coding region of *Chlamydomonas RbcS2* cDNA (Goldschmidt-Clermont and Rahire 1986). In other words, this new gene has wild-type 5' and 3' genomic flanking sequences and encodes the transit peptide, but it lacks the three introns that are characteristic of the *Chlamydomonas RbcS* genes (Goldschmidt-Clermont and Rahire 1986). The objective was to exploit engineered *Xma*I and *Xba*I restriction sites for the routine, but precise, exchange of foreign *RbcS* cDNA in place of the mature protein coding region of *Chlamydomonas RbcS1*. However, initial attempts at transforming the *RbcS* deletion mutant with the *Chlamydomonas RbcS2* cDNA construct failed. Attempts with spinach *RbcS* cDNA (Martin et al. 1996) and the cyanobacterial gene (Kleman et al. 1996) precisely replacing the *Chlamydomonas* mature small-subunit coding region also proved unsuccessful. To determine that our constructions were error free, we replaced the *Chlamydomonas RbcS2* cDNA sequence with the missing genomic sequence and observed transformation at the usual frequency.

Only recently have we succeeded in obtaining several *Chlamydomonas RbcS2* cDNA transformants. This was accomplished by providing a long expression time after transformation to allow for the accumulation of Rubisco to maximal levels prior to selection (e. g., Spreitzer and Chastain 1987). However, the transformation frequency may be too low for the recovery of foreign *RbcS* cDNA transformants, which we assume will be expressing Rubisco at lower levels or in compromised conditions. Other approaches may be necessary for expressing foreign small subunits in *Chlamydomonas*. Lumbreras et al. (1998) have recently found that introns 1 and 2 of *Chlamydomonas RbcS2* can increase the transformation frequency and level of mRNA for a bacterial gene expressed in *Chlamydomonas*. However, when we eliminated only intron 3 during the engineered replacement of the longer βA/βB-loop region of *Chlamydomonas RbcS1* with the shorter βA/βB region of spinach *RbcS*, no photosynthesis-competent transformants were obtained. Either the longer βA/βB loop is required for assembly or function of *Chlamydomonas* Rubisco, or intron 3 also plays a significant role in small-subunit expression.

PUBLICATIONS ACKNOWLEDGING THE AWARD

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