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DESIGN OF A BIOLOGICAL SYSTEM TO SELECT FOR ALTERATIONS IN THE KEY KINETIC PROPERTIES OF RIBULOSE 1,5-BISPHOSPHATE CARBOXYLASE/OXYGENASE (RUBISCO)

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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*****

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ABSTRACT

A system for biological selection of randomly mutagenized RubisCO genes was designed in which the photosynthetic bacterium *Rhodobacter capsulatus* served as a host. For these studies, the RubisCO genes (*rbcLS*) originating from the cyanobacterium *Synechococcus* PCC6301 were used to complement a RubisCO deletion mutant of *R. capsulatus* which could not grow anaerobically in minimal salts medium under a CO₂/H₂ atmosphere (photoautotrophy), or aerobically in the dark under a CO₂/H₂/O₂ atmosphere (chemoautotrophy). When provided with the *rbcLS* genes in trans, *R. capsulatus* could grow photoautotrophically with 5% CO₂, but not with 1.5% CO₂ in the atmosphere, and it could not grow chemoautotrophically. The *Synechococcus* PCC6301 RubisCO has a poor affinity for CO₂, and it was therefore hypothesized that random mutagenesis might lead to improvements in the affinity for CO₂, if such a trait could be selected for, by isolating complemented *R. capsulatus* strains capable of photoautotrophic growth in 1.5% CO₂. Using the mutator strain *Epicurian coli* XL1-Red (Stratagene), plasmid libraries of mutant *rbcLS* were generated. When introduced into the host strain, phenotypic differences between the deletion strain complemented with wild-type *rbcLS* and transconjugates carrying mutated genes were used to identify potentially interesting mutations. Mutants that were able to complement to the positive selection condition of photoautotrophic growth with 1.5% CO₂ were isolated and characterized. They were found to lack the anticipated changes in affinity for CO₂, and were unaffected for any
kinetic properties we measured, with a single exception. One mutant, F342V, had an increased affinity for the substrate RuBP. The positive mutants were used as templates for mutagenic PCR and selection at an even lower level of CO$_2$ (0.5 %). Unique mutants capable of growth under this more stringent condition were isolated, and were also characterized for their kinetic properties. Again, the mutants lacked any significant changes in affinity for CO$_2$.

Interesting mutants, with changes in the affinity for CO$_2$, were isolated through negative selection, in which alleles incapable of complementing _R. capsulatus_ to photoautotrophic growth with 5% CO$_2$ were identified. These negative mutants typically resulted from alterations in peptide folding and solubility, holoenzyme assembly, or gene expression. However, mutations at two residues, D103 and G176, resulted in enzymes that were folding/assembly-competent, but were greatly affected for different kinetic parameters, including the $K_m$ for CO$_2$. This work demonstrated that an approach of random mutagenesis and bioselection can lead to a deeper understanding of how various residues confer important kinetic properties to RubisCO. With further refinements this system may lead to even more interesting mutant forms of enzyme for future structure-function studies.
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INTRODUCTION

The Calvin-Benson-Bassham (CBB) reductive pentose phosphate pathway is the most frequently encountered scheme by which autotrophic organisms harness inorganic carbon into the organic molecules required for life. Ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO, E.C. 4.1.1.39) is the enzyme that catalyzes CO₂ fixation in the CBB cycle. RubisCO is the most abundant enzyme on the planet, and its significance to all life cannot be overstated. Its abundance, however, may serve to compensate for its inherent inefficiency, which has puzzled scientists for decades.

RubisCO fixes CO₂ onto ribulose 1,5-bisphosphate (RuBP), at a rate of 2 to 5 molecules/sec, generating two molecules of 3-phosphoglycerate (PGA). In addition to its poor catalytic rate, another limitation of the enzyme is that O₂ competes with CO₂ for the bound enediolate of RuBP at the active site. When O₂ is fixed, one molecule of PGA and a molecule of 2-phosphoglycolate (2-PG) are generated (Figure 1). 2-PG enters an oxidative pathway, which ultimately results in carbon loss for the organism, making the oxygenase activity of RubisCO the primary limitation of plant productivity (Cleland et al., 1998). A longstanding goal of researchers, therefore, has been to alter the kinetic properties of RubisCO, which in theory could lead to increased primary productivity of important crop plants.
**Figure 1.** The general reaction catalyzed by RubisCO proceeds through an enediol intermediate of the substrate ribulose-1,5-bisphosphate. The carboxylase activity generates two molecules of phosphoglycerate, and the oxygenase activity generates a molecule of phosphoglycerate and a molecule of phosphoglycolate. Partitioning between the carboxylase and oxygenase pathways ($v_c/v_o$) is defined by the product of the specificity factor ($\Omega$), which is the ratio of the Michaelis-Menten parameters for the reactions, and the concentrations of the gases at the active site (equations in box).
Figure 1. Carboxylation and oxygenation catalyzed by RubisCO.

\[
\frac{v_c}{v_0} = \Omega \left( \frac{[CO_2]}{[O_2]} \right)
\]

\[
\Omega = \frac{V_c K_o}{K_c V_0}
\]
There are three phylogenetically distinct forms of RubisCO thus far found in nature, forms I, II, and III (Gibson and Tabita, 1977; Tabita, 1999), and recently a RubisCO-like protein (RLP) was discovered that shows homology to RubisCO but is not functional in CO₂ fixation (Hanson and Tabita, 2001). The form I enzymes, found in plants, most algae, cyanobacteria, and most photosynthetic bacteria, are hexadecamers of eight large (catalytic) subunits, of ~55 kDa each, and eight small subunits, ranging from ~12 to 15 kDa, forming an L₈S₈ enzyme with a molecular mass typically of 550 kDa (Figure 2). Form II enzymes contain only multiples of large subunit dimers, (L₂)ₓ, and are found in photosynthetic bacteria, a few chemoautotrophic bacteria, and some dinoflagellates (Tabita, 1999). The paradigm for the form II RubisCOs is the dimeric enzyme from the nonsulfur purple bacterium *Rhodospirillum rubrum*. Recently described archaeal RubisCOs, the form III enzymes, are revealing even more variant structures, including an (L₂)₅ quaternary structure with pentagonal symmetry for the enzyme from *Thermococcus* (formerly *Pyrococcus*) *kodakaraensis* KOD1, and dimeric enzymes from the archaea *Methanococcus janaschii* and *Archaeoglobus fulgidus* (Watson et al., 1999; Kitano, 2001). In spite of this diversity, the reaction mechanism and active site are conserved across taxa, though the *in vivo* function may not be (Hanson and Tabita, 2001), and it is not understood why key kinetic parameters differ greatly among these enzymes, even among closely related proteins of the same class.

In all RubisCOs described to date, the active sites are formed when large subunits dimerize in a head-to-tail arrangement, such that the C-terminus of one subunit contacts the N-terminus of its partner. These dimers are the minimal functional requirement of all types of RubisCO (Hartman and Harpel, 1994; Figure 3). The C-terminus contains an
Figure 2. Representation of α-helices and β-sheets within the large (top panel) and small (lower panel) subunits of RubisCO from Synechococcus PCC6301 are shown. C and N refer to the C-terminus and N-terminus, respectively, of each subunit. The large subunit contains all of the known active site residues, which are labeled. Note that the active site residues in the N-terminal domain (E57, T62, W63, and N120) contribute to the active site when paired with the C-terminus of an adjacent large subunit, and do not complete the active site within this large subunit monomer.
Figure 2. The large and small subunits of Rubisco.
Figure 3. The dimer of large subunits is the minimal requirement for RubisCO activity. Shown here, one large subunit, in red, is in a head-to tail arrangement with its partner, shown in white. The large subunits modeled here were from the RubisCO of *Synechococcus* PCC6301. Note that no small subunits are shown in association with this dimer.
Figure 3. A dimer of large subunits.
eight-stranded α-β barrel domain, where most of the active site residues are found in the loops between β-sheets and the adjacent α-helices (Hartman and Harpel, 1994; Cleland et al., 1998). Some residues from the N-terminus of the adjacent large subunit also contribute to the active site (Figures 2, 3). In the form I enzymes, four large subunit dimers form an octameric ring through which there is a solvent channel (Figure 4), with four small subunits decorating both the top and bottom of the large subunit core. Each small subunit contacts both large subunits within a dimer and one large subunit of the adjacent dimer (Knight et al., 1990). The small subunits are not known to contain any active site residues, yet carboxylase activity is barely detectable in preparations of recombinant large subunit octameric cores of cyanobacterial RubisCO, that lack small subunits (Lee and Tabita, 1990; Lee et al., 1991).

RubisCO function requires activation of large subunits by a non-substrate molecule of CO₂, resulting in formation of a lysyl carbamate at a completely conserved lysine residue within the active site (Lys-201) (Lorimer et al., 1976; Miziorko, 1979; Lorimer and Miziorko, 1980). In the absence of activation by CO₂, RuBP and other sugar phosphates will inhibit RubisCO activity. Many plants and some cyanobacteria possess an enzyme called RubisCO activase, which facilitates dissociation of these inhibitors in an ATP-dependent manner (Somerville et al., 1982; Hartman and Harpel, 1994). Activated RubisCO is stabilized by a Mg²⁺ cofactor, which is essential in order for the carbamate to act as a general base, abstracting a proton from the substrate RuBP, thus forming an unstable enediol intermediate (Cleland et al., 1998). Both carboxylation and oxygenation proceed through this intermediate, and it is beyond this point that the
Figure 4. Space-filling model of the octameric core \((L_8)\) of Rubisco of *Spinacea oleracea*, as prepared by Kempton Horken (Horken, 1998). Panel A shows the core as viewed from “the side”. Panel B is a view “from the top” of the ring, i.e., down the vertical axis of the image in panel A, showing the solvent channel that runs through the enzyme.
Figure 4. The octameric core of RubisCO of *Spinacea oleracea*.
chemistry diverges (Figure 1). The same active site residues, therefore, are required for each reaction.

The relative rates of CO$_2$ versus O$_2$ fixation are related by the *specificity* factor, represented by the symbol $\Omega$ in honor of William Ogren, one of the first researchers to measure this property. In the literature, specificity is frequently notated by the symbol $\tau$, and is sometimes referred to as the partitioning factor. Specificity is described by the equation: $\Omega = V_cK_c/V_cK_cV_o$ (where $K$ refers to $K_m$, $V$ the $V_{max}$, subscript $c$ refers to the carboxylation reaction, and subscript $o$ refers to the oxygenation reaction). The relative rates for carboxylation and oxygenation are thus defined by the product of $\Omega$ and the ratio of the concentrations of CO$_2$ to O$_2$ at the active site: $v_c/v_o = \Omega \cdot \left[\frac{[CO_2]}{[O_2]}\right]$ (Jordan and Ogren, 1981, 1983, 1984; Figure 1).

Among the phylogenetic groups of RubisCO, specificity varies greatly, with the form II enzymes generally having the lowest and the form I enzymes having the highest specificities. For example, *Rhodospirillum rubrum* RubisCO has a specificity of 10 to 15, but most plant enzymes (form I) have specificities of about 80, with the absolute values dependent on the mode of assay. Until recently, specificities of the form I enzymes from marine nongreen algae were thought to be the highest, including the enzyme from the red alga *Galdieria partita*, reported as 240 (Shibata et al., 1996; Tabita, 1999). The highest specificity to date, 310, was recently reported for the unusual pentagonal form III enzyme of *Thermococcus kodakaraensis* KOD1 (Maeda et al., 1999). This measurement has yet to be confirmed, with parameters such as dissolved oxygen at high temperatures to be accounted for. Other form III enzymes appear to be oxygen labile. However, since the specificity assay is independent of $k_{cat}$, a specificity value was
determined for the enzyme from *Methanococcus janschii* that was the lowest ever reported (Watson et al., 1999). Despite the wide range of specificities, the basic dimeric units of all RubisCOs are fundamentally identical in structure, with very few differences seen in the tertiary structures. Trying to understand which properties of the enzymes confer different specificities and kinetic properties has driven most site-directed mutagenesis studies of RubisCO.

Some active site residues and structural features of the enzyme have been implicated in specificity through a combination of site directed mutagenesis and crystallography. One of the primary conserved features of RubisCO is an 11-residue region of the enzyme, loop 6, which closes over the α/β barrel of the active site. Lys 334 within this loop is critical for activity and has been shown to play a significant role in specificity. However, in lower specificity enzymes, substitutions in and around this loop with sequences that resemble those of high specificity enzymes usually result in an enzyme with poor carboxylase activity and no improvement in specificity (Terzaghi et al., 1986; Chen et al., 1991; Parry et al., 1992; Read and Tabita, 1994; Madgwick et al., 1998; Ramage et al., 1998). In fact, all mutants with altered, even improved, specificities have shown unacceptable decreases in carboxylase activity.

These results raise the question as to whether or not RubisCO specificity can actually be “improved”. Using a theoretical quantum mechanical/molecular mechanical approach to model the active site and possible transition state structures, some researchers have concluded, even almost 30 years ago, that RubisCO kinetics depends solely on the concentrations of CO₂ and O₂ at the active site (Lorimer et al., 1973; Andres et al., 1999; Moliner et al., 1999). RubisCO mutants have been obtained, however. that
can catalyze enolization of RuBP but in which neither CO$_2$ nor O$_2$ can react with the enediol intermediate (Hartman and Lee, 1989). Reaction with the enediol is thus not a spontaneous process but requires amino acid side chains to either directly participate or to maintain conformation of the protein, allowing the reaction to take place (Hartman, 1992). In support of genetic data, a mathematical model recently predicted that differences in $\Omega$ must involve specific residues and/or conformations of the enzyme; therefore $\Omega$ is a mutable property of the enzyme (Schlitter and Wildner, 2000).

Surprisingly, some of the successes in altering specificity have come through studies of small subunits. Site-directed mutations in the small subunit have been made which favorably affect $K_c$, though they have a deleterious effect on $V_c$ and relatively little impact on $\Omega$ (Read and Tabita, 1992a). However, a hybrid enzyme of *Synechococcus* PCC6301 large subunits and *Cylindrotheca* sp. N1 small subunits (a higher specificity enzyme) had a specificity 60% greater than the *Synechococcus* enzyme normally exhibits, but total carboxylase activity was only about 5% of that of the wild type *Synechococcus* enzyme (Read and Tabita, 1992b). This was surprising since previous hybrid constructs always reflected the specificity of the large subunit in the hybrid, leading researchers to conclude that the large subunit alone defines specificity (Andrews et al., 1984; Andrews and Lorimer, 1985; Lee et al., 1991). A more recent report claims that substitution of alanine for proline at position 20 in the small subunit of the *Synechococcus* enzyme increased specificity of that enzyme by 25% without altering carboxylase activity (Kostov et al., 1997), the first documentation that such a mutant can even be isolated.

Aside from loop 6, the C-terminus of the large subunit is gaining attention for a possible role in specificity. Recently published crystal structures of the high specificity
RubisCO from *Galdieria partita* (Sugawara et al., 1999) and the “red-like” RubisCO of *Alcaligenes eutrophus* (now called *Ralstonia eutrophus*) (Hansen et al., 1999), have been compared to the previously published structures for the spinach and cyanobacterial enzymes (Knight et al., 1990; Newman and Gutteridge, 1993). There are major differences between the C-termini of these enzymes, with the higher specificity enzymes exhibiting a C-terminal extension that appears to affect the radius of the solvent channel leading to the active site(s).

Multiple regions of the enzyme, then, ranging from the active site to the extreme C-terminus of the large subunits, and including the small subunits, have been implicated in specificity. Site-directed mutagenesis will not be practical for examining the potential effects of mutations in all of these regions of RubisCO. Random mutagenesis, either through error-prone PCR, mutator strains, or DNA shuffling, followed by biological selection, is becoming the preferred method for finding enzymes with desirable characteristics (Cadwell and Joyce, 1992, 1994; Leung et al., 1989; Greener and Callahan, 1994; Stemmer, 1994a). This approach of “directed evolution” is replacing site-directed mutagenesis for commercially significant enzymes, and for enzymes such as RubisCO, which have questions unsolved by site-directed mutagenesis (Arnold, 1998a,b; Schellenberger, 1998; Stemmer, 1994b).

In the first Chapter of this dissertation, I describe the development of a bacterial system for random mutagenesis and bioselection of RubisCO, with the goal of identifying unique mutations that affect important kinetic properties of the enzyme. Through this system, I successfully identified several unique mutants, whose properties are described in the subsequent Chapters. Finally, I describe some improvements to the system and
initial characterization of more mutants isolated through the modified system, as well as future implications and uses of the bioselection process.
CHAPTER 1

Design and Implementation of a System for the Bioselection of RubisCO With Altered Enzymatic Properties

Introduction

Multiple sequences and crystal structures of ribulose 1, 5-bisphosphate carboxylase/oxygenase (RubisCO) from various sources are available, in some instances complexed with various combinations of activator CO₂, divalent cations such as Mg₂⁺, substrate RuBP, or inhibitors such as the transition state analog 2-carboxyarabinotol-1,5-bisphosphate (CABP). This has led to speculation that such information may eventually lead to the bio-engineering of RubisCO to improve autotrophic CO₂ fixation and photosynthesis (Gutteridge et al., 1995; Tabita, 1999; Spreitzer, 1999; Salvucci and Spreitzer, 2002). By contrast, some models propose that constraints on the enzyme’s structure leave no room for improvement of kinetic properties such as specificity (Ω) (Lorimer and Andrews, 1973; Andres et al., 1999; Moliner et al., 1999). Despite these conflicting viewpoints, there is growing support for the hypothesis that differences in Ω of structurally similar RubisCO proteins must involve specific residues and/or conformations of the enzyme, and that Ω may therefore be altered without adversely affecting the enzyme’s catalytic activity (Hartman and Lee, 1989; Hartman, 1992; Tabita, 1999; Schlitter and Wildner, 2000; Spreitzer, 2002). To date, several site-directed mutants have been shown to possess alterations in Ω. However, the changes noted never
resulted in significant improvements in catalytic behavior or efficiency; moreover, increases in $\Omega$ were invariably gained at the expense of RubisCO activity (Parry et al., 1992; Gutteridge et al., 1993; Read and Tabita, 1994; Kostov and McFadden, 1997; Ramage et al., 1998).

It is implicit from the early studies of Jordan and Ogren (1981) that $\Omega$ and other kinetic properties are mutable in different sources of RubisCO. This work led to the further realization that microbial RubisCO enzymes might greatly enhance studies of kinetic variability, as nature has somehow selected for RubisCO molecules of different catalytic efficiency and kinetic properties in microorganisms adapted to different environments, while maintaining a constant tertiary structure (Horken and Tabita, 1998; Read and Tabita, 1992b; Tabita, 1999, Watson and Tabita, 1999). Presumably, from prior site-directed mutagenesis and structural studies, answers to the kinetic efficiency problem would involve unpredicted residue changes that may or may not be localized in or near the active site. Such changes, or multiple changes, might result in different conformations of the enzyme rather than direct alterations of the active site. In any case, since it is clear that different levels of enzymatic efficiency are a fact of nature, it should be feasible to experimentally determine the molecular basis for catalytic efficiency of RubisCO.

Recognition of the complexity of this issue led to the prediction that growth of an organism under RubisCO-dependent conditions may select for improvements in the enzyme, if multiple variants of the enzyme are available in the population. At this time there is a single system available to allow one to begin such an experimental approach. This involves successful biological selection of intragenic suppressors of a RubisCO-
dependent, temperature-sensitive phenotype in the green alga *Chlamydomonas reinhardtii*, as well as complementation of a *C. reinhardtii* small subunit deletion mutant. Several prior studies with the *Chlamydomonas* system have supported the prediction that Rubisco variants may be biologically selected (Chen et al., 1991; Thow et al., 1994; Spreitzer et al., 1995; Hong and Spreitzer, 1997; Du, et al. 2000; Spreitzer et al., 2001). However, the *Chlamydomonas* system has been primarily aimed towards studies of thermostability and temperature-sensitive phenotypes, with interesting mutants occasionally exhibiting changes in catalytic efficiency that were not the direct result of selection (Spreitzer et al., 2001). Further complicating matters, Rubisco small subunit genes (*rbcS*) in green algae, like plants, are encoded by a nuclear family of *rbcS* genes, two different genes in *Chlamydomonas* and up to 12 genes in some plants (Spreitzer, 1999). Furthermore, there are technical challenges associated with chloroplastic transformations and complete segregation of transformed cells to obtain pure strains of *Chlamydomonas*.

Rubisco enzymologists have thus speculated that bioselection of Rubisco may be achieved through a bacterial system (Spreitzer, 1993; Tabita, 1999). It would be especially advantageous if this system could specifically target kinetic parameters such as $\Omega$, $k_{cat}$, or $K_c$. Towards this goal, a system has been developed for random mutagenesis of Rubisco genes using the purple, nonsulfur, photosynthetic bacterium *Rhodobacter capsulatus* as a host. *R. capsulatus* is capable of aerobic growth using organic carbon (chemoheterotrophy), and can synthesize its own organic carbon via the CBB cycle either photosynthetically (photoautotrophy) or during dark aerobic growth (chemoautotrophy). The specific strain of *R. capsulatus* used in this system, SB1-II', is a Rubisco deletion
strain incapable of autotrophic growth or photoheterotrophic growth (Paoli et al., 1998). Through a system of expression of heterologous RubisCO genes in SBI-II' and complementation of the strain to RubisCO-dependent growth, we identified the enzyme from the cyanobacterium *Synechococcus* PCC6301 as a suitable target enzyme to initiate the bioselection system.

The RubisCO of *Synechococcus* sp. strain PCC6301 was randomly mutagenized and expressed in *R. capsulatus* SBI-II'; gain of function mutants were identified that could grow under conditions where the wild-type enzyme was unable to complement SBI-II' to growth. The system was designed such that defective enzymes were also identified, by their inability to complement *R. capsulatus* SBI-II' under conditions where the wild-type enzyme was fully competent. Through this system, residues not previously known to play a significant role in the catalytic activity of the enzyme were identified, and residues previously studied were found to have roles not yet characterized. Furthermore, we used mutants from the first round of bioselection as templates for further mutagenesis and bioselection under more stringent conditions. The first steps have thus been taken towards development of a "high-throughput" system typical of directed enzyme evolution experiments. This chapter describes the design and implementation of the bioselection system.

**Materials and Methods**

*Strains and growth.* Construction of the RubisCO deletion strain of *R. capsulatus*, SBI-II', from wild-type strain SB1003, was previously described (Paoli et al., 1998). *R. capsulatus* was grown aerobically on peptone yeast extract (PYE) plates (Weaver and Tabita, 1983) or in a broth containing Ormerod’s basal salts (Ormerod et al.,
1961), 6 g/l peptone, 5 g/l yeast extract, 10 mM NaCl, 3 mM KCl, and 0.1 μg/ml biotin. Both media were supplemented with 1 μg/ml nicotinic acid and 1 μg/ml thiamine-hydrochloride. Antibiotics in PYE plates were used at the following concentrations: 100 μg/ml rifampicin, 2 μg/ml tetracycline, 10 μg/ml spectinomycin, 5 μg/ml kanamycin.

Photosynthetic cultures were grown in Ormerod’s minimal medium supplemented with 1 μg/ml nicotinic acid and 1 μg/ml thiamine-hydrochloride (Ormerod et al., 1961). Liquid photoautotrophic cultures were bubbled with 1.5% CO₂/98.5% H₂. Liquid photoheterotrophic cultures were grown in completely filled 10-ml screwcap tubes, to maintain anaerobiosis. DL-malate was added to 0.4% for photoheterotrophic growth, and dimethylsulfoxide (DMSO) was added to 30 mM in liquid cultures when appropriate. Liquid chemoautotrophic cultures were grown in Ormerod’s medium and bubbled with 10% CO₂/90% H₂ gas mixed 1:1 with air, to achieve a 5% CO₂/45% H₂/10% O₂ atmosphere. All growth was at 30°C.

Minimal medium plates for “high CO₂” photoautotrophic growth, and minimal malate medium plates (0.4% malate) for photoheterotrophic growth, were incubated in jars containing a CO₂/H₂-generating system (5-6% CO₂, BBL GasPak system, Becton Dickson Microbiology Systems, Cockeysville, Maryland). Photoautotrophic plates grown under the “low CO₂” condition were incubated in jars that were flushed for 15 min with premixed 1.5%CO₂/98.5% H₂. For bioselection of mutagenic PCR products (see below), the minimal medium plates were incubated photoautotrophically in jars that were flushed for 15 min with premixed 0.5% CO₂/99.5% H₂. All phototrophic jars contained a palladium catalyst to remove O₂ from the jar’s atmosphere, and all jars were incubated in

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water baths in front of lights. Chemoautotrophic jars contained the BBL GasPak pouches, but no palladium catalyst, and were incubated in the dark.

*Eschericia coli* JM109 (F' traD36 lacI5 Δ(lacZ)M15 proA' B'1 Δ(lac-proAB) thi gyrA96 (Nalr) endA1 hsdR17 (rK1 mK1) relA glnV44 supE44 mcrA) cultures were grown in Luria-Bertani medium at 37°C using 100 µg/ml ampicillin and 12.5 µg/ml tetracycline when appropriate (Maniatis et al., 1982; Yanisch-Perron et al., 1985). For overexpression of Rubisco genes in pUC19, 25 ml cultures were inoculated with overnight cultures, and then induced after 3 h of growth with 0.5 mM IPTG for 16 h.

**Plasmids.** *E. coli* JM109 and plasmid pUC19 were used for routine cloning (Yanisch-Perron et al., 1985). Plasmid pRPS-1 was used to express genes in *R. capsulatus* SBI-II for initial experiments in which heterologous Rubisco genes were used to complement (Falcone and Tabita, 1991). Plasmid pRPS-MCS3 was constructed specifically for this system, as described below.

The promoter region of *cbbM* from *Rhodospirillum rubrum*, including its cognate transcriptional activator *cbbR*, was amplified with primers designated AseREV (5'CGG CAT TAA TCT TGG GCT TCA TGA TAT AGG3') and AseFWD (5'GGC GATT AAC TTC TCC TGA TGG GTG GGA GGG3') (oligonucleotides from Integrated DNA Technologies, Inc., Coralville, IA). The primers contained restriction sites for the enzyme AseI (underlined; New England Biolabs, Beverly, MA) to facilitate cloning. Amplification reactions contained a blend of Taq and Pfu polymerases to minimize errors without sacrificing yields (Barnes, 1994). The template DNA, plasmid pRPS-75 (Falcone and Tabita, 1991), was linearized by digestion with EcoRV (GibCO BRL, Rockville, MD). Pfu Turbo polymerase was diluted to 0.0625 U/ul in 1X cloned Pfu
buffer (Stratagene, La Jolla, CA). Each PCR reaction contained 1 mM each of dNTPs, 6.2 pmol each of AseFWD and AseREV primers, 2 mM MgSO₄, 2 mM MgCl₂, 1 µl (0.0625 U) diluted *Pfu*, 1 µl (5 U) *Taq* polymerase (Gibco BRL, Rockville, MD), 1 µl DMSO, 10-20 ng template DNA, and 50 mM KCl in 20 mM Tris-HCl (pH 8.4) buffer (Gibco BRL, Rockville, MD), in a final volume of 100 µl. Cycling parameters were as follows: denaturation at 94°C for 2 min, followed by 25 cycles of 94°C for 30 s, 52°C for 30 s, 72°C for 1 min, followed by 2 min at 72°C. This and all subsequent PCR reactions were carried out in a Perkin Elmer Gene Amp PCR System 2400 thermalcycler (Perkin Elmer, Norwalk, CT). A high yield of the 1.4 kb product was obtained, and it was digested with Asel (New England Biolabs, Beverly, MA), and ligated with pBBR1-MCS3 (Kovach et al., 1995) that had also been digested with Asel. T4 DNA ligase was used to perform this step (Gibco BRL, Rockville, MD). Proper orientation of the promoter with respect to the polylinker was verified by restriction digestion with several enzymes.

A pUC19 clone of *rbcLS* from *Synechococcus* PCC6301 that would facilitate directional cloning into pRPS-MCS3 was constructed by amplification of *rbcLS* on pBgl710 (Lee et al., 1991) using primers Pst/Sac (5'-GCA TCT GCA GAG CTC ATC TGA CAT ATC TCT A3') and SSUXba (5'-AAA TCT AGA GGC AGA GCA GCT ATC AAG ACA3') (Integrated DNA Technologies, Inc., Coralville, IA), which contained *PstI* and *XbaI* sites (underlined), respectively. The PCR reaction contained the same components described above, with the following modifications: MgCl₂ was at 4 mM and each primer was at 7 pmol/reaction. Cycling parameters were: 1 min denaturation at 94°C, 25 cycles of 94°C for 1 min, 55°C for 30 s, 72°C for 3 min, final elongation at 72°C for 5 min, and incubation at 4°C upon completion. The 1.8 kb
product, which contained a mutation resulting in a K11R substitution, was ligated with pUC19 after both had been digested with PstI and XbaI (MBI Fermentas, Amherst, NY), generating pUCLS. The same protocol was used to generate a wild type subclone of rbcLS, called pUC6301. The plasmids pRPS-LS (pRPS-K11R) and pRPS-6301 were constructed by subcloning rbcLS from pUCLS and pUC6301, respectively, using the PstI and XbaI sites of pRPS-MCS3. All pRPS plasmids carrying potential mutant genes were constructed by subcloning mutant library DNA (see below) digested with PstI and XbaI into pRPS-MCS3 digested with the same enzymes.

*Mutagenesis.* Initial random mutagenesis was achieved via transformation of *Epicurian coli* XL1-Red (E. coli: endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac mutD5' mutS mutT Tn10 (Tet'); Stratagene, La Jolla, CA) with pUCLS (a K11R mutant). About 200 XL1-Red transformants were used to inoculate 10 ml of LB, which was grown for 16 h. Plasmid DNA was isolated from 3 ml of the 10 ml culture, using the QIAprep miniprep protocol (Qiagen, Valencia, CA), was designated as Library 1. The remaining 7 ml was used to inoculate 250 ml of LB, which was grown for 24 h. A QIAprep maxiprep of the 250 ml culture (Qiagen, Valencia, CA) resulted in plasmid Library 2.

The wildtype and the positive mutants acquired from XL1-Red were used as templates for mutagenic PCR. Mutagenic PCR was carried out using high concentrations of Taq DNA polymerase, in the absence of Mg²⁺, but the presence of Mn²⁺ ions, and unbalanced concentrations of dNTPs. The extension time and cycle number were also longer than standard for PCR. Each 100 μl reaction contained: 100-300 ng linearized pUC19 plasmid containing the appropriate template (wild-type or mutant rbcLS), 0.5 μM each M13 universal forward and reverse primers, 1 mM MnCl₂, 2 μl Taq Polymerase (10
U, GibcoBRL), 1 μl DMSO, 1 mM each of dATP, dCTP, dTTP, and 0.2 mM dGTP, in 1X Taq polymerase buffer (GibcoBRL, 20 mM Tris-HCl, pH 8.4, 50 mM KCl). The DNA was denatured for 2 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 55°C, and 3 min at 72°C. Following thermal cycling, reactions were incubated at 4°C until products were examined by agarose gel electrophoresis.

Screening and selection of XL1-Red mutants. The XL1-Red plasmid library DNA was digested with PstI and XbaI (MBI Fermentas) for subcloning into pRPS-MCS3, and ligations were used to transform E. coli JM109. These reactions contained the pUC19 library and pRPS-MCS3 plasmids in one digest, rather than gel-purifying the 1.8 kb band representing rbcL.S. After transformation, ~1000 tetracycline resistant, white colonies of E. coli were hand-picked and pooled into 10 ml of LB containing tetracycline. The 10 ml cultures were grown at 37°C for approximately 4 h, when 1 ml was removed to use in a triparental mating with 5 ml of logarithmic phase R. capsulatus SBI-II' and 1 ml of helper strain E. coli HB101 (Δ(gpt-proA)62 leuB6 thi-1 lacY1 hsdS20 recA rpsL20 (Str^r) ara-14 galK2 xyl-5 mtl-1 supE44 mcrB8, Boyer and Roulland-Dussoix, 1969), which harbors the transfer genes required for conjugation on pRK2013 (Figurski and Helinski, 1979). E. coli JM109, HB101, and R. capsulatus SBI-II' cells were washed twice with phosphate buffer before mixing them in about 100 μl of buffer, and spotted directly onto PYE with no antibiotics. Matings were incubated for 36 h and resuspended with 1 ml of phosphate buffer. Ten-fold serial dilutions of the resuspended mating mixtures were prepared. 100 μl of the 10^-1 thru 10^-4 dilutions were plated onto PYE with rifampicin and tetracycline (PYE rif-tet). These plates were incubated for 3 days before colonies were large enough to pick.
Transconjugates were picked from the PYE rif-tet plates onto a minimal medium plate and onto a replicate PYE rif-tet plate (the “master plate”). The minimal medium plates were incubated under high CO$_2$ photoautotrophic conditions, while the PYE rif-tet plate was incubated aerobically at 30°C. Isolates that were capable of growth on the minimal medium were picked again, from the master plate, onto minimal medium and incubated under both low CO$_2$ photoautotrophic and chemoautotrophic conditions. Mutants capable of growth under these conditions were called positive mutants.

Isolates incapable of high CO$_2$ growth were picked from the master plate onto PYE rif-tet plates. To verify the presence of $rbcLS$ in transconjugates of $R.~capsulatus$, colony hybridizations with a randomly labeled fluorescent probe for the small subunit DNA ($rbcS$) were performed using the Vistra fluorescence labeling and detection kit (Amersham Life Science, Buckinghamshire, England). The blots were visualized using a Molecular Dynamics Storm 840 scanner (Molecular Dynamics, Sunnyvale, CA).

The remaining isolates were grown photoheterotrophically with DMSO so that RubisCO activity could be screened. The cultures were also used to verify RubisCO polypeptide synthesis by SDS-PAGE and Western blots (Laemmli, 1971). Western blots were developed and visualized by chemifluorescence as previously described (Paoli et al., 1998). To identify those mutant enzymes deficient in holoenzyme assembly, extracts from some of the strains were further screened by Western blotting of nondenaturing gels.

Isolates that synthesized no RubisCO polypeptides were discarded. The remaining isolates, called negative mutants, therefore contained pRPS-MCS3 carrying.
mutated rbcLS, which encoded a RubisCO incapable of complementing R. capsulatus SBI-II to photoautotrophic growth.

**Screening and selection of mutagenic PCR mutants.** The positive selection condition used for the mutagenic PCR products was photoautotrophic growth under an atmosphere of 0.5% CO₂/99.5% H₂, a condition under which neither the wild type nor the XL1-Red positive mutants were capable of complementation. PCR products were digested with PstI and XbaI, ligated into pRPS-MCS3, and conjugated into R. capsulatus SBI-II as described above. Transconjugates were selected two ways; on malate plates incubated photoheterotrophically, or directly on minimal medium plates incubated photoautotrophically with 0.5% CO₂/99.5% H₂. The photoheterotrophic plates were incubated for four days, and colonies from these plates were screened for the ability to grow photoautotrophically with 5% CO₂ or 0.5% CO₂. Isolates that could not grow with 5% CO₂ were considered "PCR negative mutants", and isolates that grew with only 0.5% CO₂ were called "0.5% positive mutants".

**Identifying point mutations.** Once positive and negative mutants were identified, pRPS-MCS3 was back-mated from R. capsulatus into E. coli JM109. The plasmid was isolated and the rbcLS genes subcloned into pUC19 to facilitate cloning and screening in E. coli. The Thermo Sequenase II dye terminator cycle sequencing kit (Amersham Pharmacia, Cleveland, OH), and the ABI PRISM dye terminator cycle sequencing kit (Perkin Elmer, Foster City, CA) were both used for automated sequencing with either an ABI PRISM 310 or 3700 Genetic Analyzer. In order to completely sequence both strands of the DNA for the 1.8 kb region, 7 primers were routinely used in addition to the M13 Universal forward and reverse primers:
A: 5'TCTCCCCCAGCCTTTCGACTT3'
B: 5'CCCCCAGCGATAGTCAGAGGCTCC3'
C: 5'GGGGTGACCCAAGGTGCCGCCACC3'
D: 5'GGTCGGCGCGGTCACGTTCAGGTAGTGACC3'
E: 5'GGTCGAGCGGGTAAGCGATGAACG3'
F: 5'CCGCTTCCCCCCGTGCGCTTGGTCAAAACCTTCC3'
G: 5'GGTGCCGCGACACGACGCGGTCTCTGC3'

An ABI Prism 3700 DNA sequencer became available through the new Plant-Microbe Genomics Facility at The Ohio State University (Columbus, OH), allowing for more efficient sequencing with greater read lengths, directly from pRPS-MCS3. Primers specifically designed for pRPS-MCS3, along with primer B shown above, were typically used for sequencing at this Facility:

RPSFWD: 5'GACGGCCAGTGAGCGCGTAATACGAC3'
RPSREV: 5'GGTCGACCGTATCGATAAGCTTGATATCGAATTC3'

When necessary, primers A-G listed above were used to verify point mutations on pRPS-MCS3 clones. All of the sequencing primers were supplied by MWG Biotech, Inc. (High Point, NC).

Preparation of cell extracts and assays. Cells from both *R. capsulatus* and *E. coli* cultures were washed twice with 100 mM Tris-HCl, 1 mM EDTA, pH 8.0 (TE). Cell pellets were stored at -80°C. Prior to assays, frozen cells were thawed on ice, and resuspended in TE containing 5 mM β-mercaptoethanol. Cells were either disrupted by sonication or permeabilized by addition of the permeabilization agent hexadecyltrimethylammonium bromide (C-TAB, Sigma, St. Louis, MO) to 1 µg/ml for
whole-cell assays. Low-speed supernatants were obtained by microcentrifugation for 10 min at 4°C. High-speed supernatants from *E. coli* extracts were obtained by ultracentrifugation at 45,000 rpm for 1.5 h at 4°C.

Rubisco activity was measured by incorporation of $^{14}$CO$_2$ into acid-stable products (Whitman and Tabita, 1976). Whole-cell assays were used for screening purposes, using C-TAB rather than toluene treatment to permeabilize cells (Tabita et al., 1978). Protein was measured in whole-cell samples and extracts by a modification of the Lowry assay (Markwell et al., 1978).

**Results**

*Design of the system.* In addition to *R. capsulatus* strain SBI-II', four major components were required to select for alterations in Rubisco: a suitable target enzyme; a positive selection condition for that enzyme; an efficient means of cloning and expressing the mutated genes in *R. capsulatus* SBI-II'; and a method of random mutagenesis.

The target enzyme and selection conditions were discovered by complementation of *R. capsulatus* SBI-II' with heterologous Rubisco genes under photoheterotrophic, photoautotrophic, and chemoautotrophic conditions. Photoheterotrophic or photoautotrophic growth was achieved by incubating complemented strains either on malate minimal medium or minimal (autotrophic) medium plates, respectively, in sealed GasPak jars. Jars contained a CO$_2$-H$_2$ generating pouch and a palladium catalyst to remove O$_2$, which resulted in an anaerobic atmosphere with an estimated 5-6% CO$_2$. The sealed jars were incubated in water baths in front of lights. Chemoautotrophic conditions...
were achieved by incubating minimal medium plates with the same CO$_2$-H$_2$ pouches, no palladium catalyst, and incubation in a dark, 30°C incubator. For low-CO$_2$ photoautotrophic growth conditions, jars containing the O$_2$-scavenging palladium catalyst were manually flushed with a 1.5% CO$_2$/98.5% H$_2$ mix, and then sealed and placed in front of lights.

Using plasmid pRPS-1, genes were expressed under various growth conditions through activation of the *Rhodospirillum rubrum* RubisCO promoter (pchbM) by its cognate transcriptional activator, CbbR, also carried on this plasmid (Falcone and Tabita, 1991, 1993). Several constructs, containing different bacterial RubisCO genes, were available (Falcone and Tabita, 1991, 1993) and these were conjugated into *R. capsulatus* SBI-II' (Paoli et al., 1998). When complemented with the genes (*rbcL,S*) encoding RubisCO from the cyanobacterium *Synechococcus* PCC6301, SBI-II' was unable to grow either photoautotrophically in the presence of 1.5% CO$_2$, or chemoautotrophically. Form I and form II RubisCOs from three photosynthetic bacteria, *R. capsulatus*, *R. rubrum*, and *Rhodobacter sphaeroides*, could complement under all growth conditions. These initial studies led to the hypothesis that the poor affinity of the cyanobacterial enzyme for CO$_2$ (i.e., its high K$_c$) was in part responsible for the growth phenotype (Table 1.1). This was the premise for choosing the cyanobacterial enzyme as the target enzyme for positive selection under low CO$_2$ (1.5%) photoautotrophic conditions. Furthermore, negative selection was available, in which mutants unable to complement SBI-II' to high CO$_2$ photoautotrophy (5% CO$_2$) could also be examined.

Plasmid pRPS-1, used in the above described complementation studies, was not an efficient cloning vector because (1) it lacked sites for directional cloning, and (2) blue-
<table>
<thead>
<tr>
<th>Rubisco</th>
<th>( K_c ) (( \mu \text{M} ))(^a)</th>
<th>( \text{PH} )(^b)</th>
<th>Low CO(_2) PA(^b) (1.5%)</th>
<th>High CO(_2) PA(^b) (5-6%)</th>
<th>( \text{CA} )(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SB1003 (forms I and II)</td>
<td>n/a(^c)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SBI-II- (no Rubisco)</td>
<td>n/a</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Synechococcus</em> PCC6301 form I</td>
<td>175</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>R. rubrum</em> form II</td>
<td>100</td>
<td>+</td>
<td>not tested</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>R. sphaeroides</em> form I</td>
<td>25</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>R. sphaeroides</em> form II</td>
<td>100</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>R. capsulatus</em> form I</td>
<td>29</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>R. capsulatus</em> form II</td>
<td>not determined</td>
<td>+</td>
<td>not tested</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

\(^a\) As measured by Kempton Horken (Horken, 1998).
\(^b\) PH = photoheterotrophic, PA = photoautotrophic, CA = chemoautotrophic. All growth was on minimal medium plates.
\(^c\) n/a = not applicable (SB1003 has both endogenous form I and form II Rubisco, SBI-II has no Rubisco); + = growth; ++ = heavy growth; - = no growth.

Table 1.1. Growth of *R. capsulatus* SBI-II complemented with Rubisco genes carried on the plasmid pRPS-1.
white screening for gene insertion was rendered ineffective by its construction. The plasmid constructed in this study, pRPS-MCS3, also relied on *R. rubrum* *pcbbM*, with its cognate transcriptional activator, CbbR, to drive gene expression in *R. capsulatus*. pRPS-MCS3 was constructed by amplification of the *cbbRM* region, and insertion of the PCR product upstream of the *lacZα* gene on the broad host range vector pBBR1MCS-3. The features of blue-white screening and directional cloning were preserved in this construct (Figure 5).

The genes encoding *Synechococcus* PCC6301 RubisCO were amplified by PCR with primers containing *PstI* and *XbaI* restriction sites to facilitate directional cloning into pUC19, generating plasmid pUCLS. From pUCLS the genes were subcloned into pRPS-MCS3, and the plasmid pRPS-LS was introduced into *R. capsulatus* SBI-II' by triparental conjugation to verify that the same phenotypes observed with the pRPS-1 clone would be seen with a pRPS-MCS3 subclone (Figurski and Helinski, 1979). A mutation resulting in a K11R substitution (nucleotide 110 was changed from a to g; Figure 6), presumably derived from PCR amplification, was later discovered on pRPS-LS; however, a second attempt to amplify and clone the wild type genes into pUC19 succeeded, generating pUC6301. From pUC6301 the genes were cloned onto pRPS-MCS3, resulting in pRPS-6301. The phenotype of *R. capsulatus* SBI-II' was identical with pRPS-K11R, pRPS-6301, and the original pRPS-1 clone used in complementation. It was thus concluded that (1) the pRPS-MCS3 plasmid would be an effective means of cloning and expression and (2) the K11R mutation had no discernible effect on the complementation properties of cyanobacterial RubisCO. Thus, positive selection conditions of low CO₂
Figure 5. Expression plasmid pRPS-MCS3 was derived from pBBR1MCS-3 (Kovach et al., 1995). Important features were a tetracycline resistance gene (Tet), a mobilization site (Mob), a lacZα gene with a multiple cloning site (lacZ-MCS), and the *R. rubrum* RubisCO promoter region (pcbbM). In *E. coli*, the lacZα gene appeared to be intact for blue/white screening, indicating its expression was not disrupted by the upstream insertion of the *R. rubrum* sequences. Genes were therefore directionally cloned into the plasmid, with blue-white screening for insertion. The plasmid was mobilized into *R. capsulatus* with the transfer genes provided in trans (Figurski and Helinski, 1979). In *R. capsulatus*, the promoter of *R. rubrum cbbM* (pcbbM) was presumably activated by its cognate transcriptional activator, CbbR, to drive expression of genes cloned into the multiple cloning site. This drawing is not to scale, but merely represents the relative positions of genes and the unique sites within the MCS.
Figure 5. Expression plasmid pRPS-MCS3.
Figure 6. The nucleotide sequence of \( rbcL \) of *Synechococcus* PCC6301. The atg start codon for both genes is in bold, as are the taa stop codons. The numbering in this Figure is the basis for the nucleotide numbers given in all subsequent tables and text in this dissertation.
1 actgcagctt tacaggtcgc cctgccccag aatctctgaa ctgctgacat atctgacata
61 tctctagggg gagacgacat gcccaagacg caaatctggcc caggctataa ggccggggtg
121 aaggactaca aactcactta ttacacccccc gattacaccc ccaagacac tcgacctgtcg
181 gcgggcc gccgcaagct ctcgccgctgc cgcggctgtg gccgcctctgc gcggctgtg
241 gcggctgaat cttcgacggc tacctggacc accgtgtgga ccgacgttga caggacatag
301 gatcgctaca aagcgaagct ctaccacctc gacccgggtg aagcgaaga gaactctctac
361 tttgctgctc taacgccccc gcgcgacact gttgaagagg ggtggcttcac caacattctg
421 acctcgatacg cggcgtatct cttggggttc aagactatcc gttcggctgc cttggaagac
481 atccgcttcg cgcctctcgg ctgctaaacc tctcccaaggg cttcccaaggtc tatacaaagtg
541 gcggcggacc tcgctgaaccc gtacggccgg cggacctcttg gcggctgtgc gcggctgtgc
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661 gcacccacac caagcggcao gaaaatacgg tccgaacgag aaacgccctga aatacgaaggt
721 tttcctgtttct ggctgtatgc cttgcacacag aagacgggtcc aaaacgggtg aaacccgggtg
781 cacgtacctgc acgtgaccgg gcggacccgg gcgggtgtggc tggcgctggcg tggcgctggcg
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901 accacctttg ccacgttcag gcgccgaccc gcggctgtgc gcggctgtgc gcggctgtgc
961 cacgcggtgtg ctcgggctcga gcgcgtacca gcgcgttctct tctgcgctct gcgcgttctct
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1081 gccacagctc gccacagtcc ctgttcgggt gaagccgaagat ccacgctggtgc aaggccggtga
1141 cgaggtgctgg cgctgctggtg gatgtgctggt gcggctgtgc gcggctgtgc gcggctgtgc
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1261 gcgggcgccg atggcggtgg gcggacgcct gcgggcgcgc gcgggcgcgc gcgggcgcgc
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1501 gcgcgtgctg gcgcgtgctg gcgcgtgctg gcgcgtgctg gcgcgtgctg gcgcgtgctg

Figure 6. The nucleotide sequence of \textit{rbcLS} of \textit{Synechococcus} PCC6301 (continued on next page).
Figure 6 (continued).

1561 gacttaacct ttcaggattt ctgaatcatg agcatgaaaa ctctgccccaa agagctcgt
1621 ttcgagacct ttcgtaacct gcctccccctc agcgatgcgc aaatcgctgc acaaatcag
1681 tacatgatcg agcaaggctt ccaccccttg atcgatgctg atcgagttca acgagcaetc gaatccgaa
1741 gaggctctact ggacgatgtg gaagctcccc ctgtttgact gcaagagccc tcagcaagtc
1801 ctgatgaaag tgcgtgaatg ccgcagcggaa taccgtgtatt gctacatccg tgtgcgtggc
1861 ttcgacaaaac tcaagcagtg ccaccccgtcg agctttcatcgt ttcatcgcc cggcctgtaac
1921 taaagccctga ttttccttga tagcttgtct gcctttgacc

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photoautotrophy and of chemoautotrophy could be used with pRPS-MCS3 to detect alterations in RubisCO.

As a means of randomly mutagenizing \textit{rbcLS}, prior to any knowledge that pUCLS harbored the K11R mutation, the mutator strain \textit{Epicurian coli} XL1-Red (Stratagene, La Jolla, CA) was transformed with the plasmid; all resultant transformants were pooled and inoculated into liquid LB. Sequential rounds of growth of XL1-Red, as described in Materials and Methods, generated two plasmid libraries of mutagenized pUCLS, Library 1 and Library 2. Library 1 was isolated after 16 h of growth of XL1-Red, and Library 2 was isolated after a further 24 h of growth. Note that the pUC19 clone, pUCLS, rather than pRPS-LS, was mutated to avoid potential false negatives or positives resulting from promoter or plasmid mutations on pRPS-MCS3. Thus, the plasmid libraries provided a pool of mutants that were subcloned from pUCLS into pRPS-MCS3, and the phenotypes observed were due only to the properties of the subcloned genes.

\textit{Screening and selection of XL1-Red mutants.} Mutated genes were subcloned from pUCLS into pRPS-MCS3, and white, tetracycline-resistant colonies of \textit{E. coli} JM109 were selectively pooled and introduced into SBI-II' by triparental conjugation, with the helper plasmid pRK2013 in \textit{E. coli} HB101 (Figurski and Helinski, 1979). Transconjugates were selected on chemoheterotrophic plates, with the antibiotic rifampicin to select against \textit{E. coli}, and tetracycline to select for SBI-II' that contained pRPS-MCS3 (Figure 7).

Colonies were next tested for the ability to grow photoautotrophically with 5% CO$_2$. Negative mutants were defined as those unable to grow under the high CO$_2$ photoautotrophic growth condition (Figure 7). From Library 1, 177/1900 (9.3\%) of the
Figure 7. A general scheme of the selection process using the XL1-Red strain. I, rbclS genes from the pUC19, XL1-Red mutant plasmid libraries were subcloned into pRPS-MCS3, and transformed E. coli JM109 was plated onto LB with tetracycline (Tc). II, white colonies were preferentially chosen for mating into R. capsulatus SBI-II'. III, transconjugates were incubated on rich medium containing rifampicin (Rif) to select against E. coli, and tetracycline to select for R. capsulatus containing pRPS-MCS3. IV, transconjugates were picked onto a chemoheterotrophic (CH) “master” plate for future use, and screened for their ability to grow photoautotrophically (PA) with “high” CO₂ (5-6% CO₂ in the atmosphere). Isolates incapable of growth were potential “negative” mutants. V, colonies capable of high CO₂ photoautotrophic growth were taken from the master plate and incubated under the low CO₂ condition (1.5% CO₂). The mutants that grew were potential “positive” mutants. VI, all positive and negative mutants were eventually backmated into E. coli for further study.
Figure 7. The selection process.
colonies screened did not grow photoautotrophically, and from Library 2, 370/3290 (11.2%) of the colonies picked did not grow. The overall negative mutation rate was therefore estimated to be 10.5%. Although white colonies were selectively pooled for conjugation, a negative phenotype frequently resulted from lack of an \textit{rbcL.S} insert. These false negative mutants were screened out by colony hybridizations (Figure 8). 37% of the negative mutants did not carry \textit{rbcL.S} on pRPS-MCS3; when corrected for this the negative mutation rate was estimated at 6.9%.

Mutants capable of high CO$_2$ photoautotrophic growth were screened for the ability to grow chemoautotrophically or photoautotrophically with 1.5% CO$_2$. Five positive mutants, defined as those capable of low-CO$_2$ photoautotrophic growth, chemoautotrophic growth, or both, were isolated from the Round 2 library, resulting in a positive mutation rate of 0.09%.

\textit{Screening out false negative mutants.} For initial measurements of RubisCO activity, negative mutants that contained \textit{rbcL.S}, as identified by colony hybridizations, were grown photoheterotrophically in liquid malate minimal medium. Dimethyl sulfoxide (DMSO) was added to the medium as an exogenous electron acceptor, so that all strains would be able to grow, even when they lacked a fully functional RubisCO (Falcone and Tabita, 1991). The addition of DMSO as an exogenous electron acceptor allowed for RubisCO-independent growth, but the promoter on pRPS-MCS3 was sufficiently activated under such photoheterotrophic conditions to express the cloned genes. These cultures were used to screen RubisCO activity in whole cells and to screen for the presence of normally-migrating large subunits using Western blots. There was no detectable RubisCO activity in 179/329 of the cultures screened for activity. Five of
Figure 8. False negative mutants that lacked *rbcLS* were identified by colony hybridization. Putative negative mutants of *R. capsulatus* SBI-II were grown on PYE with rifampicin and tetracycline. A positive control, SBI-II with pRPS-LS ("+"), was included on each plate, as well as a negative control, the wild type strain SB1003 ("-"). The gene encoding the small subunit from *Synechococcus* PCC6301, *rbcS*, was used as a randomly labelled chemifluorescent probe.
Figure 8. Colony hybridization.
these possessed a visibly truncated large subunit polypeptide. Among the strains that had no activity, 86 did not contain large subunit polypeptides at all (Figure 9). The 225 negative mutants that had a normally migrating large subunit polypeptide were categorized as having no activity, partial activity, or wild-type activity (Table 1). Negative mutants from each category, as well as the positive mutants, were chosen for further characterization.

Identification of mutations (I). On the basis of colony hybridizations, Western blots, and RubisCO activity results for photoheterotrophically grown *R. capsulatus* SBI-II', 33 mutants (5 positive and 28 negative) were conjugated back into *E. coli*, subcloned into pUC19, and sequenced. The pUC19 subclones were also used for over-expression in *E. coli* to compare the relative RubisCO activity of the mutants to the wild type and K11R enzymes in crude extracts (Figure 6, Table 1.3). Several of the mutants in this first set were identical, and we refer to them as "siblings". The redundancy inherent in the system was the result of multiple cloning, conjugation, and subcloning steps. When corrected for siblings, there were 15 unique negative mutants and 3 unique positive mutants from the 33 sequenced.

Identification of mutations (II). An ABI Prism 3700 DNA sequencer became available through the new Plant Molecular Genomics Facility at The Ohio State University (Columbus, OH), allowing for high-throughput sequencing of mutants. The improved quality of the sequencing reactions and increased length of output allowed use of the pRPS-MCS3 plasmids, rather than pUC19 subclones, to obtain sequence data. Using this system, 16 unique mutations, out of 72 plasmids sequenced, were rapidly identified. Once again, redundancy in the system was apparent, as most clones had
Figure 9. Western blot of an SDS-PAGE gel containing extracts from negative mutants grown photoheterotrophically with DMSO. Lane 1, *R. capsulatus* SBI-II containing pRPS-K11R; mutants in lanes 2 and 5-7 were unable to maintain a large subunit polypeptide; the mutant in lane 9 had a truncated large subunit polypeptide. Therefore mutants in lanes 3, 4, 8, 10, 11, and 12 were potential activity mutants.
Figure 9. Western blot for screening negative mutants.
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<th>Activity category</th>
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</tr>
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<td>None</td>
<td>88 (39)</td>
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<tr>
<td>Partial</td>
<td>74 (33)</td>
</tr>
<tr>
<td>Wild type</td>
<td>63 (28)</td>
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</table>

*None*=less than 1% of the activity measured for the wild type in the same assay, *partial*=1-95% of the activity measured for the wild type in the same assay, *wild type*=96% or greater than the activity measured for the wild type in the same assay.

Table 1.2. RubisCO specific activity categories of negative mutants.
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<td>agga</td>
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<td>1576</td>
<td>S</td>
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Table 1.3. Summary of the XL1-Red negative mutants (continued, next page).
(Table 1.3, continued)

| a | the residue altered as numbered in the *Synechococcus* PCC6301 sequence |
| b | the original amino acid |
| c | the amino acid in the mutant |
| d | the original codon |
| e | the codon in the mutant |
| f | the nucleotide number within *rbcLS*, with the a of the atg initiation codon at position 79. |
| g | the number of mutants sequenced that had this exact change |
| h | L=large subunit, S=small subunit |
| i | assembled holoenzyme as visualized on nondenaturing Western blots. +=visible holoenzyme, -= no visible holoenzyme, +/-=faintly visible band |
| j | activity relative to the wild type (100%) measured at the same time |
| k | na=not applicable, since a complete enzyme could not be assembled |
mutations previously identified. Also, from both sets of sequenced mutants, ~1/3 of the negative mutants sequenced were not the result of point mutations, but instead resulted from coinsertion of pUC19 into pRPS-MCS3 in the ligation reactions. This coinsertion likely prevented effective expression of \( rbcLS \) in \( R. \) capsulatus SBI-II.

Overall, 34 unique mutations were identified among all the XL1-Red mutants sequenced. The mutator strain *Epicurian coli* XL1-Red was an effective means for generating mutants that had only one or two point mutations per 1.8 kb \( rbcLS \) region, and there were only two insertion/deletion mutants identified. However, there was heavy favoritism for transitions over transversions, and among the transitions over 1/3 were g to a substitutions (Table 1.4).

Both pUC19 subclones and pRPS-MCS3 clones in *E. coli* were induced with IPTG to express the \( rbcLS \) genes, allowing for activity measurements to be made with the resultant recombinant proteins, and comparisons to be made with the nonmutated \( rbcLS \) genes expressed from the same plasmids (Table 1.3). *E. coli* extracts were also used for Western blot analyses of nondenaturing polyacrylamide gels to assay for properly folded and assembled holoenzyme. In addition, Western blots of SDS-containing gels assured that there were no gross changes in the migration of individual large subunits and that normal amounts of protein were present. Most negative mutants had no detectable activity in *E. coli*, and many were shown to be folding mutants, as identified by lack of detectable holoenzyme on nondenaturing PAGE (Figure 10). A few of the negative mutants were defective for catalysis, rather than folding. These included residues that had been previously studied and characterized (such as K198 and to some extent G176), as well as residues that had not previously been shown to influence activity (A47, D103).
<table>
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<tr>
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<tr>
<td>t to c</td>
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<td><strong>Total mutations</strong></td>
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Table 1.4. Point mutations in the XL1-Red mutants.

50
Figure 10. Western blots of nondenaturing PAGE gels were used to identify negative mutants that were unable to form holoenzyme. On the example shown, high-speed soluble extracts from *E. coli* expressing the K11R enzyme (lane 1) and some negative mutants (lanes 2-6) were compared. Mutants in lanes 4 and 5 were defective for holoenzyme assembly, and had no RubisCO activity in photoheterotrophic cultures of *R. capsulatus.*
Figure 10. Nondenaturing Western blot to screen for holoenzyme.
The characterization of two catalytic negative mutants, D103V and G176D, and one folding mutant, R347H, is discussed in Chapter 3. Mutations that resulted in F342V, F342I, or M259T substitutions yielded enzymes capable of complementing *R. capsulatus* SBI-II* to low-CO₂ photoautotrophic growth, and to weak CA growth. The F342V and M259T substitutions were studied in detail, and are discussed in Chapter 2.

**Mutagenic PCR and bioselection.** Mutagenesis by XL1-Red produced, on average, a single change per 1.8 kb *rbcLS*, and transitions were heavily favored over transversions. It was desirable to achieve a higher mutagenic rate and a more even distribution of mutation types. Previously, mutagenic PCR had been optimized for a small subunit template to achieve a point mutation rate of 0.25% (data not shown). The same conditions were used in mutagenic PCR using the entire *rbcLS* region, containing the F342V mutation, as a template.

Rather than picking and sequencing individual clones to see if the mutagenic PCR was working, a “quick” negative selection scheme was used to enrich for mutants that were defective for catalysis. Due to the difference in the functional significance of Rubisco to the cell under photoheterotrophic conditions, it seemed possible that a mutant that could grow photoheterotrophically, but not photoautotrophically, could be isolated. The isolation of such a mutant would quickly indicate that 1) the mutagenic PCR was effective, and 2) photoheterotrophically-competent cells could be directly selected from the conjugations, rather than plating mating mixtures onto chemoheterotrophic plates as was done in the original scheme. This modification could save time in future attempts to isolate mutants, by enriching for those which are not defective for folding/assembly (Figure 11, see Figure 7 for comparison).
Figure 11. Modified selection scheme to enrich for positive mutants during bioselection of PCR products with 0.5% CO$_2$. Mutagenic PCR products were cloned into pRPS-MCS3 (stage I), and all transformants were pooled for conjugations with \textit{R. capsulatus} SBI-II' (stage II). Dilutions of matings were plated directly onto photoheterotrophic plates (stage III) to select against plasmids with no inserts and assembly mutants, and to enrich for potential positive mutants. Portions of each mating were also plated directly onto photoautotrophic plates for incubation in an atmosphere of 0.5% CO$_2$/99.5% H$_2$ (stage IV). Colonies were picked from the malate plates onto a minimal medium plate for incubation at 0.5% CO$_2$, and onto a chemoheterotrophic “master” plate for future use (also stage IV). Positive mutants were identified by growth on the photoautotrophic plate, and were picked from the master plate for re-screening and back-matings. Negative mutants capable of growth on malate plates, but unable to grow with 5% CO$_2$/bal H$_2$ were also identified (not shown in this scheme).
Figure 11. The modified selection process.
Products of a single PCR reaction, using the F342V mutant as a template, were cloned into pRPS-MCS3. The matings were spread onto malate plates (photoheterotrophic) to test the new enrichment scheme, and onto 0.5% CO$_2$ plates to see if positive mutants could be selected directly. From the malate plates, 50 colonies were picked onto minimal medium plates that were incubated with high CO$_2$ (5%) in the atmosphere. Two of these isolates were unable to grow photoautotrophically. The first PCR negative mutant had three point mutations in addition to F342V. One was silent, one resulted in an L317M substitution in the large subunit, and one resulted in an S22G substitution in the small subunit. The second negative mutant had 7 new point mutations, one silent, one that changed the stop codon at the end of \textit{rbcL} from taa to tga, one that was in the intergenic region between \textit{rbcL} and \textit{rbsS}, and 4 mutations in the 5' region of the operon, resulting in L19H, T27A, M74V, and Q88R substitutions in the N terminus of the large subunit.

The second set of plates, incubated at 0.5% CO$_2$, were incubated for 11 days (longer than usual). The resulting colonies (174 total) were picked and re-screened for their ability to grow at 0.5% CO$_2$. Four of these mutants grew well after a 7 day incubation with 0.5% CO$_2$. The first 0.5% positive mutant had 8 point mutations, 4 silent, one intergenic, and three resulting in substitutions in both the large and small subunits (#1, Table 1.5). Two (#2 and #4) were identical, and had a silent mutation in each subunit. The third (#3) had two silent mutations in the large subunit (Table 1.5).

Using F342V, M262T, and wild type \textit{rbcLS} as templates, the products of eleven more PCR reactions were cloned into pRPS-MCS3. From all of these, ~2100 colonies were picked from malate plates and incubated photoautotrophically with 0.5% CO$_2$. A
<table>
<thead>
<tr>
<th>PA+ mutant number&lt;sup&gt;a&lt;/sup&gt;</th>
<th>LSU mutations&lt;sup&gt;b&lt;/sup&gt;</th>
<th>SSU mutations&lt;sup&gt;c&lt;/sup&gt;</th>
<th>silent mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>E90D, P68S, D84N</td>
<td></td>
<td>3 in LSU, 1 in SSU, 1 intergenic</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td>1 in LSU, 1 in SSU</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td>2 in LSU</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td>1 in LSU, 1 intergenic</td>
</tr>
<tr>
<td>7</td>
<td>R24S, A27T</td>
<td></td>
<td>1 in LSU</td>
</tr>
<tr>
<td>9</td>
<td>R439H, E35K</td>
<td></td>
<td>2 in LSU</td>
</tr>
<tr>
<td>10</td>
<td>S80N</td>
<td></td>
<td>1 in LSU</td>
</tr>
<tr>
<td>11</td>
<td></td>
<td></td>
<td>1 in LSU</td>
</tr>
<tr>
<td>12</td>
<td></td>
<td></td>
<td>3 in LSU, 1 intergenic</td>
</tr>
<tr>
<td>13</td>
<td>F217L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>K29N</td>
<td></td>
<td>3 in LSU</td>
</tr>
<tr>
<td>15</td>
<td>F469Y</td>
<td></td>
<td>1 in LSU</td>
</tr>
<tr>
<td>16</td>
<td></td>
<td></td>
<td>1 in LSU</td>
</tr>
<tr>
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<td>K29I, L21H</td>
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</tr>
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<td>K29N</td>
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</tr>
<tr>
<td>19</td>
<td>F311Y</td>
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<td>5 in LSU</td>
</tr>
<tr>
<td>20</td>
<td></td>
<td></td>
<td>4 in LSU</td>
</tr>
<tr>
<td>29</td>
<td>T365N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>31</td>
<td></td>
<td></td>
<td>1 in LSU</td>
</tr>
<tr>
<td>45</td>
<td></td>
<td></td>
<td>4 in LSU</td>
</tr>
<tr>
<td>47</td>
<td></td>
<td></td>
<td>1 in LSU</td>
</tr>
<tr>
<td>50 (M259T)</td>
<td></td>
<td></td>
<td>1 in LSU</td>
</tr>
</tbody>
</table>

<sup>a</sup>The number assigned to each isolate as it was sequenced.

<sup>b</sup>LSU=large subunit

<sup>c</sup>SSU=small subunit

<sup>d</sup>intergenic refers to the region between rbcL and rbcS

Table 1.5. 0.5% positive mutants.
portion of each mating was also plated onto 0.5% CO$_2$ photoautotrophic plates for direct positive selection. Altogether, 52 new positive mutants, 44 originally from malate plates and 8 directly from 0.5% photoautotrophic plates, were isolated and sequenced. There were 22 unique mutants, and all were in the F342V background, except for one M262T mutant with an additional silent mutation (#50, Table 1.5). Several had only silent mutations, mutations in the intergenic region, or both (Table 1.6). Most that had amino acid substitutions also had silent mutations. Thus, there were only two positive mutants with substitutions in the absence of any silent mutations (#13 and #29, Table 1.5).
<table>
<thead>
<tr>
<th>nucleotide #(^a)</th>
<th>mutant #(^b)</th>
<th>from(^c)</th>
<th>to(^c)</th>
<th>amino acid</th>
<th>preferred codon (%)(^d)</th>
</tr>
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<tr>
<td>234</td>
<td>45</td>
<td>gcG</td>
<td>gcT</td>
<td>A</td>
<td>gcc or gcg (46% each)</td>
</tr>
<tr>
<td>897</td>
<td>1</td>
<td>gcC</td>
<td>gcT</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>1176</td>
<td>9</td>
<td>gcG</td>
<td>gcA</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>1857</td>
<td>2</td>
<td>gcT</td>
<td>gcC</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>1350</td>
<td>19, 31</td>
<td>tgC</td>
<td>tgT</td>
<td>C</td>
<td>tgc (88%)</td>
</tr>
<tr>
<td>168</td>
<td>19, 20</td>
<td>gaC</td>
<td>gaT</td>
<td>D</td>
<td>gac (62%)</td>
</tr>
<tr>
<td>675</td>
<td>9</td>
<td>gaC</td>
<td>gaT</td>
<td>D</td>
<td></td>
</tr>
<tr>
<td>1245</td>
<td>1</td>
<td>gaA</td>
<td>gaG</td>
<td>E</td>
<td>gaa (48%)</td>
</tr>
<tr>
<td>666</td>
<td>15</td>
<td>ttC</td>
<td>ttT</td>
<td>F</td>
<td>ttc (86%)</td>
</tr>
<tr>
<td>447</td>
<td>12</td>
<td>ggC</td>
<td>ggT</td>
<td>G</td>
<td>ggc (66%)</td>
</tr>
<tr>
<td>768</td>
<td>47</td>
<td>ggT</td>
<td>ggG</td>
<td>G</td>
<td></td>
</tr>
<tr>
<td>1080</td>
<td>17</td>
<td>ggC</td>
<td>ggT</td>
<td>G</td>
<td></td>
</tr>
<tr>
<td>1317</td>
<td>50</td>
<td>ggT</td>
<td>ggA</td>
<td>G</td>
<td></td>
</tr>
<tr>
<td>1371</td>
<td>1</td>
<td>ggT</td>
<td>ggA</td>
<td>G</td>
<td></td>
</tr>
<tr>
<td>429</td>
<td>17</td>
<td>atC</td>
<td>atT</td>
<td>I</td>
<td>atc (92%)</td>
</tr>
<tr>
<td>972</td>
<td>7</td>
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<td>atT</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>672</td>
<td>45</td>
<td>aaA</td>
<td>aaG</td>
<td>K</td>
<td>aag (74%)</td>
</tr>
<tr>
<td>1086</td>
<td>14</td>
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<td>aaG</td>
<td>K</td>
<td></td>
</tr>
<tr>
<td>501</td>
<td>19, 20</td>
<td>ttG</td>
<td>ttA</td>
<td>L</td>
<td>ctg (64%)</td>
</tr>
<tr>
<td>877</td>
<td>11</td>
<td>Ttg</td>
<td>Ctg</td>
<td>L</td>
<td></td>
</tr>
</tbody>
</table>

Table 1.6. Silent mutations in the 0.5% CO\(_2\) positive mutants (continued, next page).
Table 1.6 (continued).

<table>
<thead>
<tr>
<th>nucleotide #</th>
<th>mutant #</th>
<th>from</th>
<th>to</th>
<th>amino acid</th>
<th>preferred codon (%)</th>
</tr>
</thead>
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<td>ctA</td>
<td>L</td>
<td></td>
</tr>
<tr>
<td>1113</td>
<td>17</td>
<td>ttG</td>
<td>ttA</td>
<td>L</td>
<td></td>
</tr>
<tr>
<td>930</td>
<td>6, 16</td>
<td>aaC</td>
<td>aaT</td>
<td>N</td>
<td>aac (82%)</td>
</tr>
<tr>
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<td>19</td>
<td>aaC</td>
<td>aaT</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>150</td>
<td>12</td>
<td>ccC</td>
<td>ccT</td>
<td>P</td>
<td>ccg (61%)</td>
</tr>
<tr>
<td>525</td>
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<td>ccA</td>
<td>P</td>
<td></td>
</tr>
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<td>45</td>
<td>ccG</td>
<td>ccA</td>
<td>P</td>
<td></td>
</tr>
<tr>
<td>1299</td>
<td>3</td>
<td>ccC</td>
<td>ccA</td>
<td>P</td>
<td></td>
</tr>
<tr>
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<td>1</td>
<td>ccC</td>
<td>ccT</td>
<td>P</td>
<td></td>
</tr>
<tr>
<td>630</td>
<td>19, 20</td>
<td>cgT</td>
<td>cgA</td>
<td>R</td>
<td>cgc (55%)</td>
</tr>
<tr>
<td>1386</td>
<td>3</td>
<td>cgT</td>
<td>cgA</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>90</td>
<td>2</td>
<td>acG</td>
<td>acA</td>
<td>T</td>
<td>acc (62%)</td>
</tr>
<tr>
<td>147</td>
<td>45</td>
<td>acC</td>
<td>acT</td>
<td>T</td>
<td></td>
</tr>
<tr>
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<td>20</td>
<td>acC</td>
<td>acA</td>
<td>T</td>
<td></td>
</tr>
<tr>
<td>936</td>
<td>10</td>
<td>gtc</td>
<td>gtc</td>
<td>V</td>
<td>gtc (47%)</td>
</tr>
<tr>
<td>129</td>
<td>12</td>
<td>taC</td>
<td>taT</td>
<td>Y</td>
<td>tat (42%)</td>
</tr>
<tr>
<td>156</td>
<td>15</td>
<td>taC</td>
<td>taT</td>
<td>Y</td>
<td></td>
</tr>
<tr>
<td>1499</td>
<td>1</td>
<td>g</td>
<td>a</td>
<td>intergenic</td>
<td></td>
</tr>
<tr>
<td>1570</td>
<td>6</td>
<td>t</td>
<td>c</td>
<td>intergenic</td>
<td></td>
</tr>
<tr>
<td>1572</td>
<td>12</td>
<td>t</td>
<td>a</td>
<td>intergenic</td>
<td></td>
</tr>
<tr>
<td>1584</td>
<td>11</td>
<td>a</td>
<td>g</td>
<td>intergenic</td>
<td></td>
</tr>
</tbody>
</table>

\(^{a}\)nucleotide number as assigned in Figure 6.

\(^{b}\)mutant number, assigned as each mutant was sequenced. See table 1.5.

\(^{c}\)from= the original codon, t= the codon in the mutant. The bases that changed are in capital letters.

\(^{d}\)the preferred codon for the given amino acid in *R. capsulatus* (Wu and Saier, 1991)
Discussion

These results describe the first bacterial system developed for random mutagenesis and biological selection for changes in RubisCO. Due to its complementation properties in *R. capsulatus* SBI-II*, the Synechococcus PCC6301* RubisCO was the enzyme used in these studies (Table 1.1). This enzyme's extremely high $K_c$ (~173 µM, Horken and Tabita, 1999) was suspected to be a factor its inability to complement when the level of CO$_2$ in the atmosphere was lowered to 1.5%. By comparison, form I RubisCO of *R. capsulatus*, which shares 72% and 37% identity with the large and small subunits of the cyanobacterial enzyme, respectively, has a $K_c$ of approximately 29 µM (Horken and Tabita, 1999), and can complement the deletion strain to both chemoautotrophy and to photoautotrophy with 1.5% CO$_2$. Lower expression of the cyanobacterial genes in *R. capsulatus* was not believed to be the cause of the observed phenotypes, since the cyanobacterial enzyme complemented the deletion strain to photoautotrophic growth with higher levels of CO$_2$, and in liquid medium that was continuously bubbled with 1.5%CO$_2$/98.5% H$_2$. These results led to the hypothesis that the $K_c$ of the cyanobacterial enzyme was responsible for the observed differences, and that a mutant enzyme capable of complementing SBI-II* to growth under these conditions could conceivably have a lower $K_c$.

There were other advantages to using the cyanobacterial RubisCO. First, the protocols for overexpression and purification of the enzyme from *E. coli* were well established in our laboratory. Next, there was a wealth of information about this enzyme in both our laboratory and the literature, including the sequence and crystal structure, the
properties of site directed mutants and hybrid enzymes, and studies of the unique kinetic
properties of this enzyme.

Some of the properties which make the cyanobacterial enzyme unique include its
close identity with primary and tertiary structures of the plant enzymes (the Form IB
"green" enzymes; Tabita, 1995), its resistance to increasing inhibition by slow, tight-
binding inhibitors with reaction time, a phenomenon that affects several RubisCOs
("fallover"; Andrews and Ballament, 1984; Edmondson et al., 1990a, b, c), and, as
described above, its poor affinity for CO$_2$. The latter property has important
physiological consequences, creating a requirement for a carbon concentrating
mechanism (reviewed in Kaplan and Reinhold, 1999). This characteristic led us to try
complementation studies at decreased concentrations of CO$_2$ in the atmosphere, the
condition that defined the first positive selection condition for mutants.

Random mutagenesis of the enzyme was achieved with the mutator strain
_Epicurian coli_ XL1-Red (Stratagene, La Jolla, CA). This technique was chosen for its
simplicity, and because it generates about 1 mutation in 2000 bp, a size roughly similar to
the size of the _rbcLS_ target (1800 bp). Though it was suggested in the Introduction that
the kinetic changes desired in RubisCO will likely require multiple substitutions
throughout the enzyme, this technique, which would generate about 1 mutation per
enzyme, was chosen to test the utility of the system for finding mutants, especially
negative mutants. XL1-Red proved to be an effective means for generating single-site
mutants with interesting properties, and even with the relatively low level of mutagenesis
some positive mutants were isolated. Contrary, however, to the manufacturer's claims,
there was a strong bias for transition point mutations over transversions. The majority of

62
all mutations were g to a transitions (Table 1.4). Preference for this change could be the consequence of the relatively high GC content (58%) of the genes targeted here.

Mutagenic PCR was used as a more aggressive method of mutagenesis in later rounds of selection. In directed evolution experiments, it is typical to use selected mutants for further rounds of mutagenesis, leading to further “improvements” by increasing the stringency of selection. The XL1-Red positive mutants isolated with 1.5% CO$_2$ were discovered to be incapable of growth when CO$_2$ was lowered to 0.5% in the atmosphere. Attempts to “improve” these mutants thus required a technique that would induce more mutations than XL1-Red, and allow for selection under photoautotrophic growth conditions with 0.5% CO$_2$.

The selection was modified to enrich for positive mutants and catalytic (as opposed to folding) negative mutants, while avoiding false negative mutants (i.e., plasmids with no inserts). To do this, matings were spread onto photoheterotrophic plates, rather than chemoheterotrophic plates. This enriched for plasmids that had inserts, enzymes that properly assembled, and enzymes that had at least some RubisCO activity (Figure 10). Mutants capable of photoheterotrophic growth, but not photoautotrophic growth with high CO$_2$, were excellent candidates for negative catalytic activity mutants. However, for the first time evidence was obtained that silent mutations within the \textit{rbcL:S} region could result in changes in positive mutants, indicating that even the mutants with defined amino acid substitutions may not necessarily be the result of kinetic changes in the enzyme (Chapter 2).

For both mutagenic techniques, there was an even distribution of mutations throughout the sequence, indicating that all regions of the enzyme were potential targets.
(Tables 1.3, 1.5, 1.6; Figure 12). The effectiveness of the selection was also verified, through isolation of mutants such as K198R. This residue receives the “activator” CO$_2$ molecule to form a lysyl carbamate, and it is present and required by every RubisCO known (Lorimer et al., 1976; Miziorko, 1979; Lorimer and Miziorko, 1980). The K198R mutant was isolated as an XL1-Red negative mutant, consistent with its invariant role in catalysis.

The primary limitation of the system as described in this Chapter was the amount of time elapsed between mutagenesis and mutation identification. Of course, there were limitations as to how many colonies could be picked onto the minimal medium plates. The selection jars were incubated for one week at a time, and when potential negative mutants were identified they had to be screened for the presence of $rbcL$, a process that took another week. Initially pRPS-MCS3 clones in XL1-Red positive and negative mutants were back-mated from SBI-II’ to *E. coli* JM109, and then subcloned into pUC19 prior to sequencing. One improvement that arose during the implementation of the system was the opening of the new Plant-Microbe Genomics Facility, which allowed for excellent, rapid sequencing results that could be obtained directly off of the pRPS-MCS3 clones, abrogating the need for a subcloning step into pUC19 and reducing the number of sequencing reactions needed to cover the entire 1.8 kb region. Finally, sequencing data revealed that there was great redundancy in the system, meaning that many mutants sequenced were previously identified. This redundancy resulted in overall decreased efficiency with which unique mutants could be identified, and was the consequence of multiple subcloning and conjugation steps.
Figure 12. Summary of the positive and negative large subunit substitutions identified through bioselection of XL1-Red plasmid libraries. Residues within α-helices are doubly underlined, and residues within β-sheets are singly underlined. *Italicized* residues were changed as a consequence of mutagenesis with *E. coli* XL1-Red (refer to Table 1.3), and these include the positive mutants M259T, F342V, and F342I. Note that K11R was present in all mutants isolated, but it was on the plasmid used to transform XL1-Red (pUCLS), and therefore is not shown in this Figure. Active site residues are in **bold**.
Figure 12. Summary of the XL1-Red large subunit mutations.
In spite of these limitations, this system has allowed for the identification of several mutants that would not have been examined by site-directed mutagenesis, simply because they are in regions that would not be predicted to play a major role in catalysis or as determinants of kinetic properties. The advantages of this bacterial system, and considerations for its future use, include the availability of multiple target enzymes, the ability to mutagenize either the entire enzyme or only regions of the enzyme, the increased efficiency with which mutants can be selected, and the potential to select various types of mutants, including folding, catalytic, or stability mutants. Chapters 2 and 3 demonstrate the utility of the system not only for the isolation of interesting positive and negative mutants, respectively, but also for directing future use of site-directed mutagenesis to study regions of the enzyme discovered to have unpredicted effects.
CHAPTER 2

Characterization of Positive Selection Mutants

Introduction

Complementation of *R. capsulatus* SBI-II' to photoautotrophic growth at low CO₂ concentrations, or to aerobic chemoautotrophic growth, provided the rationale for positive selection of cyanobacterial RubisCO with an “improvement” in its affinity for CO₂, i.e., a lower K_c (Chapter 1). As a consequence of a lowered K_c, such mutants could potentially possess improvements or alterations in the specificity (Ω) for CO₂ and O₂.

Unlike site directed mutants previously found to affect Ω that invariably possess low inherent activity, biological selection has the potential to enrich for enzymes with “improved” specificity and relatively good activity since such enzymes would of necessity support CO₂-dependent growth. Such a mutant has eluded researchers for decades; its implications for bioengineering of the CO₂ assimilatory process are enormous. Thus, the risks associated with this type of selection were well worth taking.

Two of the positive mutants isolated through bioselection of the XL1-Red plasmid libraries, F342V and M259T, were purified and their kinetic properties were characterized to ascertain whether these parameters were targets of bioselection (Figure 13). Furthermore, several positive mutant enzymes, whose genes were altered via mutagenic PCR protocols and isolated through subsequent bioselection, were also purified and analyzed kinetically. The following pages illustrate that the organism
Figure 13. Ribbon diagram showing the positions of F342 and M259 in the large subunit (in green). Residue F342 is in α-helix 6 that is immediately adjacent to loop 6. M259 is in the C-terminal domain, but is distant from the active site, in a loop that contacts a small subunit within the holoenzyme.
Figure 13. The positions of residues F342 and M259 in the large subunit.
found unpredicted ways to deal with the selection conditions chosen. Irregardless, the fact that some of the mutations obtained did not negatively affect the enzyme's function is in itself informative, and one mutant, F342V, was found to have an increased affinity for the substrate RuBP. Thus, the utility of this system has been demonstrated while also illustrating the fact that specific residues could be discovered that play heretofore unknown roles in catalysis. The initial studies reported here also demonstrated that modifications in the selection parameters will be required for future efforts to experimentally identify mutations that produce improvements in $K_c$, $\Omega$, and other properties.

**Materials and Methods**

*Strains, plasmids, and growth.* All *R. capsulatus* and *E. coli* strains were grown as described in the “Materials and Methods” section of Chapter 1. Growth curves and growth rates of photoautotrophic cultures were determined by removing 1 ml samples at 6-12 h intervals for absorbance readings at 660 nm. Beyond an $OD_{660}$ of 0.6, samples were diluted 1.5 for such measurements. Growth rates were calculated from the early to mid exponential phase of the growth curve (usually an $OD_{660}$ ranging from 0.1 to 0.4). When cultures reached an $OD_{660}$ between 1.2 and 1.5, a range known to have maximum RubisCO specific activity (data not shown), 40 ml were removed, the cells were washed with TE (100 mM Tris-HCl, 1 mM EDTA, pH 8.0), and then the cell pellets were stored at –80°C until used for assays.

*E. coli* JM109 cultures were grown in Luria-Bertani medium at 37°C, with shaking at ~200 rpm, and 100 $\mu$g/ml ampicillin and 12.5 $\mu$g/ml tetracycline when appropriate. For overexpression of RubisCO subclones in pUC19, 25 ml cultures were
inoculated with 2 ml overnight cultures. After 3 hours of growth, IPTG was added to a concentration of 0.5 mM, and incubation and growth continued for 16 h.

Construction of single site mutants. The F342V and M259T substitutions were constructed for study of the mutations in the absence of K11R, a mutation present in all XL1-Red mutants due to its presence on the plasmid pUC-LS (Chapter 1). The F342V mutation was isolated by digestion of the pUC19 clone of K11R/F342V with EcoRI and XbaI, which excised the 3' coding region encompassing the C terminus of the large subunit and all of the small subunit. This fragment was used to replace the wild type fragment in pUC6301, generating a clone that had the wild type N-terminus (with no K11R), and the F342V C-terminus of the protein. For M259T, site-directed mutagenesis was used to introduce the change into the wild type gene, using the QuikChange Site-Directed Mutagenesis Kit of Stratagene (La Jolla, CA), and the following complementary primers:

5' GCTAAAGAACTCGGCACGCCGATCATCATGCAT3' and 5' CATGCATGATGATCGGCGTGCCGAGTTCTTTAGC3'. Fragments containing the single mutations were cloned into pRPS-MCS3 and conjugated into R. capsulatus SBI-II for comparative studies with the original K11R/F342V and K11R/M259T mutants, as well as K11R and the wild type.

Preparation of cell extracts and assays. Cells from both R. capsulatus and E. coli cultures were washed twice with TE. Pellets were stored at -80°C. Prior to assays, frozen cells were thawed on ice, and resuspended in TE containing 5 mM β-mercaptoethanol. Cells were disrupted by sonication, and low-speed supernatants were obtained by microcentrifugation for 10 min at 4°C. High-speed supernatants from E. coli...
extracts were obtained by ultracentrifugation at 45,000 rpm, in a Beckman 70Ti rotor, for 1.5 h at 4°C. RubisCO activity was measured by incorporation of $^{14}$CO$_2$ into acid-stable products (Whitman and Tabita, 1976). Protein was measured by a modification of the Lowry assay (Markwell et al., 1978).

**Purification of RubisCO.** Buffer TEM (25 mM Tris-HCl, 1 mM EDTA, 10 mM MgCl$_2$, pH 8.0) was prepared and stored at 4°C several days before purification. TEMMB (25 mM Tris-HCl, 1 mM EDTA, 10 mM MgCl$_2$, 5 mM β-mercaptoethanol, 50 mM NaHCO$_3$, pH 8.0) was freshly prepared for each purification by adding NaHCO$_3$ and β-mercaptoethanol to TEM.

For the XL1-Red positive mutants, 1 L cultures of *E. coli* JM109 containing the appropriate pUC19 subclone were induced with 0.5 mM IPTG for 16 hours. For the 0.5% positive mutants, cells were grown in 5 L fermentors, and induced in the same manner. Cells were harvested by centrifugation and washed twice with TE before storage at -80°C. The cell pellets from the fermentor cultures were split into three separate pellets before being frozen. Cells from 1 L shake cultures or from 1/3 of a 5 L fermentor were thawed on ice and resuspended in 10 ml ice-cold TEMMB, then disrupted by sonication. The lysate was centrifuged at 10,000 rpm for 10 min at 4°C, in a JA-20 rotor for a Beckman Model J2-21 centrifuge (Beckman, Palo Alto, CA). The low-speed supernatant was transferred to an ultracentrifuge tube for a 70Ti rotor (Beckman) and centrifuged at 45,000 rpm for 1.5 h at 4°C. A 100 µl sample of the high-speed supernatant (crude extract) was removed for assays, and the remainder was loaded onto a Green A column.
The Green A dye-affinity column (Amicon, Beverly, MA) was equilibrated with TEMMB at 4°C. RubisCO was eluted with a 0-1 M NaCl gradient in TEMMB while fractions were collected at 0.4 min/fraction using a Model 203 micro-fraction collector (Gilson, Middleton, WI). Fractions with the highest RubisCO activity were combined and a 200 μl sample was removed. The pooled column fractions were concentrated by the addition of solid ammonium sulfate to 70% saturation. The concentrated sample was resuspended in TEMMB, and desalted with an Econo Pak 10DG disposable chromatography column (BioRad Laboratories, Hercules, CA) that had been equilibrated with TEM. The enzyme was eluted with TEM.

The desalted sample, in a volume of about 4 ml, was loaded onto a sucrose density step gradient (0.2, 0.4, 0.6, 0.8 M sucrose in TEM) and centrifuged in a Beckman SW28 swinging bucket rotor at 25,000 rpm for 20-22 h at 4°C. Fractions of 1 ml were manually collected and the fractions with the highest RubisCO activity were pooled. A 50 μl sample was removed (sucrose pool) and the remainder was dialyzed against TEM for 2 h; the buffer was exchanged and dialysis continued for 2 h. The third and final 2 h of dialysis was against TEM containing 20% glycerol. A 50 μl sample was removed (final sample), and the remainder was stored at -80°C.

**Kinetic measurements.** Samples removed at the end of purification, before storage of the enzyme, were used to measure the $V_{\text{max}}$ and $k_{\text{cat}}$ of the enzymes in 2 min RubisCO assays. The $K_m$ for CO$_2$ ($K_c$) was determined under strict anaerobic conditions (100% N$_2$ atmosphere). Dilutions of $^{14}$C-bicarbonate were prepared in 50 mM bicine-NaOH buffer with 10 mM MgCl$_2$. The pH of the buffer was usually around 8.0, and the exact pH was recorded for each assay. This measurement was used to calculate the
concentration of CO₂ in each assay, as derived from the Henderson-Hasselbach relationship: pH = pKₐ + log [HA] - log [A]. The concentration of CO₂, [A], was calculated using a pKₐ of 6.12 for bicarbonate, where [HA] is the concentration of bicarbonate in the assay. In an assay volume of 250 µl, CO₂ concentrations ranged from 42 to 2000 µM CO₂, and assays contained 10 U of pure enzyme. Assays were initiated by the addition of anaerobic RuBP to 1 mM, and terminated after 30 sec by addition of 100 µl of propionate. Samples were dried at 65°C overnight, under vacuum, and products were redissolved in 2 N HCl, and added to 3 ml of scintillation cocktail. Results were plotted using Sigma Plot 2000, and the Kₘ was derived by fitting values to a hyperbolic curve.

The Kₘ for RuBP was measured with concentrations of RuBP ranging from 16 to 100 µM, which was used to initiate a 30 sec reaction that was terminated with propionic acid. Products were dried and counted as described above for the Kₛ assays, and results were plotted using Lineweaver-Burke reciprocal plots.

Specificity was measured under conditions of saturating O₂ (1.23 mM) with 200 mM NaHCO₃ in 50 mM bicine buffer, pH 8.0, and 10 mM MgCl₂. Concentration of CO₂ was calculated from the Henderson-Hasselbach relationship, as described above for Kₛ. Reactions were initiated by addition of [1-³H] RuBP, and incubated at 23°C for 1-3 h. Reaction products were separated with a MonoQ resin using a Dionex DX500 chromatography system (Dionex Corporation, Sunnyvale, CA) and detected with an in-line scintillation counter (IN/US β-Ram, Tampa, FL), as previously described (Harpel et al., 1993).
Thermostability assays. Thermostability of the purified, activated enzymes was assayed by a previously described method (Du et al., 2000). 5 μg of purified enzyme in a final volume of 30 μl containing 80 mM Hepes-NaOH, pH 8.0, 10 mM MgCl₂, 50 mM NaHCO₃, 1 mM DTT buffer was incubated at the appropriate temperatures (40, 45......75 °C) for 20 minutes in a Perkin-Elmer Thermalcycler (Perkin Elmer, Norwalk, CT). Samples were cooled on ice for 5 min, then 25 μl, containing about 4 μg of enzyme, was added directly to a 225 μl assay mixture of 80 mM Hepes, 20 mM NaH¹⁴CO₃ (~5 Ci/mol), 10 mM MgCl₂, 0.8 mM RuBP, that had been equilibrated at 30°C. Assays were incubated for 2 min at 30°C, and terminated by the addition of 100 μl of propionic acid. After centrifugation, 200 μl of each assay was added to 3 ml of Ecoscint-A scintillation cocktail (National Diagnostics, Atlanta, GA). The relative activity was calculated as the counts per minute divided by the counts per minute in the 40°C assay.

Thermostability with prolonged incubation at 55°C was measured by the same method, but 200 μl of enzyme in activation buffer was incubated in the thermalcycler at 55°C, and 25 μl aliquots were removed at 20 min intervals, cooled for 5 min, then assayed as described. Relative activity was calculated as the counts per minute for each assay divided by the counts per minute in a sample that had not been incubated at 55°C (t=0).

One thermostability assay was done without preactivation of the enzymes prior to thermal incubation. Purified enzymes in the storage buffer, TEM + 20% glycerol, were incubated directly at the temperatures indicated, then added to 100 μl of 80 mM Hapes-NaOH buffer, pH 8.0, and incubated for 5 min at 30°C. The standard RubisCO assay was...
then carried out as previously described, by adding the activation buffer containing
NaH\textsuperscript{14}CO\textsubscript{3} and MgCl\textsubscript{2} 5 min prior to addition of the substrate RuBP (Whitman and
Tabita, 1976).

\textit{Proteinase K digestion.} Proteinase K was dissolved in distilled H\textsubscript{2}O to a
concentration of 0.5 \(\mu\)g/ml, and the protease inhibitor phenylmethylsulfonyl fluoride
(PMSF, Sigma, St. Louis) was prepared as a 22 mM solution in 100\% ethanol. Each
assay contained 2 \(\mu\)g enzyme in 8 \(\mu\)l TEM. To this, 2 \(\mu\)l of proteinase K was added. 1 \(\mu\)l
of PMSF was added to terminate reactions at 5 and 10 min intervals. Immediately
following addition of PMSF, SDS-PAGE sample loading buffer was added. To the \(t=0\)
control, PMSF was added prior to proteinase K, then loading buffer was added. All
samples were electrophoresed in 12.5\% acrylamide gels.

\textbf{Results}

\textit{Overexpression studies of the XL1-Red positive mutants in E. coli.} When the first
XL1-Red positive mutant, K11R/F342V, was identified through subcloning and
sequencing of \textit{rbcL} in pUC19, studies were performed to compare the relative activities
of the mutant enzyme and the wild type. After construction of the single mutant, F342V,
the pUC19 subclones of the wild type, K11R, K11R/F342V, and F342V were used to
overexpress the genes in \textit{E. coli} JM109. Cultures initially were grown to an \(OD_{595}\) of
0.3-0.4, and then induced with 0.5 mM IPTG for 16 h. Occasionally, but not
consistently, the F342V and K11R/F342V cultures had higher activity than the wild type
and K11R cultures, which were always very similar to one another.
Shorter induction times were examined to determine if there was any appreciable difference in the specific activity of these cultures. When inductions were shortened to 12 and 9 hours, there appeared to be higher RubisCO specific activity in the F342V mutant cultures (Table 2.1). Once the next two positive mutants, K11R/F342I and K11R/M259T, were identified, an experiment was done in which the *E. coli* cultures were induced for 9 h. The mutant cultures had higher specific activities than the wild type and K11R cultures (Figure 14). Whole cells and extracts from these cultures were examined on Western blots. Interestingly, it did not appear qualitatively that there were higher levels of large subunit polypeptides in the whole cells of mutant cultures (Figure 14). However, when the high-speed soluble extracts were compared, the mutant extracts appeared to have slightly more large subunit polypeptide. The fact that there was no visible difference in the whole cells indicated that the mutant cultures did not simply contain more total RubisCO than the wild-type cultures. This suggested that some property of the mutant enzymes had resulted in the increased levels of enzyme in soluble extracts and, likewise, the higher specific activities. The relevance of this observation to the growth phenotype of *R. capsulatus* SBI-II when complemented with these enzymes was examined.

*Construction of the single mutants, F342V and M259T.* In the context of K11R, the additional changes of F342V, F342I, or M259T resulted in enzymes capable of complementing *R. capsulatus* SBI-II to low-CO₂ photoautotrophic growth, and to weak chemoautotrophic growth on minimal medium plates. The F342V and M259T substitutions were studied in the absence of K11R. The single mutants were cloned into
Rubisco expressed<sup>a</sup> Specific Activity<sup>b</sup>

<table>
<thead>
<tr>
<th></th>
<th>16 h induction&lt;sup&gt;c&lt;/sup&gt;</th>
<th>12 h induction&lt;sup&gt;d&lt;/sup&gt;</th>
<th>9 h induction&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>0.21</td>
<td>0.27</td>
<td>0.23</td>
</tr>
<tr>
<td>K11R</td>
<td>0.22</td>
<td>0.20</td>
<td>0.15</td>
</tr>
<tr>
<td>F342V</td>
<td>0.21</td>
<td>0.49</td>
<td>0.44</td>
</tr>
<tr>
<td>K11R/F342V</td>
<td>0.16</td>
<td>0.37</td>
<td>0.35</td>
</tr>
</tbody>
</table>

<sup>a</sup>The genes were expressed on pUC19.

<sup>b</sup>Expressed as μmol CO₂ fixed/min/mg protein in the crude extract.

<sup>c</sup>The amount of time the culture was grown after the addition of IPTG to 0.5 mM.

<sup>d</sup>The 9 and 12 h samples were taken from duplicate cultures, grown at the same time. The 16 h cultures shown here were also in duplicate, but were grown and assayed at an earlier date. The numbers shown here are the average of the duplicate cultures.

Table 2.1. Rubisco activity in E. coli expressing wild-type and mutant Rubisco genes.

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Figure 14. Western blot showing *E. coli* extracts from cultures expressing wild-type and mutant RubisCO genes on pUC19. Cultures were induced for 9 hours with 0.5 mM IPTG. 5 μg of protein was loaded into each lane, and the table indicates which samples were in each set of lanes. “A” lanes are whole cells, “B” lanes are high speed soluble extracts. RubisCO specific activities for the soluble extracts are given below each set of lanes, in μmol CO$_2$ fixed/min/mg protein.
Figure 14. Western blot and RubisCO specific activity for the wild type and positive mutants expressed in *E. coli*.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>wt</th>
<th>K11R</th>
<th>K11R/ F342V</th>
<th>K11R/ M259T</th>
<th>K11R/ F342I</th>
</tr>
</thead>
<tbody>
<tr>
<td>specific activity</td>
<td>0.17</td>
<td>0.12</td>
<td>0.25</td>
<td>0.21</td>
<td>0.27</td>
</tr>
</tbody>
</table>
pRPS-MCS3 and conjugated into *R. capsulatus* SBI-II'. The growth phenotypes of the wild type and of the single mutants on minimal medium plates were identical to the phenotypes of the mutants in the K11R background. In fact, the single mutants had identical properties in all respects to the original K11R double mutants, which were tested in parallel, in the experiments discussed below (Figure 15).

*Growth of the XL1-Red 1.5% CO$_2$ positive mutants in liquid cultures.* The growth phenotypes of *R. capsulatus* SBI-II' expressing mutant and wild type *rbcLS* were studied in liquid cultures. In photoautotrophic cultures, the positive mutant enzymes invariably enabled the strain to grow faster than when it was complemented with either the wild-type or the K11R enzyme (Table 2.2, Figure 16). Furthermore, the wild-type cultures had a longer lag phase, around 150 hours, relative to that of the mutant cultures, around 100 hours after inoculation. RubisCO activity in these cultures varied greatly, ranging from 0.1 to 0.8 μmol CO$_2$ fixed/min/mg protein, with the mutants usually, but not always, having higher activity than the wild type (Table 2.2).

Even when the mutant and wild-type cultures differed very little in RubisCO specific activity, the mutant cultures grew faster. For example, in one set of wild type and K11R/M259T cultures grown simultaneously, the wild-type culture had a doubling time of 43 h and the mutant had a doubling time of 25 h. RubisCO activity, however, varied little, with 0.48 μmol CO$_2$ fixed/min/mg protein in the wild-type culture, and 0.45 μmol CO$_2$ fixed/min/mg protein in the K11R/M259T culture (Figure 17). This lack of correlation was also evident on Western blots of extracts, with mutant cultures usually, but not consistently, appearing to have increased levels of RubisCO (Figures 18 and 19)
Figure 15. Growth of *R. capsulatus* SBI-II' photoautotrophically in liquid cultures bubbled with 1.5% CO₂/98.5% H₂, complemented with the wild-type and K11R enzymes. This curve demonstrates that the K11R mutation did not alter growth. For the specific cultures shown here, the calculated doubling times were approximately 39 hours.
Figure 15. Photoautotrophic growth of the wild type and the K11R mutant.
<table>
<thead>
<tr>
<th>RubisCO expressed</th>
<th>Photoautotrophic&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Chemoautotrophic&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>doubling time&lt;sup&gt;c&lt;/sup&gt;</td>
<td>specific activity&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>wild type</td>
<td>42</td>
<td>0.24</td>
</tr>
<tr>
<td>F342V</td>
<td>25</td>
<td>0.36</td>
</tr>
<tr>
<td>M259T</td>
<td>30</td>
<td>0.41</td>
</tr>
<tr>
<td>K11R</td>
<td>41</td>
<td>0.20</td>
</tr>
<tr>
<td>K11R/F342V</td>
<td>28</td>
<td>0.30</td>
</tr>
<tr>
<td>K11R/M259T</td>
<td>34</td>
<td>0.36</td>
</tr>
</tbody>
</table>

<sup>a</sup>liquid minimal medium was bubbled with 1.5% CO₂/98.5% H₂, incubated in front of lights at 30°C
<sup>b</sup>liquid minimal medium was bubbled with 5% CO₂/10% O₂/85% air in the dark at 30°C
<sup>c</sup>doubling time is in hours, this is the average of at least two cultures for each
<sup>d</sup>specific activity is expressed as μmol CO₂ fixed/min/mg protein, this is the average of at least two cultures for each.
<sup>e</sup>ng=no growth

Table 2.2. Doubling times and RubisCO specific activity in photoautotrophic and chemoautotrophic cultures.
Figure 16. Cultures of *R. capsulatus* SBI-II containing the wild-type, F342V, or M259T enzymes were simultaneously grown photoautotrophically in liquid minimal medium that was continuously bubbled with 1.5% CO$_2$/98.5% H$_2$. The early exponential growth phase is shown here, the point at which the culture OD$_{660}$ was between 0.2 and 0.4. This is the OD$_{660}$ range over which the growth rates were calculated for all samples. Note that for construction of this graph, the X-axis represents the hours over this range of OD$_{660}$, and not the hours after inoculation. This was necessary because of the variable lag times for the cultures (see text). For these specific samples, the lag times were: WT, 153 h; F342V, 95 h; and M259T, 118 h. The doubling times, in hours, were: WT, 43; F342V, 28; M259T, 30.
Figure 16. Photoautotrophic growth of the wild type and the F342V and M259T mutants.
Figure 17. Coomassie-stained SDS-PAGE gel of extracts of photoautotrophically grown *R. capsulatus* SBI-II- containing wild-type or mutant *rbcL S*. This gel demonstrates the lack of correlation often encountered between activity and growth rate, irrespective of which enzyme was complementing the strain to growth. Lane 1, purified wild-type enzyme; lanes 2 and 3, wild-type; lanes 4 and 5, K11R/M259T; lane 6, K11R/F342V; lanes 7 and 8, F342V. The accompanying table shows the doubling times and specific activities (µmol/min/mg protein) for the cultures shown on the gel. In lane 4, this K11R/M259T culture, in spite of its very high activity, grew slower than usual due to complications maintaining consistent bubbling of this culture with the gas mixture. In spite of this, the strain still grew faster than the wild type cultures.
<table>
<thead>
<tr>
<th>Lane</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>doubling time (h)</td>
<td>42</td>
<td>43</td>
<td>38</td>
<td>25</td>
<td>24</td>
<td>22</td>
<td>24</td>
</tr>
<tr>
<td>specific activity</td>
<td>0.28</td>
<td>0.48</td>
<td>0.78</td>
<td>0.45</td>
<td>0.57</td>
<td>0.63</td>
<td>0.74</td>
</tr>
</tbody>
</table>

Figure 17. Coomassie-stained polyacrylamide gel of wild type and positive mutant photoautotrophic extracts.
**Figure 18.** Nondenaturing Western blot showing the wild-type and mutant genes expressed in *R. capsulatus* SBI-II grown in photoautotrophic cultures, bubbled with 1.5% CO₂/98.5% H₂. 5 μg protein were loaded into each lane. Lane 1, wild type; lane 2, F342V; lane 3, M259T; lane 4, K11R; lane 5, K11R/F342V; lane 6, K11R/M259T.
Figure 18. Nondenaturing Western blot of wild type and mutant photoautotrophic extracts.
Figure 19. Chemifluorescent Western blot of photoautotrophically grown *R. capsulatus* SB1-II\textsuperscript{+} containing wild type and mutant *rbcLS* in plasmid pRPS-MCS3. Cells were harvested at an OD\textsubscript{660} between 1.2 and 1.5, washed, and stored at -80°C prior to sonication. The gels were run under denaturing conditions (with SDS), and 5 µg of extract were loaded in each lane. The arrows indicate large subunits (L) and small subunits (S). Lane 1, wild type; lane 2, F342V; lane 3, M259T; lane 4, K11R; lane 5, K11R/F342V; lane 6, K11R/M259T.
Figure 19. Western blot of wild type and mutant photoautotrophic extracts in the presence of SDS.
The positive mutant enzymes had also conferred the ability of the strain to grow on chemoautotrophic plates, while the wild type enzyme was incapable of complementation under this growth condition. Chemoautotrophic liquid cultures were inoculated with photoautotrophically grown cells, and bubbled with a gas mixture of about 5% CO₂/10% O₂/balance air. In liquid chemoautotrophic cultures, the mutant enzymes enabled the strain to grow, with doubling times between 25 and 29 h, while the wild-type enzyme was unable to support the growth of strain SBI-II' (Table 2.2, Figure 20). RubisCO activity was measured in *R. capsulatus* SBI-II containing the wild type enzyme after it had been incubated in chemoautotrophic culture for one week, and had not grown. The RubisCO specific activity was around 0.12 μmol CO₂ fixed/min/mg protein, comparable to that measured in the photoautotrophic inoculum, but the strain did not grow chemoautotrophically. By comparison, the RubisCO activity in the mutant cultures was typically from 0.04-0.06 μmol CO₂ fixed/min/mg protein, a lower level of activity that is typical of chemoautotrophic growth of complemented *R. capsulatus*.

The chemoautotrophic growth experiments were repeated using chemoheterotrophic inocula, rather than photoautotrophically grown cells. Under chemoheterotrophic conditions, RubisCO levels were undetectable. Thus, *de novo* synthesis was required for growth, and the possibility that the chemoautotrophic phenotype was the consequence of higher RubisCO activity in the mutant photoautotrophic inocula could be excluded. Even with chemoheterotrophic inocula, the mutant cultures were capable of growth under these conditions, albeit after a longer lag period (7-10 days, rather than 1-3 when photoautotrophic inocula were used), while the wild type did not grow at all.
Figure 20. The XL1-Red positive mutant enzymes were capable of complementing *R. capsulatus* SBI-II to chemoautotrophic growth in liquid cultures bubbled with an estimated gas mixture of 5% CO$_2$/10% O$_2$/ balance air. The specific cultures shown here were inoculated with late logarithmic phase photoautotrophic cells, and grew with doubling times of approximately 25 h.
Figure 20. Chemoautotrophic growth of the wild type and positive mutants.
**Kinetic properties of the mutant enzymes.** Rubisco wild type and mutant enzymes were purified as recombinant protein from *E. coli*, and pure enzymes were used for all kinetic studies. The results of these measurements indicated that there was no improvement in the $K_c$ of the positive mutant enzymes. In fact, the wild type and K11R enzymes had slightly better affinities for CO$_2$ than the mutants. The specificities, likewise, were slightly lower for the mutant enzymes. The only major difference seen in kinetic parameters tested was that the F342V mutants had a better affinity for the substrate RuBP than the wild type, K11R, or the M259T enzymes (Table 2.3).

**Resistance of 1.5% mutants to thermal inactivation and protease degradation.** In the absence of any clear explanation for the ability of the F342V and M259T mutations to confer the positive phenotype, thermostability was examined as a general indicator of enzyme stability. The F342V enzyme and wild type enzyme, in TEM +20% glycerol (the storage buffer), were incubated at temperatures ranging from room temperature to 70°C for 10 minutes. Both enzymes were very stable, and F342V did not differ from the wild type at all in this assay (Figure 21). In light of a recent publication concerning the effects of residue 259 on thermostability of Rubisco from *Chlamydomonas reinhardtii* (Du et al., 2000), the thermostability of the F342V and M259T enzymes was tested as described in this paper and compared with the wild type enzyme. With 20 min incubations of fully activated enzyme, at temperatures ranging from 40-75°C, the mutant enzymes displayed no significant thermostability over that observed with the wild type enzyme. The apparent stability of the F342V mutant at 70°C in Figure 21B was not reproducible. Furthermore, with prolonged incubation at 55°C, the mutant enzymes retained no more activity than the wild type enzyme.
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{cat}$&lt;sup&gt;a&lt;/sup&gt; (s&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>$V_c$	extsuperscript{a} (µmol/min/mg)</th>
<th>$K_c$	extsuperscript{b} (µM)</th>
<th>$\Omega$	extsuperscript{b}</th>
<th>$K_{RuBP}$&lt;sup&gt;c&lt;/sup&gt; (µM)</th>
<th>$k_{cat}/K_c$&lt;sup&gt;b&lt;/sup&gt; (s&lt;sup&gt;-1&lt;/sup&gt; µM&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>$k_{cat}/K_{RuBP}$ (s&lt;sup&gt;-1&lt;/sup&gt; µM&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>3.5</td>
<td>3.0</td>
<td>186 ± 15</td>
<td>42 ± 5</td>
<td>38 ± 6</td>
<td>0.02</td>
<td>0.09</td>
</tr>
<tr>
<td>K11R</td>
<td>3.8</td>
<td>3.3</td>
<td>204 ± 36</td>
<td>38 ± 3</td>
<td>39 ± 8</td>
<td>0.02</td>
<td>0.10</td>
</tr>
<tr>
<td>F342V</td>
<td>2.4</td>
<td>2.1</td>
<td>256 ± 15</td>
<td>39 ± 3</td>
<td>21 ± 3</td>
<td>0.01</td>
<td>0.11</td>
</tr>
<tr>
<td>K11R/F342V</td>
<td>2.3</td>
<td>1.9</td>
<td>260 ± 38</td>
<td>41 ± 1</td>
<td>27 ± 6</td>
<td>0.01</td>
<td>0.09</td>
</tr>
<tr>
<td>M259T</td>
<td>3.4</td>
<td>2.9</td>
<td>244 ± 16</td>
<td>37 ± 5</td>
<td>38 ± 7</td>
<td>0.01</td>
<td>0.09</td>
</tr>
<tr>
<td>K11R/M259T</td>
<td>2.3</td>
<td>1.9</td>
<td>227 ± 19</td>
<td>45 ± 4</td>
<td>38 ± 4</td>
<td>0.01</td>
<td>0.06</td>
</tr>
</tbody>
</table>

<sup>a</sup>Averages of two separate preparations, except for M259T and K11R/M259T, which were purified once.

<sup>b</sup>Averages of at least three independent assays of a bicarbonate-free enzyme preparation.

<sup>c</sup>Averages of at least three independent assays of two separate enzyme preparations, except for M259T and K11R/M259T, which were purified once.

Table 2.3. Kinetic data for the wild type, K11R, and XI1-Red positive mutant enzymes.
Figure 21. Thermostability experiments with the XL1 Red positive mutants and wild type purified enzymes. In panel A, samples were incubated in storage buffer (TEM + 20% glycerol) at the given temperatures for 10 min, then assayed for RubisCO activity by the standard protocol. The relative activity is the proportion of activity remaining relative to that measured after a 10 min room temperature incubation. In panel B, purified enzymes were incubated at the indicated temperatures for 20 min in buffer containing HCO$_3^-$, Mg$^{2+}$, and DTT, and were thus fully activated (Du et al., 2000). Relative activity was calculated as the activity remaining relative to the activity at 40°C. In panel C, enzymes were incubated at 55°C in the activated state, for the indicated times. The relative activity is the proportion of activity remaining relative to that measured before incubation at 55°C.
Figure 21. Thermostability experiments with the positive mutants.
The expression studies in *E. coli* indicated that there was a slight increase in the levels of mutant enzymes in soluble extracts relative to the wild type (Figure 14). A possible explanation for this could be increased resistance to protease degradation. This was examined by SDS-PAGE of samples that were exposed to proteinase K for 5 or 10 min incubations. The mutant enzymes did not qualitatively appear to be any more resistant to proteinase K degradation than the wild type enzyme (Figure 22).

*Mutagenic PCR and 0.5% positive selection mutants.* Though the basis for the positive phenotype of the XL1-Red positive mutants was not clear, it was evident that an obstacle had been overcome, allowing the enzymes to complement the *R. capsulatus* host strain to photoautotrophic growth with 1.5% CO₂, or to aerobic chemoautotrophic growth. With the goal in mind of improving the $K_c$ of this enzyme, selection was continued with even lower levels of CO₂ in the atmosphere. When CO₂ was lowered to 0.5%, the wild type, F342V, and M259T enzymes could not complement *R. capsulatus* SBI-II' to photoautotrophic growth. In a control experiment, both the parent strain SB1003 and SBI-II' complemented with the *R. capsulatus* form I RubisCO genes were capable of growth under this condition, indicating that the lack of growth was not a limitation of the strain itself. In fact, these latter two strains were capable of photoautotrophic growth with CO₂ as low as 0.1%. Mutagenic PCR was thus carried out using the wild type, F342V, and M259T variants of *rbcL*S as templates, as described in Chapter 1. 22 unique mutants were isolated that were capable of photoautotrophic growth in an atmosphere of 0.5% CO₂/99.5% H₂. All but one of these were in the F342V background, with the single exception having a silent mutation in the M259T background. No 0.5% CO₂ positive mutants in the wild type context were isolated.
Figure 22. The purified wild type (a), F342V (b), and M259T (c) enzymes were digested with proteinase K. In lanes 1-3, the inhibitor PMSF was added prior to proteinase K, followed by addition of SDS-PAGE sample loading buffer. In lanes 4-6, samples were digested with proteinase K for 5 min before addition of PMSF and sample loading buffer. In lanes 7-9, samples were digested for 10 min before PMSF and loading buffer were added. All samples were immediately electrophoresed in 12% polyacrylamide gels. LSU indicates the large subunit polypeptide, and SSU indicates the small subunits, which ran with the dye front in this gel.
Figure 22. Digestion of the wild type and mutant enzymes with proteinase K.
In order to avoid the time limitations experienced when studying the XL1-Red positive mutants, no studies of activity in *E. coli* were done with the new set of mutants. Rather, the mutant genes were subcloned into pUC19, overexpressed in 5 L fermentors in the Fermentation Facility (OSU Department of Microbiology, Columbus, OH), and recombinant proteins purified for kinetic studies. Only 0.5% positive mutants with amino acid substitutions were chosen for purification and kinetic studies. For direct comparison, the wild type enzyme was also purified from fermentation growth. The purified enzymes showed no outstanding kinetic properties, with most having specificities and $K_c$ comparable to the parent F342V (Table 2.4).

During this process, most of the 0.5% positive mutants, including those with silent mutations only, were grown in liquid photoautotrophic cultures for comparison with the wild type and F342V strains. Almost all of these strains grew faster than the wild type and F342V strains under these conditions, in keeping with the mode of selection (Table 2.5). Consistent with observations of growth of the XL1-Red positive mutants, there was not a correlation between doubling time and Rubisco specific activity, though several of the mutants had activity higher than the wild type and F342V measured in parallel. These activities, however, were not outside the range of what had been seen previously with the F342V or M259T mutants (Table 2.2). In addition, these cultures and measurements were done only once to screen for potentially interesting anomalies.
<table>
<thead>
<tr>
<th>RubiscoC³</th>
<th>Description</th>
<th>$k_{cat}$ ($s^{-1}$)</th>
<th>$K_c$ (µM)</th>
<th>$\Omega$</th>
<th>$K_{RuBP}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td></td>
<td>3.0</td>
<td>183</td>
<td>42±5.3</td>
<td>40</td>
</tr>
<tr>
<td>parent</td>
<td>F342V</td>
<td>2.1</td>
<td>-----</td>
<td>40±3.4</td>
<td>25</td>
</tr>
<tr>
<td>PA+7</td>
<td>R24S, A27T in SSU</td>
<td>3.0</td>
<td>246</td>
<td>38±3.3</td>
<td>21</td>
</tr>
<tr>
<td>PA+9</td>
<td>R439H in LSU</td>
<td>2.9</td>
<td>242</td>
<td>41±4.1</td>
<td>28</td>
</tr>
<tr>
<td>PA+10</td>
<td>S80N in SSU</td>
<td>3.5</td>
<td>225</td>
<td>42±3.3</td>
<td>17</td>
</tr>
<tr>
<td>PA+13*</td>
<td>F217L</td>
<td>1.6</td>
<td>203</td>
<td>47±2.7</td>
<td>19</td>
</tr>
<tr>
<td>PA+14</td>
<td>K29N</td>
<td>2.2</td>
<td>242</td>
<td>44±1.8</td>
<td>26</td>
</tr>
<tr>
<td>PA+15</td>
<td>F469Y</td>
<td>2.7</td>
<td>223</td>
<td>37±6.2</td>
<td>22</td>
</tr>
<tr>
<td>PA+17</td>
<td>K29I in LSU</td>
<td>2.9</td>
<td>261</td>
<td>35±1.6</td>
<td>17</td>
</tr>
<tr>
<td>PA+19</td>
<td>F311Y</td>
<td>4.2</td>
<td>233</td>
<td>39±1.2</td>
<td>16</td>
</tr>
<tr>
<td>PA+29*</td>
<td>T365N</td>
<td>1.9</td>
<td>259</td>
<td>38±1.2</td>
<td>27</td>
</tr>
</tbody>
</table>

*a The mutant numbers may be cross-referenced with Table 1.5.

*b LSU=large subunit, SSU=small subunit. All mutants also contain the F342V mutation in the large subunit.

*c All kinetic measurements for the mutants were done once, except for specificity ($\Omega$), which was measured in triplicate. The wild type was an internal control for every assay.

*There are no silent mutations in this mutant.

Table 2.4. Kinetic measurements for the 0.5% positive mutant enzymes.
<table>
<thead>
<tr>
<th>LSU mutations&lt;sup&gt;b&lt;/sup&gt;</th>
<th>SSU mutations&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Doubling time&lt;sup&gt;a,d&lt;/sup&gt;</th>
<th>Specific activity&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td></td>
<td>45</td>
<td>0.12</td>
</tr>
<tr>
<td>F342V</td>
<td></td>
<td>24</td>
<td>0.30</td>
</tr>
<tr>
<td>E90D, 3 silent</td>
<td>P68S, D84N, 1 silent,</td>
<td>13</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>1 intergenic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 silent</td>
<td>1 silent</td>
<td>17</td>
<td>0.26</td>
</tr>
<tr>
<td>2 silent</td>
<td></td>
<td>18</td>
<td>0.33</td>
</tr>
<tr>
<td>1 silent</td>
<td>1 intergenic</td>
<td>17</td>
<td>0.22</td>
</tr>
<tr>
<td>1 silent</td>
<td>R24S, A27T</td>
<td>16</td>
<td>0.21</td>
</tr>
<tr>
<td>R439H, 2 silent</td>
<td>E35K</td>
<td>17</td>
<td>0.36</td>
</tr>
<tr>
<td>1 silent</td>
<td>S80N</td>
<td>15</td>
<td>0.27</td>
</tr>
<tr>
<td>1 silent</td>
<td></td>
<td>15</td>
<td>0.40</td>
</tr>
<tr>
<td>3 silent</td>
<td>1 intergenic</td>
<td>15</td>
<td>0.47</td>
</tr>
<tr>
<td>F217L</td>
<td></td>
<td>21</td>
<td>0.44</td>
</tr>
<tr>
<td>K29I, 3 silent</td>
<td>L21H</td>
<td>24</td>
<td>0.29</td>
</tr>
<tr>
<td>F311Y, 5 silent</td>
<td></td>
<td>16</td>
<td>0.20</td>
</tr>
<tr>
<td>T365N</td>
<td></td>
<td>16</td>
<td>0.28</td>
</tr>
<tr>
<td>4 silent</td>
<td></td>
<td>14</td>
<td>0.40</td>
</tr>
<tr>
<td>1 silent</td>
<td></td>
<td>16</td>
<td>0.27</td>
</tr>
</tbody>
</table>

<sup>a</sup>Cultures grown in liquid minimal medium, bubbled with 1.5% CO<sub>2</sub>/98.5% H<sub>2</sub>.

<sup>b</sup>Large subunit mutation in addition to the F342V mutation.

<sup>c</sup>Small subunit mutations.

<sup>d</sup>Doubling time, in hours, for a single photoautotrophic culture grown in parallel with the wild type and F342V.

<sup>e</sup>RubisCO specific activity in μmol CO<sub>2</sub> fixed/min/mg protein in the same culture for which the doubling time is reported.

Table 2.5. Photoautotrophic growth of the 0.5% positive mutants<sup>a</sup>.
Discussion

At the outset of these studies, positive selection was believed to be the most promising route for isolation of RubisCO mutants that might have the desired "improvements" of lower $K_c$ and/or higher $\Omega$. What was discovered instead was that many changes in the $rbcL.S$, whether they resulted in silent mutations or amino acid substitutions, led to a positive mutant phenotype. The basis for the phenotype may thus be attributable to unforeseen factors that may include increased levels of $rbcL.S$ transcript, increased translational efficiency, or increased stability of the enzyme in vivo. This does not rule out the possibility that the improvements sought (lower $K_c$ or improved $\Omega$) might not result from the positive selection. However, from the results of these studies, it appears that isolation of such a mutant by the current selection method is highly improbable until the other (albeit unknown) requirements for the observed positive phenotype are satisfied.

Rather than the predicted improvement in $K_c$, the first positive mutant identified through bioselection of the XL1-Red plasmid libraries exhibited an approximately two-fold increase in its affinity for the substrate RuBP. The original isolate, K11R/F342V, and the single mutation construct, F342V, were capable of complementation under the low CO$_2$ selection condition, and could confer the ability to grow on chemoautotrophic plates. When overexpressed in E. coli JM109, cultures containing the F342V enzyme typically had higher RubisCO specific activity than cultures containing the wild-type enzyme, and extracts from the F342V strain usually appeared to have higher levels of soluble large subunit polypeptides than were observed in the wild type cultures (Figure 14). These differences were only apparent when the induction with IPTG was shortened.
to 9 or 12 h rather than the usual 16 h. The higher levels of enzyme could have resulted from increased expression of the mutant genes, through increased transcript stability or increased translational efficiency. The increased levels could also have resulted from an enzyme that is more stable in the cell or more stable through the preparation of extracts, though this latter scenario seems unlikely since the stability of the enzyme through preparation would also have been a factor when the cultures were induced for 16 h.

Either way, the preliminary observations in *E. coli* seemed relevant to the phenotype of *R. capsulatus* SBI-II, since the photosynthetic cultures often had higher RubisCO specific activities when the mutant enzymes were used to support the growth of the host *R. capsulatus* strain. In addition, the mutant enzymes appeared to be more abundant than the wild-type enzyme in complemented strains grown simultaneously (Figures 17 and 18). This suggests that something in common between *E. coli* and *R. capsulatus* can account for the slightly higher levels of enzyme, though it does not necessarily suggest that the positive phenotype of *R. capsulatus* is simply the result of increased expression.

Unsuccessful attempts were made to accurately quantitate the amount of RubisCO in wild-type and mutant photoautotrophic extracts, by competitive enzyme-linked immunosorbent assays (ELISAs) or through quantitative chemifluorescent Western blots. In both procedures, the purified enzyme gave an excellent linear response when suspended in buffer. However, the photoautotrophic crude extract samples, when diluted to absorb in the range of the linear standard curves in the same buffer, rarely gave consistent, reliable results. For instance, a set of 2-fold dilutions, whose absorbances both fell within the range of the linear curve on a Western blot, would not yield results that were different by 2 fold. Likewise, on ELISAs, a control experiment was conducted...
in which purified enzyme was added to photoautotrophic extracts that did not contain the cyanobacterial Rubisco. Thus, the concentration of Rubisco was known in this control sample. The results for the control garnered from the assay, however, were off by as much as 5-fold in some assays, and in no instance was it ever off by less than 50%. It appeared, then, that these techniques would not be reliable for drawing conclusions about the levels of wild type and mutant enzymes in photoautotrophic cultures. If the question of enzyme or transcript levels is to be pursued in the future, the ELISAs may be attempted with a cleaner preparation of antibodies, or the transcript levels may be measured directly by Northern hybridizations. It is important to note, however, that simply demonstrating a difference in levels of transcript and/or Rubisco would not necessarily allow one to conclude that these were the only factors responsible for the positive selection phenotype, in the case of mutants with amino acid substitutions. A better way to ascertain this for some mutants, such as the 0.5% positive mutants, would be to make the acquired silent mutations in the wild type background, and compare the transcript levels in the wild type, the silent mutants, and the mutants with only amino acid substitutions after silent mutations have been removed.

It is possible that the decreased $K_{\text{RuBP}}$ of the K11R/F342V and the F342V mutants contributed to their ability to complement. Since, however, no other mutants selected, either through XL1-Red mutagenesis or mutagenic PCR, exhibited a change in this parameter, it seems unlikely that there was heavy selection for this change. Nonetheless, the discovery that F342 influences $K_{\text{RuBP}}$ was a fortunate finding. This residue, found in $\alpha$-helix 6, and just downstream of the catalytically significant loop 6, is well conserved in the form I green enzymes, and is typically only substituted with aromatic residues.
tyrosine or tryptophan, in other form I enzymes. However, F342 has never been studied through site-directed mutagenesis, though residues within and immediately adjacent to loop 6 have been studied in some detail. For example, alignment of the primary sequence of the *Synechococcus* enzyme with those from higher plants revealed that a region just upstream of F342, residues 335-338 (residues 338-341 of the spinach enzyme), is the only region within loop 6 that varies significantly among these enzymes (Parry et al., 1992). Substitutions within this region resulted in slight increase in specificity, with a coincidental slight decrease in carboxylase activity. Interestingly, most mutations made in the experiments of Parry, et al. had deleterious effects on the affinity for RuBP.

Likewise, a different group reported that replacement of alanine 337 with histidine resulted in a 12-13% increase in specificity, a 25-33% decrease in carboxylase activity (V_c), and a decrease in RuBP affinity. However, increases in both specificity and carboxylase activity, as well as an increase in the affinity for RuBP, were gained with an asparagine substitution at this site (Madgwick et al., 1998). Another study of the region found that substitutions of the glycine residue immediately adjacent to F342 with a serine suppressed the photosynthetic incompetence of a strain of *C. reinhardtii* that carried a V328A mutation (V331  in *C. reinhardtii*). While the K_{RuBP} was not reported for this mutant, it did have an enhanced ability to bind to the transition state analogue, 2-carboxyarabinoitol bisphosphate (CABP). Finally, a residue just downstream of F342, M349 in the *C. reinhardtii* enzyme (M346 in the *Synechococcus* enzyme), was changed to leucine to reflect the sequence of the higher plant enzymes. This substitution did not affect the K_{RuBP}, and had little effect on other kinetic parameters (Zhu and Spreitzer, 1996).
Unlike the F342V mutants, the M259T mutants exhibited no outstanding kinetic properties when examined in vitro. A recent report that the residue at this position in the chloroplastic RubisCO of *C. reinhardtii* was mutated to suppress a temperature sensitive phenotype prompted us to study the thermostability of the purified enzyme in vitro (Du and Spreitzer, 2000). Unlike the V262L mutant isolated by Du and Spreitzer, the M259T mutant exhibited no change in its thermostability relative to the wild type enzyme; these experiments included tests using procedures outlined by Du and Spreitzer (Figure 21). Residue 259/262 is quite variable across taxa, but it is interesting to note that it resides in a loop at the interface between a large and small subunit (Knight et al., 1990). In common with F342V, M259T accommodated faster photoautotrophic growth of *R. capsulatus* SBI-II*, often had higher specific activity in photoautotrophic cultures, and appeared in *E. coli* to result in increased levels of soluble large subunit polypeptides.

Enhanced accumulation of the mutant enzymes cannot be ruled out as a contributing factor to the ability of these alleles to complement SBI-II* to low CO$_2$ photoautotrophic growth. However, the ability of the alleles to complement to chemoautotrophic growth in liquid cultures, where the wild type enzyme is completely incompetent, suggests that some other issues contribute to the phenotype. RubisCO specific activity alone cannot explain this phenotype, since a culture of SBI-II* complemented with the wild type enzyme, and inoculated with photoautotrophically grown cells, still had a specific activity of 0.1 μmol CO$_2$ fixed/min/mg protein after a week of incubation under chemoautotrophic conditions, with no growth. Furthermore, the specific activity of the chemoautotrophic cultures was not much higher than was typically seen in the past, when SBI-II* was complemented to chemoautotrophic growth.
with heterologous RubisCO genes on pRPS-1 (data not shown, Paoli et al., 1998). For example, when *R. capsulatus* SBI-II was complemented to chemoautotrophic growth with the form I RubisCO of *Rhodobacter sphaeroides* in liquid cultures, it had RubisCO specific activity of 0.08 μmol/min/mg protein, comparable to that observed in the positive mutant cultures that were inoculated with chemoheterotrophic cells (Table 2.2). This suggests, then, that a property other than enzyme levels, as reflected by RubisCO specific activity, is affecting the growth of these cultures. It is certainly conceivable that some other inherent property of the enzyme is affected by these mutations.

While the underlying reason for the positive phenotype remained unresolved, the XL1-Red positive mutants were found to be incapable of complementation when CO₂ was lowered to 0.5% in the atmosphere for photoautotrophic growth on minimal medium plates. The genes encoding the mutant enzymes, as well as wild type *rbcLS*, were randomly mutagenized by error-prone PCR (Leung et al, 1989; Cadwell and Joyce, 1992). The resultant mutant enzymes proved to be even more disposed to noncatalytic changes, as evidenced by the selection of several mutants that had only acquired silent mutations within the coding region of *rbcLS*. While improved transcript stability or translational efficiency seemed the only probable explanation for the 0.5% positive phenotype of these silent mutants, it is surprising to note that many of them, when grown photoautotrophically in liquid cultures, had RubisCO activity that was comparable to that observed in their parent, F342V, even though they typically grew faster than the F342V strain. Note, however, that unlike the growth studies with the 1.5% mutants, the 0.5% positive mutant plasmids were not isolated, subcloned, and re-mated back into *R. capsulatus*. Thus, it is possible that the growth phenotypes of some of these mutants may
be strain-associated, i.e., the result of chromosomal mutations in *R. capsulatus* SBI-II'. In spite of this, it appeared that, even in the unlikely event that SBI-II' had acquired chromosomal changes, the only background in which these changes and/or the silent mutations had any effect were in the context of F342V, as only a single mutant was isolated in the M259T background and no mutants were isolated in the wild type context. This supports the notion that some of the mutations acquired in initial rounds of selection will be required if positive selection is to continue.

There are several properties of RubisCO that were not tested in these experiments that could lead to explanations of the positive phenotypes. These include resistance to inhibitors that may be present in the cell, or the rate of activation of the mutant enzymes by CO₂. Furthermore, one should not assume that what is allowing one mutant to grow is the same for the next. In other words, perhaps the F342V mutant *does* grow because of its increased affinity for RuBP, but the M259T mutant has found another solution to the selection question. It would also be interesting to see if any of the silent mutations would be capable of generating the positive phenotype in the absence of either the F342V or M259T mutations. If so, they may provide a starting point for further selection.

Of course, whether further selection in this manner will be successful is questionable. There are clearly undefined factors that affect the ability of the *Synechococcus PCC6301* RubisCO to complement SBI-II' to autotrophic growth at lower levels of CO₂. The level at which these factors operate, (i.e., transcription, translation, or lack of a carbon concentrating mechanism) is irrelevant to the question: Can a mutant of *Synechococcus PCC6301* RubisCO with an improved affinity for CO₂ and/or an improved specificity, without a negatively affected carboxylase activity, be isolated by
these means? The data presented here indicate that the answer is either no, or that the system will have to be modified and the selection parameters changed to more exhaustively identify mutants, since in the end less than 10,000 colonies total were screened or selected. Clearly, a new target enzyme, a new host, or a different basis for positive selection may be required to improve the probability of isolating the desired mutant; all of these possibilities will be discussed further in Chapter 4. Surprisingly, however, interesting catalytic mutants, i.e., those that were affected in $k_{\text{cat}}$ and $K_c$, resulted from the negative selection process. These mutant enzymes are discussed in detail in Chapter 3.
CHAPTER 3

Characterization of Negative Selection Mutants

Introduction

As described in the Introduction, most RubisCO mutants described to date possess decreased carboxylase activity. These mutants have helped researchers identify residues that are required for the enzyme's activity, and have facilitated development of a catalytic mechanism that is conserved for all RubisCO enzymes (reviewed in Cleland et al., 1998). In spite of an excellent understanding of the active site chemistry required for carboxylation and oxygenation of RuBP, site-directed mutants have not clearly defined what determines such important parameters as $K_c$ and $\Omega$, or the basis for failover. While such parameters might be better understood through isolation and characterization of mutations that positively affect complementation of *R. capsulatus* SBI-II* (i.e., positive selection mutants, see Chapter 2), it seemed very likely that such information might also be revealed through isolation of negative mutants (i.e., mutants whose activity was deleteriously affected). The latter type of altered RubisCO protein would be in keeping with patterns observed over years of mutagenizing RubisCO genes; such mutations affect the Michaelis-Menten parameters and typically alter carboxylase activity in a negative fashion.

In this study, negative mutants were functionally identified by the inability to complement *R. capsulatus* SBI-II* to photoautotrophic growth on minimal medium plates.
a condition where the wild type enzyme of *Synechococcus* PCC6301 was fully capable of complementation. Such mutants were easily isolated, but, as described in Chapter 1, were typically the result of improper folding and/or assembly of holoenzyme. The crystal structure indicated that the affected residues of such folding/holoenzyme assembly mutants were in or near large subunit-small subunit contacts or large subunit dimer-dimer interfaces (Chapter 1, Table 1.3). However, it was found that alterations in a residue distal from such subunit contacts, R347, resulted in a negative mutant phenotype. This residue is buried in the interior of the enzyme, and is near α-helix 6, a region previously shown to be catalytically significant (Figure 23; Ramage et al., 1998). It was not expected, then, that the K11R/R347H mutant we isolated through negative selection would be unable to fold properly. Site-directed mutants were therefore constructed at this residue for comparison with the original isolate, as described in this Chapter.

Negative mutants that appeared to result from catalytic deficiencies, instead of or in addition to folding changes, were also isolated. These negative mutants appeared to properly form holoenzyme, as assayed by nondenaturing PAGE, and had detectable activity that ranged from about 10% (K11R/G176D) to as high as 80% (K11R/A47T) of wild type activity, when assayed in crude extracts of *E. coli* (Chapter 1, Table 1.3). Two of the residues identified in negative mutants, G176 and D103, were chosen for further study (Figure 24).

While G176 had been mutated before, it was not described beyond its decreased catalytic capability or its characterization on nondenaturing Western blots (Cheng and McFadden, 1998). Due to its inability to enable *R. capsulatus* SBI-II' to grow, we chose to characterize it more thoroughly, in hopes of better understanding the properties of
Figure 23. Ribbon diagram indicating the position of R347 (green) in the large subunit. Notice that this residue is in α-helix 6, the same helix that contained the F342V mutation described in Chapter 2 (see Figure 13 for comparison).
Figure 23. Position of residue R347 within the large subunit.
Figure 24. Ribbon diagram showing the positions of D103 and G176 within the large subunit. Notice that both of these residues are in loops, rather than α-helices or β-sheets. D103 is in the N-terminal domain of the enzyme, quite distant from the active site, and near a region involved in contacts between large subunits to form dimers. G176 is in the C-terminal domain, near the region that will contact the N-terminus of the partner large subunit to form the complete active site within the large subunit dimer.
Figure 24. Position of residues D103 and G176 within the large subunit.
Rubisco affected by this residue. Furthermore, we wanted to distinguish whether our prediction that decreased carboxylase activity alone was sufficient to confer the negative phenotype. As shown below, several other parameters of the enzyme were interestingly affected in this mutant, so this distinction was made. This also demonstrated that despite the fact that mutations had been previously made at this residue, it was well worthwhile to study it further (Cheng and McFadden, 1998).

D103 was chosen for further study because this residue is quite distant from any amino acid known to play a role in catalysis, and has never been directly studied. It was therefore surprising to observe the dramatic effect a valine substitution had on the phenotype of R. capsulatus SBI-II, and, as described below, on the kinetic properties of Rubisco. Both the G176D and D103V mutants provided support for the prediction that the selection system could facilitate identification of residues affected in Michaelis-Menten parameters. Thus, negative selection might be useful in future efforts to isolate additional interesting catalytic mutants.

**Materials and Methods**

All procedures for growth, purification of enzymes, assays, PAGE and Western blotting were identical to those described in Chapters 1 and 2, with the additional experiments described below.

*Site-directed mutagenesis*. The following sets of complementary primers were used to construct the site-directed mutants, with the QuikChange kit from Stratagene (Stratagene, La Jolla, CA). The mutant constructed is listed in **bold**, followed by the primer sequences.
R347A:
5'GGGCTTTGTTGACTTGATGGCCGAAGACACCACATCGAAGCTG3'
5'CAGCTTCGATGTGGTCCTCCGATACATCAGTCAACAAAGGCCC3'

R347K:
5'GGGCTTTGTTGACTTGATGAAGGAAGACCACATCGAAGCTG3'
3'CAGCTTCGATGTGGTCCTCCGATACATCAGTCAACAAAGGCCC3'

D103E:
5'CGCTTACCCCGCTCGAGCTTGTTTGAAGAAGGG3'
3'CCCTTTCTTCAAAACAGCTCGAGCGGGGTAAGCG3'

D103N:
5'CGCTTACCCCGCTCAACCTGTTTGAAGAAGGG3'
5'CCCTTTCTTCAAAACAGGTGTGAGCGGGGTAAGCG3'

D103V:
5'GCCTTCATCGCTTACCCGCTCGTTCCTGGTTGATCGTGC3'
5'GGTGACCGACCCCTTCTTCAAAACAGGACGAGCGGGTAAGCGATGAACGC3'

G176A:
5'CCGTAGTTTTTCGCCGACAGACAGATTTTGTGGTTTGATCGTGC3'
5'GCACGATCAAAAACAACTCGCTCTGTCGCGGAAAAACTACG3'

G176D:
5'GCACGATCAAAAACAACTCGATCTGTCGCGGAAAAACTACG3'
5'CCGTAGTTTTTCGCCGACAGATCGAGTTTGTGGTTTGATCGTGC3'
**G176N:**

5'CGTAGTTTTTGCACACCTCAGGTTCAGTTTTGGTTTGATCGTGC3'

5'GACGATCAAACCAAACTCAATCTGTCGCGAAAACTACGG3'

*Thermostability assays.* All enzymes were diluted to 0.5 μg/ul, in TEM (25 mM Tris-HCl, 1 mM EDTA, 10 mM MgCl₂, pH 8.0) with 20% glycerol, the same buffer the enzymes had been stored in at −80°C. 20 μl of each were used for a standard RubisCO assay, which served as the reference assay for calculation of relative activity. 200 μl samples were placed in a thermocycler (Perkin Elmer, Foster City, CA) heated to 55°C. After 10, 20, 30, 40, 60, 80, 100, and 120 min of incubation, 20 μl aliquots were removed and immediately assayed by the standard protocol. The wild type, K11R, and G176A enzymes were assayed for 1 min, the other mutants were assayed for 5 minutes. The different lengths of the assays were necessary to stay within the linear range of enzyme activity with respect to time. Relative activity was calculated as the percent of specific activity in the incubated sample relative to the specific activity in the reference assay prior to incubation.

*Assays of concentration dependence.* All enzymes were diluted to 0.5 μg/ul, in TEM (25 mM Tris-Cl, 1 mM EDTA, 10 mM MgCl₂, pH 8.0) with 20% glycerol, so that the same volume of enzyme would be added to each assay. Thus 10 μl was added for 5 μg RubisCO/assay, and so on. Samples were assayed by the standard protocol, with 1 minute assays for the wild type, K11R, and G176A enzymes and 5 min assays for the K11R/G176D, G176D, and G176N mutants. Relative activity was calculated as the
percentage of activity in the assay relative to the assay that contained 5 μg of the same Rubisco.

**Results**

*Characterization of a negative mutant altered in proper folding.* K11R/R347H is a negative mutant that was unable to form a soluble holoenzyme, as assayed by nondenaturing PAGE (Figure 25). Assuming its folding deficiency was due to the drastic R-H substitution, R347K and R347A mutants were constructed by site-directed mutagenesis using the QuikChange kit from Stratagene (La Jolla, CA). Neither of these substitutions was tolerated, as both mutants were assembly incompetent, exactly as the original K11R/R347H isolate (Figure 25). The R347K mutant had only about 1% of wild type activity, the R347A mutant had no detectable activity. It was verified that the mutant enzymes were synthesized, since large and small subunit polypeptides were easily detected when whole cell extracts were loaded onto SDS-PAGE gels. Little or no soluble large subunits were visible on SDS Westerns of the soluble fraction from the same cells (Figure 26).

*Characterization of the D103 mutants.* A mutant containing K11R/D103V substitutions was identified by its inability to complement strain SBI-II' to high-CO₂ photoautotrophic growth on minimal medium plates, but appeared to form soluble holoenzyme when the mutated genes were overexpressed in *E. coli*. In these *E. coli* cultures, the K11R/D103V mutant cultures had only about 25-30% of the Rubisco specific activity observed in *E. coli* cultures harboring the wild-type enzyme. Residue 103 was thus chosen for further study, and D103E and D103N mutants were constructed.
Figure 25. Nondenaturing PAGE followed by Western blotting to compare the K11R/R347H and K11R/D103V negative mutants (lanes 2 and 5, respectively) and the site-directed mutants (lanes 3, 4, 6, and 7). Cultures of *E. coli* JM109 containing pUC19 clones of each mutant were induced for 16 h with 0.5 mM IPTG. Soluble extracts containing 5 µg of protein were loaded onto a nondenaturing polyacrylamide gel (7% acrylamide) for this Western blot. Lane 1, wild type; lane 2, K11R/R347H; lane 3, R347A; lane 4, R347K; lane 5, K11R/D103V; lane 6, D103E; lane 7, D103N. RubisCO specific activity (nmol CO$_2$ fixed/min/mg protein) in each lane; lane 1, 151.3; lane 2, 0; lane 3, 0; lane 4, 0; lane 5, 48.9; lane 6, 82.3; lane 7, 60.2.
Figure 25. Nondenaturing Western of the R347 and D103 mutants in *E. coli* extracts.
Figure 26. Western immunoblots of SDS-containing gels were used to compare extracts of *E. coli* JM109 containing the pUC19 clones of the wild type RubisCO (lanes 1, 6). K11R (lanes 2, 7), K11R/R347H (lanes 3, 8), R347A (lanes 4, 9), and R347K (lanes 5, 10) enzymes. Cultures were induced with 0.5 mM IPTG for 16 h. Whole cell extracts (lanes 1-5) were loaded to examine total RubisCO polypeptide expression in the cultures. Supernatants from a low-speed centrifugation, in a microfuge at 4°C, were loaded into lanes 6-10, to examine the solubility of the polypeptides. 5 μg of protein were loaded into each lane.
Figure 26. Western blot of whole cell extracts and soluble fractions from R350 mutants expressed in *E. coli*. 

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by site-directed mutagenesis. The D103E and D103N alleles likewise had about 50% and 40% of wild-type specific activity, respectively, when expressed in *E. coli*. As expected from the specific activity in cultures, both site-directed mutants were capable of forming a holoenzyme (Figure 25), though the D103N mutant protein appeared to migrate slightly differently.

The D103 site-directed mutants, including a D103V mutant constructed for study in the absence of Kl1R, were introduced into *R. capsulatus* SBI-II' on plasmid pRPS-MCS3. On minimal medium plates, the D103V, D103E, and D103N mutants behaved exactly as the original Kl1R/D103V isolate, with each showing only slight growth after a week of incubation, while the wild type grew normally on the same plates. The mutants were grown in liquid photoautotrophic cultures that were continuously bubbled with 1.5% CO₂/98.5% H₂ to test their performance relative to the wild type. The mutants were capable of very slow growth, and the D103E mutant grew faster than the others, though still slower than SBI-II' complemented with the wild type enzyme (Figure 27). Another notable difference was that the wild-type and D103E cultures experienced a shorter lag phase (~135 h) than either the D103N (156 h) or the D103V (204 h) cultures. RubisCO activity in the mutant cultures was lower than that measured for the wild-type culture, but on Western blots it did not appear that there were lower levels of enzyme protein in cultures containing the mutant enzymes (Table 3.1, Figure 28).
Figure 27. Photoautotrophic growth of *R. capsulatus* SBI-II complemented with the wild type and D103 mutant enzymes in liquid cultures. Cultures were inoculated with photoheterotrophically grown cells, then bubbled with 1.5% CO₂/98.5% H₂. Samples were taken approximately every 24 h. When cultures reached an OD₆₆₀ of 0.6, samples were diluted 1:5 in minimal medium for the OD measurement. This graph was constructed as described for Figure 16 (Chapter 2). The lag times for these cultures were: WT, 135 h; D103E, 134 h; D103N, 156 h; D103V, 204 h. The doubling times, in hours, were: WT, 35; D103E, 47; D103N, 74; D103V, 76.
Figure 27. Photoautotrophic growth of the D103 mutants.
<table>
<thead>
<tr>
<th>Complementing RubisCO</th>
<th>Doubling time&lt;sup&gt;a&lt;/sup&gt; (h)</th>
<th>Specific activity&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td>wild type</td>
<td>35</td>
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</tr>
<tr>
<td>K11R/D103V</td>
<td>68</td>
<td>0.12</td>
</tr>
<tr>
<td>D103V</td>
<td>76</td>
<td>0.14</td>
</tr>
<tr>
<td>D103E</td>
<td>47</td>
<td>0.18</td>
</tr>
<tr>
<td>D103N</td>
<td>74</td>
<td>0.19</td>
</tr>
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<td>K11R/G176D</td>
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<td>0.02</td>
</tr>
<tr>
<td>G176D</td>
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<tr>
<td>G176N</td>
<td>90</td>
<td>0.07</td>
</tr>
<tr>
<td>G176A</td>
<td>42</td>
<td>0.19</td>
</tr>
</tbody>
</table>

<sup>a</sup> calculated from the early logarithmic stage of growth, between an OD<sub>660</sub> of 0.2 and 0.4  
<sup>b</sup> RubisCO specific activity in μmol CO<sub>2</sub> fixed/min/mg protein

Table 3.1. Photoautotrophic doubling time and RubisCO specific activity for the negative mutant enzymes.
Figure 28. Soluble extracts from photoautotrophically grown *R. capsulatus* SBI-II containing the wild type and D103 mutants were used in Western blotting to qualitatively examine the levels of RubisCO in each culture. Lane 1, 2 μg of purified enzyme; lane 2, wild type; lane 3, K11R/D103V; lane 4, D103V; lane 5, D103N; lane 6, D103E. In lanes 2-6, 5 μg of soluble crude extract was loaded.
Figure 28. Western blot of photoautotrophic extracts from D103 mutants.
Each of the D103V mutant enzymes was purified to study the kinetic properties \textit{in vitro}. Surprisingly, the mutants were only modestly affected for $k_{\text{cat}}$, $K_{\text{RabP}}$, and $\Omega$ (Table 3.2), with the lower $k_{\text{cat}}$ being consistent with the lower specific activity measured in photoautotrophic cultures (Table 3.1). However, the mutant enzymes were greatly affected for their affinity for CO$_2$, with each mutant having a much higher $K_c$ than the wild type enzyme measured in parallel (Table 3.2).

\textit{Characterization of the G176 mutants.} The K11R/G176D isolate was incapable of complementing \textit{R. capsulatus} SBI-II to photoautotrophic growth, but on nondenaturing gels of extracts from both \textit{R. capsulatus}, grown photoheterotrophically with DMSO, and \textit{E. coli} JM109, expressing the protein from pUC19, there appeared to be normal levels of soluble holoenzyme. It appeared that the enzyme had slightly aberrant migration on nondenaturing gels, while on SDS-PAGE the subunits looked normal. Thus, the enzyme was purified, while site-directed mutants G176D, G176N, and G176A were constructed for comparison.

Measurements of the $V_{\text{max}}$ were attempted at the end of purification of the original K11R/G176D mutant when it was noticed that two different concentrations of enzyme yielded differing $V_{\text{max}}$ (and thus $k_{\text{cat}}$) values. Thus, the $V_{\text{max}}$ appeared to range from 0.1 to 0.3, about 3-10% of wild type $V_{\text{max}}$. It was verified that the activity measurements were linear with respect to time. This apparent protein concentration-dependence on activity was subsequently studied further using the site-directed mutant enzymes, with the wild type as a control. Each of the mutant enzymes displayed this protein concentration-dependence characteristic, albeit to differing degrees, with the G176D mutant having the
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{\text{cat}}$ $^a$ (s$^{-1}$)</th>
<th>$V_c$ $^a$ (μmol/min/mg)</th>
<th>$K_c$ $^b$ (μM)</th>
<th>$\Omega$ $^b$</th>
<th>$K_{\text{RuBP}}$ $^b$ (μM)</th>
<th>$k_{\text{cat}}/K_c$ $^b$ (s$^{-1}$μM$^{-1}$)</th>
<th>$k_{\text{cat}}/K_{\text{RuBP}}$ (s$^{-1}$μM$^{-1}$)</th>
</tr>
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<tbody>
<tr>
<td>WT</td>
<td>3.5</td>
<td>3.0</td>
<td>198 ± 7.0</td>
<td>39 ± 5</td>
<td>38 ± 5</td>
<td>0.02</td>
<td>0.09</td>
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<td>K11R</td>
<td>3.8</td>
<td>3.3</td>
<td>197 ± 6.0</td>
<td>38 ± 3</td>
<td>39 ± 3</td>
<td>0.02</td>
<td>0.10</td>
</tr>
<tr>
<td>K11R/D103V</td>
<td>1.8</td>
<td>1.5</td>
<td>443 ± 9.0</td>
<td>36 ± 2</td>
<td>36 ± 7</td>
<td>0.004</td>
<td>0.05</td>
</tr>
<tr>
<td>D103V</td>
<td>2.4</td>
<td>2.0</td>
<td>459 ± 31</td>
<td>38 ± 1</td>
<td>34 ± 2</td>
<td>0.005</td>
<td>0.07</td>
</tr>
<tr>
<td>D103N</td>
<td>1.8</td>
<td>1.5</td>
<td>325 ± 17</td>
<td>36 ± 1</td>
<td>36 ± 1</td>
<td>0.005</td>
<td>0.05</td>
</tr>
<tr>
<td>D103E</td>
<td>1.3</td>
<td>1.1</td>
<td>301 ± 11</td>
<td>35 ± 4</td>
<td>33 ± 3</td>
<td>0.004</td>
<td>0.04</td>
</tr>
</tbody>
</table>

$^a$ Average of at least two separate preparations.

$^b$ Average of at least three independent assays.

Table 3.2. Kinetic properties of the D103 mutants.
most pronounced effect, followed by the G176N and G176A mutant proteins (Figure 29). The G176D mutant (and the original K11R/G176D) typically had a 1.5-3 fold increase in activity, that plateaued when about 40 µg of enzyme were used in the assay. In fact, beyond 50 µg of enzyme, activity sometimes decreased, which could be the consequence of adding too much sample to the 250 µl assay. The G176N and G176A mutants showed this effect to a much lesser extent, with activity of about 130% being reached at concentrations of about 40 µg of enzyme, relative to an assay containing 5 µg of enzyme. The addition of up to 40 µg of BSA/assay, in addition to 5 µg of RubisCO, had no effect on the specific activity of either the original K11R/G176D mutant or the wild type, so the phenomenon of concentration dependence appeared to be RubisCO-dependent (Figure 30).

Since G176 is near an intra-dimer interface, between large subunits (Knight et al., 1990), the observed RubisCO concentration-dependency suggested a possible effect on the stability of the holoenzyme at differing concentrations in the assays. While not a direct measurement of the association into dimers, it was predicted that the potentially weakened quaternary structure might result in a less thermostable enzyme. Indeed, each of the G176 mutant enzymes was much less thermostable than the wild type enzyme, losing up to 50% of their activity within 20 min after incubation at 55°C (Figure 31). The wild type enzyme required 120 min of incubation before 50% of its activity was lost at 55°C, by which time the mutant proteins exhibited only traces of activity. Consistent with the hypothesis that an unstable or conformationally affected holoenzyme resulted from the residue changes at position 176, the mutant enzymes migrated differently on
Figure 29. Concentration-dependence of RubisCO specific activity in the G176 mutants.

The RubisCO specific activity was measured with concentrations of purified enzyme ranging from 5 to 40 μg in each assay. Note that the y-axis for the G176D mutant (on the right side of the graph) differs, since the increase in its relative activity was much greater than in the other mutant enzymes. The specific activity for the 5 μg assay for each enzyme was: wild type, 3.8 μmol CO₂ fixed/min/mg protein; G176D, 0.07 μmol CO₂ fixed/min/mg protein; G176N, 0.55 μmol/min/mg protein; and G176A, 1.0 μmol/min/mg protein. Due to the range of activities, the wild type and G176A mutant were assayed for 1 min, and the G176D and G176N mutants were assayed for 5 min.
Figure 29. RubisCO specific activity vs. concentration.
Figure 30. The K11R/G176D and wild type enzymes were used in assays with increasing concentrations of BSA. Each assay had 10 μg of Rubisco. The wild type assays were incubated for 1 min, and the K11R/G176D assays for 5 min. In the absence of BSA, the specific activity for the wild type was 2.7 μmol CO$_2$ fixed/min/mg protein, and the specific activity of K11R/G176D was 0.06 μmol CO$_2$ fixed/min/mg protein.
Figure 30. Rubisco specific activity with addition of BSA.
Figure 31. Thermal stability of the wild type and G176 mutant enzymes. All samples were diluted to a concentration of 0.5 μg/μl in TEM + 20% glycerol (the same buffer the enzymes were stored in). Samples were incubated at 55°C. One assay was done prior to incubation of the samples (t=0), and this was the reference assay for calculating relative activity after incubation. At the indicated times, samples were removed from the incubations and assayed for RubisCO activity.
Figure 31. Thermal instability of the G176 mutants.
nondenaturing polyacrylamide gels compared to the wild type and K11R enzymes. However, the subunit structure appeared normal after SDS-PAGE (Figure 32).

The site-directed mutants were introduced into *R. capsulatus* SBI-II' on plasmid pRPS-MCS3. None of the mutants could complement this strain to photoautotrophic growth on minimal medium plates to the levels observed with the wild type enzyme, though the G176A mutant appeared to be capable of very weak growth. The growth of the strains was compared in liquid photoautotrophic cultures that were continuously bubbled with 1.5% CO$_2$/98.5% H$_2$. Under these conditions, the G176A mutant grew almost as fast as the wild type strain, while the G176D and G176N mutants grew much slower with a pronounced lag (Figure 33). RubisCO activity in these cultures ranged from 20 to 189 nmol CO$_2$ fixed/min/mg protein (Table 3.1). Western blots of the same extract revealed that RubisCO levels did not qualitatively differ in extracts from cultures containing genes that encode the mutant and wild type proteins; thus the low activity measured in the mutant cultures was a property of the enzyme and not of its expression (Figure 34). Nondenaturing Western blots of the extracts containing the mutant proteins revealed the same aberrant migration that was observed with the purified enzymes (data not shown).

The kinetic properties of the purified G176 mutant enzymes were compared with the wild type and K11R enzymes. All of the mutant enzymes had lower $k_{cat}$, with the G176A enzyme being the least affected. The affinities for the substrates CO$_2$ and RuBP were greatly affected, however, in the G176D and G176N mutants. These mutants had a better affinity for CO$_2$ than the wild type enzyme, but they had a lower affinity for RuBP.
Figure 32. PAGE of purified wild type Rubisco (lanes 1 and 7), K11R (lane 2), K11R/G176D (lane 3), G176D (lane 4), G176N (lane 5), and G176A (lane 6) enzymes in the presence (upper panel) or absence (lower panel) of SDS. The gel with SDS contained 15% acrylamide, and the gel without SDS contained 7% acrylamide. Approximately 2 μg of each purified sample were loaded onto the SDS gels, while 3 μg were loaded onto the nondenaturing gels. Both were stained with Coomassie. LSU indicates the large subunit, and SSU indicates the small subunit.
Figure 32. PAGE of the purified G176 mutants in the presence or absence of SDS.
Figure 33. Photoautotrophic growth of cultures containing plasmids with the wild-type and G176 mutant Rubisco genes. The cultures were inoculated with photoheterotrophically-grown cells, and bubbled with 1.5% CO₂/98.5% H₂. At approximately 24 h intervals, 1 ml samples were removed to measure OD₆₆₀. After cultures reached an OD₆₆₀ of 0.6, samples were diluted 1:5 and absorbance was measured. This graph was constructed as described for Figure 16 (Chapter 2). The lag times for these cultures were: WT, 136 h; G176A, 300 h; G176N, 280 h; G176D, 280 h. The doubling times, in hours, were: WT, 35; G176A, 42; G176N, 90; G176D, 102.
Figure 33. Photoautotrophic growth of the G176 mutants.
Figure 34. SDS-PAGE and Western immunoblots of extracts for *R. capsulatus* cultures. Extracts were from photoautotrophic cultures containing plasmids that expressed the wild type and G176 mutant genes, to assess the relative levels of RubisCO. 5 μg of soluble crude extract were loaded into lanes 2-6. Lane 1, 2 μg of purified wild type enzyme; lane 2, wild type; lane 3, K11R/G176D; lane 4, G176D; lane 5, G176N; lane 6, G176A.
Figure 34. Western blot of photoautotrophic extracts from G176 mutants.
The G176A mutant possessed a somewhat higher K_c but the K_RubP did not differ greatly from the wild type enzyme (Table 3.3). In spite of the lower K_c, the G176D and G176N mutants did not appear to have a higher specificity, and in fact it appeared to be slightly lower relative to the specificity of the wild type.

**Discussion**

For the most part, negative mutants that were isolated were defective for proper folding and assembly into holoenzyme. The quaternary structure of the enzyme predicts that this would be the most common type of mutant, since most residues are involved in interactions between large subunits, large subunit dimers, or small and large subunits. One folding mutant we isolated (K11R/R347H), however, was in a catalytically significant region (α-helix 6) of the enzyme that is buried in the interior. Note that the positive mutant described in Chapter 2, F342V, was also localized to this region. This helix was previously studied in detail in our laboratory, with mutations found to affect all catalytic parameters except Ω (Ramage et al., 1998). In fact, when residues 343-345 were changed by Ramage et al. in a background that included other substitutions within the helix, the resultant mutants were folding/assembly deficient. As observed for the original K11R/R347H mutant, it was subsequently found that site-directed mutants R347A and R347K were also defective for folding. The selection and isolation of this negative mutant, and the results obtained with the previously constructed mutant proteins, demonstrated that this region is very important for the folding and assembly of RubisCO, even though it does not appear to be directly involved in subunit interactions. These data indicated that the C-terminal portion of α-helix 6 affected folding of the large subunits in
<table>
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<tr>
<th>Enzyme</th>
<th>$k_{\text{cat}}^a$ (s$^{-1}$)</th>
<th>$V_c^a$ (μmol/min/mg)</th>
<th>$K_c^b$ (μM)</th>
<th>$\Omega^b$</th>
<th>$K_{\text{RuBP}}^b$ (μM)</th>
<th>$k_{\text{cat}}/K_c$ (s$^{-1}$μM$^{-1}$)</th>
<th>$k_{\text{cat}}/K_{\text{RuBP}}$ (s$^{-1}$μM$^{-1}$)</th>
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<tbody>
<tr>
<td>wt</td>
<td>3.5</td>
<td>3.0</td>
<td>183 ± 20</td>
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<td>K11R</td>
<td>3.8</td>
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<td>44 ± 5</td>
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<td>0.09</td>
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<td>K11R/G176D</td>
<td>0.3</td>
<td>0.2</td>
<td>93.0 ± 3</td>
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<td>96 ± 22</td>
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<tr>
<td>G176D</td>
<td>0.3</td>
<td>0.2</td>
<td>97.0 ± 7</td>
<td>33 ± 3</td>
<td>100 ± 14</td>
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<td>0.4</td>
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<td>237 ± 21</td>
<td>38 ± 6</td>
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<td>0.03</td>
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* The $k_{\text{cat}}$ and $V_c$ measurements are for a single preparation of each of the G176 mutants. The values for the wild type and K11R are the average of two enzyme preparations.

*Average of at least three independent assays.

**Table 3.3. Kinetic properties of the G176 mutants.**
some way, while the N-terminal 2/3 of the helix, adjacent to loop 6, seemed to more
directly affect catalytic parameters. Therefore, Rubisco catalysis would appear to be
very sensitive to positioning of this helix relative to the rest of the C-terminus of the
protein.

As one of only three catalytic negative mutants thus far isolated, K11R/D103V
was studied in detail. Residue 103 (residue 106 in spinach Rubisco) is found in a loop
between the fourth β-sheet and third α-helix of the N-terminal domain, near a stretch of 6
amino acids that are completely conserved in all form I and form II Rubisco (Knight et
al., 1990). The region is important for association of the large subunits into dimers,
which was consistent with the properties of a F105L mutant also isolated, which was
incapable of assembly into holoenzyme (Chapter 1, Table 1.3). In contrast to the
K11R/F105L mutant, the K11R/D103V mutant was fully competent in folding/assembly,
as demonstrated on multiple Western blots of nondenaturing gels.

It was considered that the introduction of the branched, hydrophobic valine
residue at position 103 might perhaps be responsible for disrupting interactions between
large subunits, inducing a conformational change that decreased the enzyme’s catalytic
capabilities. However, the $k_{cat}$ of the K11R/D103V mutant was only modestly affected.
Likewise, the site-directed mutants, D103V, D103N, and D103E, had activity that was
not much lower than that seen in one of the positive mutants, F342V. Thus, the turnover
capability of the negative mutant enzymes was not likely the primary reason why SBI-II
cultures complemented with Rubisco genes containing these mutations were
photoautotrophically incompetent. The most likely culprit was the surprisingly high $K_c$
as measured with the purified enzymes (Table 3.2). This discovery is significant because
residue 103, which is not even in the C-terminal domain that contains the active site, would not be predicted to affect $K_c$, especially since the mutations only modestly affected the $k_{cat}$. This finding supported the notion that changes away from the active site may be required in future approaches to positively affect $K_c$ and $\Omega$, of course such changes would be important if substantial decreases in rates of enzymatic turnover did not occur.

A second catalytic negative mutant, K11R/G176D, was studied and found to have several interesting properties. G176 (residue 179 in spinach RubisCO) is one of 22 glycine residues that are completely conserved in RubisCO. Note that another of these glycines, G401 (residue 404 in spinach) was identified through the negative selection process, but yielded a folding/assembly-incompetent enzyme (G401S, Chapter 1, Table 1.3). G176 lies within a stretch of 7 amino acids that are completely conserved, near an intradimer interface, and in a loop preceding the first $\alpha$-helix of the C-terminal domain of the enzyme (Knight et al, 1990; Newman and Gutteridge, 1993).

G176 was studied previously by Cheng and McFadden (1998), but its properties were not thoroughly described, since the paper presented a broad survey of the effects of substitutions at conserved glycy1 residues. However, G176A was one of the mutants made by Cheng and McFadden, and its properties seem to differ slightly from the results obtained for the G176A mutant in this study. Cheng and McFadden describe this mutant as having about 18% of wild-type activity, while results described here show it is closer to 40-50% of wild type-activity. A possible explanation for this discrepancy could be the concentration-dependent effect on activity (Figure 28). The amount of enzyme used in the assays performed by Cheng and McFadden was not given; possibly if a higher concentration had been used the reported activity would more closely resemble that
which is reported here. Cheng and McFadden also described the mutant as mildly
defective for assembly, but did not show it on any of several nondenaturing Western blots
in the paper. It is likely that the slightly aberrant migration observed in this study (Figure
31) was observed by Cheng and McFadden, justifying the classification of G176A
(G179A in their paper) as defective for folding/assembly.

The concentration-dependent effect described here could be a consequence of
forcing an equilibrium towards association of large subunits into dimers (Figure 28).
Likewise, weak association of large subunits could also account for the thermal
instability of enzymes with substitutions at this residue (Figure 30). If this is true, then
the results of the kinetic measurements for the G176 mutants are especially interesting,
because they support the hypothesis that the $K_c$, as well as $K_{rubp}$, are determined by
conformational properties at a tertiary (and possibly quaternary) level, and not just the
positioning of side chains of a few specific residues that might interact with the
substrates. This helps to reconcile the seemingly conflicting results of molecular models,
which predict that the CO$_2$ and O$_2$ concentrations alone determine the relative ratios of
products through the carboxylase and oxygenase pathways, with genetic studies that
clearly show that substitutions at specific residues affect the parameters of $K_c$ and
specificity (see the Introduction to this dissertation for a review).

It is especially interesting to note that in both the D103 mutants, which have an
increased $K_c$, and the G176 mutants, which have a decreased $K_c$, the mutations were
found at interfaces between the large subunits that form the dimers of the octameric core
of the enzyme. It is disheartening, however, that none of the mutations at these sites
affected specificity. At the outset of these studies, the $K_c$ was the presumed “target”
when we began positive selection with lowered CO₂, in the hopes that a mutant would be
isolated that had a lowered Kₐ, and, consequently, an increased specificity. These results,
and the results of dozens of mutant studies that precede this, reflect that there is actually
strong selection against alterations in specificity, even when mutations result in a lower
Kₐ, as with the G176D and N mutants. Why specificity is so “protected” from change
remains unexplained, but if our assumption about the requirement for large-scale
conformational changes is correct, it is likely that these changes would result in an
enzyme that is incapable of turnover. Perhaps, as described in the final chapter of this
dissertation, some of the mutants here will serve as a better starting material for further
mutagenesis and selection than the wild-type enzyme, since the G176 mutants have an
increased affinity for CO₂ already. Also for future study, a K11R/A47T mutant was
isolated as a negative mutant, and it appears that this may be an interesting catalytic
mutant. For a negative mutant, it had very high activity in extracts of E. coli (50-80% of
wild-type activity), which leads to the prediction that one of its catalytic parameters,
possibly Kₐ, has been negatively affected, resulting in the negative phenotype of R.
capsulatus SBI-II' when complemented with this enzyme.
CHAPTER 4

Summary and Future Directions

As demonstrated in the previous Chapters, an effective system was developed and made available for random mutagenesis of the RubisCO genes, *rbcLS*, from the cyanobacterium *Synechococcus* PCC6301. It was subsequently demonstrated that interesting RubisCO mutants could be selected using *Rhodobacter capsulatus* as a host. This approach to RubisCO was significant for several reasons. First, for RubisCO, the gene-directed, random mutagenic approach was novel. Systems have been designed whereby photosynthetic organisms have been affected on the whole-organism level, as described in the Introduction to Chapter 1, for the *Chlamydomonas* system. In the *Chlamydomonas* system, selection of suppressor mutations carried the risk of isolating mutations in genes other than those encoding RubisCO. While that was also a risk in the bacterial system described here, the specific mutagenesis of *rbcLS* exponentially increased the probability that only changes in RubisCO, and not in other cell functions, would serve as the basis for the host's phenotype. Furthermore, targeting *rbcLS* with random mutagenesis, rather than site-directed mutagenesis, was an inherent strength of this system. Site-directed mutagenesis has been successful for identifying the primary active site residues and structural elements, such as loop 6, which are required for RubisCO function. Site-directed mutants have not, however, clearly identified the residues or conformational requirements that serve as the basis for catalytic efficiency.
(Ω), an understanding of which could have an impact on plant primary productivity, especially among economically-significant crop species (reviewed in Tabita, 1999). With site-directed mutagenesis there are also limitations to the number of mutations and combinations of mutations that can be studied, and results so far indicate that multiple mutations will likely be required to reach the goal of understanding Ω, as well as other properties like falloover, the effects of intracellular inhibitors, and the affinity of the enzyme-RuBP complex for CO₂. Random mutagenesis can generate mutants with multiple changes in potentially all regions of the enzyme, thus increasing the likelihood of finding such mutants.

Finally, the significance of this system was also demonstrated by the speed with which interesting mutants could be identified. It would take decades to study all the mutants generated here by the classical techniques of isolating the enzyme and characterizing its kinetic properties. However, the dependence of a photosynthetic host, *R. capsulatus*, on a functional RubisCO expressed *in trans* was an ideal way to select the enzymes with the greatest potential for yielding interesting results. This selection worked in a positive context, where the organism could not grow unless mutations were acquired in *rbcLS*, as well as a negative context, where the organism lost the ability to grow due to mutations in *rbcLS*. While the speed with which such mutants were isolated and characterized has much room for improvement, as discussed below, this was still a much faster way to identify interesting mutations.

The positive selection mutants, F342V and M259T, revealed to us that there are possibly functions other than specificity or the affinity for CO₂ which should be sought out for improvement. Each of these mutations conferred the ability upon *R. capsulatus* to

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grow with 1.5% CO$_2$ in the atmosphere, but, surprisingly, neither mutation affected either \( \Omega \) or \( K_c \) in ways I predicted. Furthermore, when these positive mutants were used as the basis for further mutagenesis and selection at 0.5% CO$_2$, silent mutations were acquired in the \textit{rbcLS} sequence which enhanced the ability of the organism to grow (Tables 1.5, 1.6). While this was suggestive that something other than enzyme function served as the basis for selection, amino acid substitutions were also acquired in some of these mutants, and twice in the absence of any silent mutations (Table 1.5). This indicated that the basis for selection was likely very complex, and may have involved multiple factors that could range from stability of the mRNA to other properties of RubisCO which we did not measure. The locations of the silent mutations have been provided in hopes that, in the future, a recognizable pattern may emerge that describes why they affected the host, \textit{R. capsulatus} in a positive manner (Table 1.6).

One of the 0.5% CO$_2$ positive mutants certainly warrants further verification and study. PA+13 (Table 2.4) acquired an F217L substitution in the F342V background, and lacked any silent mutations. The \( K_c \) of this enzyme was comparable to that of the wild type enzyme. However, this may reflect a significant improvement since the parent for this mutant, F342V, had a higher \( K_c \) than either the wild type or the F342V/F217L mutant. Likewise, the specificity of the F342V/F217L mutant appeared to be higher than that of either the wild type or the F342V mutant, though the level of significance is indeterminable since this was measured on only a single preparation of this enzyme. Clearly, this mutation needs to be studied further, in both the F342V and the wild type backgrounds, and could even serve as the basis for further mutagenesis and selection.
Perhaps the most surprising results of this study were found in the negative mutants. Most negative mutants were defective for assembly or other undefined properties, but the few that had good activity had interesting kinetic properties also. Of primary interest were the contrasting effects that the G176D and D103V mutations had on the $K_c$. The G176D mutation, at an interface between large subunits paired in a dimer, resulted in an increased affinity for CO$_2$ by about 2-fold, while the D103V mutation, located near a dimer-dimer interface, resulted in a 2-fold decrease in CO$_2$ affinity. Neither of these residues would have been predicted, based on the available crystal structures, to have this effect. In fact, if the G176 residue was not one of the several highly conserved glycines in RubisCO, it is unlikely that either of these residues would ever have been chosen for study by site-directed mutagenesis (Cheng and McFadden, 1998). In light of these findings, a third negative mutant, A47T, is currently under study, as it was the only other negative mutant that was not defective for assembly and had fairly high RubisCO activity in crude extracts of *E. coli* (Table 1.3).

While several interesting mutants were isolated using this system, further modification and improvements to the original scheme will likely produce more mutant forms of the cyanobacterial enzyme or RubisCO from other sources. It is also anticipated that additional useful mutations might be used to isolate mutants in a more efficient manner in the coming months and years.

If one of the goals remains the isolation of an enzyme with an improved specificity or an improved $K_c$, the primary selection condition of low CO$_2$ (1.5%) photoautotrophic growth will need to be reexamined for the cyanobacterial enzyme. While interesting mutants were isolated under this condition (F342V, M259T), clearly
their ability to complement is something other than an effect on the $K_c$ or specificity. Furthermore, when the positive selection condition was made more stringent (0.5% CO$_2$, Chapter 2), no mutants were isolated in a wild type background, and many positive mutant isolates resulted from silent mutations only, in the F342V or M259T background. This indicates that, for most isolates, something other than the kinetic properties of the cyanobacterial RubisCO were responsible for the observed phenotype. Whatever this parameter might be, it must be overcome to continue with further selection along these lines. It would be interesting to see if some (or all?) of the silent mutations acquired would confer the positive phenotypes to the wild type genes at either 1.5% or 0.5% CO$_2$ photoautotrophic growth. This question could be further addressed by DNA shuffling of the genes containing silent mutations (and, for example, the F342V mutation) with the wild type genes, followed by selection at 0.5% CO$_2$. The functional mutations should be selected if they are required (Zhao and Arnold, 1997). Then $\text{rbcLS}$ with the silent mutations could serve as a template for random mutagenesis and bioselection under an even lower CO$_2$ atmosphere.

While direct positive selection will likely not be successful if continued in this exact manner, isolation of suppressors of the negative mutants described could very likely lead to more interesting mutants. For instance, the G176D mutant has a better affinity for CO$_2$ than the wild type enzyme, but less than 10% of wild type carboxylase activity (Chapter 3, Table 3.3). Since it already possesses one of the desirable characteristics, a low $K_c$, random mutagenesis might lead to a mutant with an improvement in $k_{cat}$, which could be identified by the ability to grow photoautotrophically in the high-CO$_2$ atmosphere. It seems possible that such a mutant
may retain the gains made in $K_c$. Of course, the most likely isolate will be a revertant of the original mutation. However, intragenic suppressors (in $rbcS$) of G176D or other negative mutants could be isolated if the mutagenesis was targeted to specific regions.

Towards this goal, a major modification to the bioselection system was recently introduced by a postdoctoral researcher in our laboratory, Dr. Stephanie Scott. Dr. Scott successfully demonstrated that the plasmids pRPS-1 and pRPS-MCS3 are compatible within *R. capsulatus*. This has enabled expression of $rbcL$, encoding the large subunit, on the pRPS-1 plasmid, and the small subunit gene, $rbcS$, on pRPS-MCS3, resulting in complementation of *R. capsulatus* SBI-II to photoautotrophic growth via reconstitution of the enzyme from its individual subunits *in vivo*. This two-plasmid system of complementation will allow for random mutagenesis of one subunit in the absence of the other. Dr. Scott’s plan is to put mutant large subunits into *R. capsulatus* on pRPS-1, randomly mutagenize $rbcS$, and clone the mutant genes into the more versatile pRPS-MCS3. A few short rounds of selection should quickly reveal the likelihood of isolating this type of suppressor mutation. Alternatively, mutagenesis may be localized to $rbcL$ to isolate intragenic suppressors, or the entire $rbcLS$ operon can be targeted. A positive control for this selection will be the isolation of revertants. Lack of such isolates will indicate that there is some other flaw in the system that must be addressed.

As suggested by this approach, targeting specific regions of RubisCO, with expression of genes on pRPS-MCS3, is another potential use of this system. For example, the results described in Chapter 2, for the F342V mutant, and in Chapter 3, for the R347H mutant, as well as numerous other accounts (see Chapter 2 Discussion), demonstrate that α-helix 6 holds great importance in both RubisCO catalysis and folding.
The technique of oligo-directed random mutagenesis, which allows for a region of several amino acids to be replaced by random oligonucleotides, has been successfully used for regiospecific random mutagenesis of several enzymes, including Taq DNA polymerase (Encell and Loeb, 1998; Suzuki, et al., 1996). I think it would be worthwhile to apply this technique to replacement of α-helix 6, followed by positive selection at 0.5% CO₂. The oligo-directed mutagenesis approach could be adapted to study any of several important regions within the enzyme.

Finally, the options for different RubisCO targets are numerous, including enzymes from other phototrophic bacteria (Chapter 1, Table 1.1). Use of a different RubisCO will require definition of new selection conditions. Once the target(s) and conditions are defined, DNA shuffling of similar enzymes, such as the form I enzyme from R. capsulatus and the Synechococcus PCC6301 enzyme, can be applied (Stemmer, 1994a,b; Lorimer and Pastan, 1995). These two enzymes have high sequence homology at the level of DNA (~60%) and high amino acid identity (85%, Horken, 1998), but differ markedly in their kinetic characteristics (Horken and Tabita, 1999).

In summary, the system described in this Dissertation has led us to new and interesting information about this well-studied enzyme, and if pursued it will continue to bring forth new information. The findings that residues at large subunit interfaces can affect the Kc of the enzyme (D103 and G176 mutants), that a concentration-dependent activity and thermal instability can result (as in the G176 mutants), and that mutations proximal or distal to the active site can affect the K_{RubP} (F342V and G176 mutants), are all novel observations and will help direct future studies of RubisCO. Most importantly, however, this system provides a foundation for multiple approaches to mutagenesis and
schemes of selection. The future bioselection of Rubisco promises to uncover the mysteries that remain about specificity, and will have broad implications for agricultural productivity and the role of this enzyme in bioengineering of photosynthesis and/or autotrophic CO$_2$ fixation for the synthesis of value-added products.
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