Dual Role of Ribulose 1,5 Bisphosphate Carboxylase/Oxygenase in Two Distinct Carbon and Sulfur Metabolic Pathways

DISSERTATION

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By

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Abstract

Phylogenetic and X-ray crystallographic studies suggest that ribulose 1,5 bisphosphate carboxylase/oxygenase (RubisCO) and RubisCO-Like-Proteins (RLPs) are structurally related, though RLPs lack the catalytic properties of bonafide RubisCO. Previous studies implicate a role for RLP in metabolism of 5-methylthioadenosine (MTA) in Bacillus subtilis, where MTA is an intermediate of a sulfur (methionine) salvage pathway. Interestingly, Rhodospirillum rubrum RubisCO weakly catalyzes an enolase reaction with 2,3-diketo-5-methylthiopentyl-1-phosphate (DKMTP), similar to the RLP of Bacillus subtilis. R. rubrum is an intriguing model as this organism contains both RubisCO and RLP and each protein seems to be utilized for MTA metabolism under distinct growth conditions.
The questions addressed in this dissertation were: (1) Is RubisCO involved in simultaneous carbon and sulfur metabolism; (2) Are all forms of RubisCO capable of performing an MTA-dependent enolase reaction; (3) Do the substrates RuBP and DKMTP share the same active site?

Results from molecular, genetic and *in vivo* experiments indicate several sources of form I, form II, and form III RubisCO complement RubisCO/RLP knockout strains in *R. rubrum* to MTA dependent growth under specified physiological conditions. All forms of RubisCO utilized in this dissertation appear to catalyze the enolization of DKMTP via a reaction that bears similarity to the enolization reaction catalyzed by RubisCO during CO₂ fixation.

*In vitro* and *in vivo* studies suggest *R. rubrum* RubisCO plays an important role in the sulfur salvage pathway that is distinct from its previously well-characterized carboxylation/oxygenation reaction. Structure-function studies in combination with molecular modeling studies of various mutant forms of RubisCO revealed that two known ligands of RubisCO (i.e., DKMTP, RuBP) do not share conserved
interactions with RubisCO, suggesting distinct residues are differentially involved in the carboxylation/oxygenation or enolase reactions.

In summary, using genetic and molecular tools, this dissertation advances our knowledge on the mechanism and regulation of novel, wide-spread sulfur salvage roles of the well characterized enzyme RubisCO. Important insight was obtained into the structure-function relationships of the enzyme in its two different metabolic roles. The discovery of RLP and the elucidation of its function, gives an entirely new dimension to the function and evolution of RubisCO, i.e., this one enzyme functions to catalyze two distinct metabolic pathways.
Dedication

My work is dedicated to my late Mother and my late Grandfather, who are my inspiration. Even though they left us, their abstract presence motivated me to work through the low tides and kept me grounded during the high tides.
Acknowledgements

I am indebted to my advisor, Dr. F. Robert Tabita. Without his guidance and mentorship, this work would not have been possible. He taught me how to develop hypotheses and transform them into theories, and showed me the value of careful, thoughtful and rigorous scientific research. I very much appreciate his encouragement and unwavering support during the challenging days of graduate training.

I am grateful to Dr. Venkat Gopalan and Dr. Warren Dick for their valuable suggestions and insight and to Dr. Olli Tuovinen for being there for me and serving on my committee on short notice. I am indebted to Dr. Rattan Lal for his kind guidance and for being a pillar of support throughout my graduate training.
I am fortunate to have such a wonderful, supporting and loving family. My sister, Jaya, has been my inspiration and role model, and my uncle, Gautam Deb, and aunt, Nabamita Deb, have set an example that I will always strive to achieve throughout my life. I am thankful to my father, Bimalendu Dey for his support and patience.

Last, but not least, my heartfelt gratitude to my biggest strength, my fiancé Deep for his patience, guidance, motivation, and for always being right beside me, giving me the courage to stand up and to move ahead when I had lost hope.
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<td>CBB</td>
<td>Calvin-Benson-Bass ham</td>
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<tr>
<td>DKMTP</td>
<td>2,3 dike to- 5 - methylthiopentyl-1-phosphate</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>HK-MTP-1P</td>
<td>2-hydroxy-3-keto-5-methylthiopentenyl-1-phosphate</td>
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<td>HB plot</td>
<td>Hydronogen Bond plot</td>
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<td>KMTB</td>
<td>2-keto-4-methylthiobutyrate</td>
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<tr>
<td>LB</td>
<td>Luria-Bertani</td>
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<td>MCS</td>
<td>Multiple Cloning Site</td>
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<td>MSP</td>
<td>Methionine Salvage Pathway</td>
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<td>MTA</td>
<td>5-Methylthioadenosine</td>
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<tr>
<td>OD</td>
<td>Optical Density</td>
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<td>OM</td>
<td>Ormerod’s Medium</td>
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<td>PA</td>
<td>Photoautotrophic</td>
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<td>Photoheterotrophic</td>
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<td>CH</td>
<td>Chemoheterotrophic</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>PYE</td>
<td>Peptone Yeast-extract</td>
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<td>RLP</td>
<td>RubisCO Like Protein</td>
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<tr>
<td>RPM</td>
<td>Revolution Per Minute</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcription-Polymerase Chain Reaction</td>
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<tr>
<td>RuBP</td>
<td>Ribulose 1,5 bisphosphate</td>
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<tr>
<td>RubisCO</td>
<td>Ribulose 1,5-bisphosphate carboxylase/oxygenase</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis</td>
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<td>SAM</td>
<td>S-adenosylmethionine</td>
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Chapter One

Introduction
Chapter 1

Introduction

Ribulose 1,5 bisphosphate carboxylase/oxygenase (RubisCO), the most abundant protein on earth, has evolved with various structural forms that predominantly perform the function of carbon fixation (Tabita, 2007). Based on its amino acid sequence, we know of four major forms of RubisCO (Figure 1.1).

Crystal structures of enzymes isolated from different organisms have provided insight into how these forms differ in their structure-function relationships. Form I is the most abundant form of RubisCO and is found predominantly in plants, eukaryotic algae, cyanobacteria and proteobacteria. Comprised of 8 large and 8 small subunits, form I is arranged as four dimeric pairs to form the catalytic core of the enzyme. The 8 small subunits decorate the top and bottom of the large subunits to form an $L_8S_8$ structure.
FIGURE 1.1: Representative structures of different forms of RubisCO

All forms of RubisCO (as illustrated above) are comprised of dimers of catalytic large subunits. Form I is comprised of four dimers with small subunits decorating the top and bottom of the L₈ octomeric core. Only form I has small subunits. Form II is comprised of dimers of L, ranging from L₂₈ depending on the source. Form III is found only in some archaea and is comprised of dimers of L in either an L₂ or (L₂)₅ arrangements as above. Form IV (the RubisCO-like Protein or RLP) appears thus far always to have an L₂ structure (Tabita et al., 2007).
The large, catalytic subunit of the holoenzyme, coded by either the \textit{rbcL} or the \textit{cbbL} gene, has a molecular weight of about 50kDa, whereas the small, noncatalytic subunit is an approximate 15kDa polypeptide and is encoded by the \textit{rbcS} or \textit{cbbS} genes.

\textbf{Figure 1.2} illustrates the RubisCO family phylogenetic tree and its different clades (forms I, II, III and IV) based on amino acid sequence identities of large subunits. Out of the four clades, forms I, II, and III catalyze bonafide \textsubscript{CO$_2$/O$_2$} fixation reactions by using RuBP as the substrate.

Form IV RubisCO (RLP) is not capable of catalyzing RuBP-dependent \textsubscript{CO$_2$/O$_2$} fixation. The RLPs are further divided into six known clades, with representatives of the type IV-YkrW (\textit{Bacillus. subtilis}) and IV-DeepYkrW (\textit{Rhodospirillum. rubrum, Rhodopseudomonas palustris}) catalyzing defined reactions (Singh and Tabita, 2010; Tabita et al., 2007).
FIGURE 1.2: Phylogenetic tree of RubisCO super family

The above schematic (Tabita et al., 2008a) is based on sequence homology and illustrates the RubisCO family, including forms I, II, III and IV as represented in a phylogenetic tree.

(next page)
Form II RubisCO, found in proteobacteria and dinoflagellates, is only comprised of dimers of large subunits which are distinct in amino acid sequence compared to form I large subunits. The active site of the enzyme is comprised of a hydrophobic pocket, which forms at the interface of the subunits between the N-terminal domain of one subunit and the $\alpha/\beta$ barrel of the C-terminal domain of the second subunit.

Compared to form I, the form II proteins are slower and more inefficient in recognizing CO$_2$ as a substrate as compared to O$_2$. Some organisms like *Rps. palustris* and *Rb. sphaeroides* have form I and form II enzymes such that form I is the major enzyme and form II either acts as a complementary enzyme (as demonstrated by *in vivo* experiments) or participates in the maintenance of the redox balance of the cell by using CO$_2$ as an electron sink (Dubbs and Tabita, 2004). In these organisms, the system of carbon fixation is highly sophisticated and selectively regulated depending on whether carbon or CO$_2$ is present in limited amounts, consistent with the form I enzymes having a higher affinity for CO$_2$ than form II proteins.
Form III is found in archaea and provides a means for removal of RuBP, which is produced during metabolism of purines and pyrimidine (Finn and Tabita, 2004; Sato et al., 2007). The form III enzymes isolated from many extremophiles are highly oxygen sensitive (Kreel and Tabita, 2007). Most often, they are present as dimers, although there are interesting exceptions (i.e., the pentamer of dimers in *Thermococcus kodakaraensis*) (Ezaki et al., 1999).

Form IV is referred to as the RubisCO-Like Protein (RLP) because it does not catalyze bonafide RubisCO CO$_2$/O$_2$ fixation reactions by using RuBP as the substrate (Hanson and Tabita, 2001). Even though the RLPs do not seem to be functionally related to RubisCO, they do, however, share a common sequence identity but do not maintain some key residues present in RubisCO that are required for catalytic activity. Thus, one may speculate that the lack of these key residues is the reason for RLP’s inability to catalyze the RubisCO CO$_2$/O$_2$ bonafide reaction.

The *Bacillus subtilis* (Ashida et al., 2003) and *Geobacillus kaustophilus* (Imker et al.,
(Carre-Mlouka et al., 2006) and RLP from the photosynthetic bacteria *Rhodopseudomonas palustris*, and *R. rubrum* show that RLPs participate in a methionine salvage pathway and catalyze the enolization of the RuBP analogue, DKMTP RLP. RLP from the green sulfur bacterium *Chlorobium tepidum* shows involvement in some aspect of thiosulphate oxidation (Hanson and Tabita, 2001; Hanson and Tabita, 2003) though the precise reaction has not yet been identified.

**Sulfur salvage pathways**

The biosynthesis of sulfur containing compounds [e.g. methionine, cysteine, Sadenosylmethionine (Vashisht et al.)] is essential for cell survival. Extensive studies in prokaryotic and eukaryotic systems have demonstrated that methionine and cysteine are essential amino acids for normal cellular function. Small molecules such as SAM are involved in at least 40 different metabolic reactions and act as a methyl transferase in the synthesis of polyamines (e.g., putrescine, spermidine, spermine), which are ubiquitous cellular polycationic
compounds. Transfer of the aminopropyl moiety of SAM, for biosynthesis of various essential molecules, results in formation of the byproduct 5-methylthioadenosine (MTA). MTA is highly toxic to the cell and different organisms handle MTA exposure in various ways to maintain cell viability. For example, in *Escherichia coli*, MTA is excreted into the medium (Schroeder et al., 1973).

In contrast, in *Klebsiella oxytoca* (earlier *K.pneumoniae*), a sparing pathway called the methionine salvage pathway (Figure 1.3) salvages MTA to form methionine (Dai et al., 2001; Heilbronn et al., 1999; Sekowska and Danchin, 1999). The pathway in *K. oxytoca* has been well described (Sekowska et al., 2004; Wray and Abeles, 1995). Growth with MTA as the sole sulfur source entails a series of reactions that lead to the formation of 5-methylthioribulose-1-phosphate (MTRu-1P) and subsequently, 2, 3-diketo-5-methylthiopentyl-P (DKMTP).
FIGURE 1.3: Anticipated Methionine Salvage Pathway in R. rubrum

The involvement of R. rubrum RubisCO in the anaerobic (left) pathway and of RLP in the aerobic (right) pathway is illustrated in the above schematic. The details of these pathways have not been fully deciphered to date.

DKMTP is then acted upon by a bifunctional enolase-phosphatase to produce, an intermediate, 2-keto-4-methylthiobutyrate, which is the precursor to the formation of methionine (Fitchen et al., 1988; Riscoe et al., 1988; Sekowska et al., 1988; Sekowska et al., 1988).
This pathway is an example of a step in the evolution of the complexity of cell processes. Because sulfur containing compounds are important for maintaining homeostasis, the MSP provides an excellent cellular pathway for conserving the sulfur pool and recycling the sulfur into a usable form in an energetically efficient manner. Compromising the MSP results in cell death (Cellarier et al., 2003; Christopher et al., 2002a; Christopher et al., 2002b).

A similar reaction and pathway has been reported in *B. subtilis*. However, in contrast to the enolase–phosphatase reaction of *K. oxytoca*, a RubisCO like protein instead catalyzes a similar step in the sulfur metabolism pathway (Ashida et al., 2003; Ashida et al., 2008; Murphy et al., 2002). In addition, RLP is involved in reactions for sulfur metabolism in *Chlorobium tepidum* (Hanson and Tabita, 2001).

**RubisCO and RLP**

By virtue of structural and phylogenetic data, we know that RLP shares many important structural features with RubisCO (Tabita et al., 2007; Tabita et al., 2008a). It is interesting that despite the structural similarities between RubisCO
and RLP, fails to demonstrate a bonafide RubisCO reaction of carbon fixation but, participates in sulfur metabolism. Based on their structural identity, substrate structure similarity and reaction mechanism similarity, one may speculate that RubisCO and RLP have some unknown functional similarity and perhaps, RubisCO also participates in sulfur metabolism.

Consistent with this hypothesis, it has been proposed that RLP evolved from an ancestral archaeal RubisCO via the substitution of key residues and structural elements required when this function became necessary in the early biosphere (Tabita et al., 2008a; Tabita et al., 2008b).

A typical MSP has an oxygen requiring step, i.e., the conversion of dihydroxyketonemethyl-thiopentene (DHK-MTPene) to ketomethylthiobutyrate (KMTB) (Tabita et al., 2007). Interestingly, preliminary results in our laboratory suggest MSP may also function under anaerobic conditions in *R. rubrum*, albeit via an unknown mechanism. This raises the possibility of an alternate sulfur salvage pathway under anaerobic conditions in these organisms. This alternate
pathway may involve a simple step that bypasses the oxygen requiring step. To
date, no information is available about an anaerobic MSP salvage pathway. If this
were to be a bonafide metabolic pathway, that would suggest RubisCO has a
dual metabolic function and could serve as a model system for future studies on
similar enzymatic reactions. To establish the existence and mechanism of an
anaerobic sulfur salvage pathway by RubisCO is an important goal.

In support of this hypothesis, the form II RubisCO from *Rhodospirillum rubrum*
has been shown to substitute for RLP in MTA-grown *Bacillus subtilis* and catalyze
the typical RLP enolase reaction *in vivo* and *in vitro*. Moreover, preliminary
studies of RubisCO in *R. rubrum* support the idea of anaerobic MTA-dependent
photosynthetic growth, but not aerobic (nonphotosynthetic) MTA-dependent
growth, which requires RLP (Singh and Tabita, 2010). Presumably, *R. rubrum*
RubisCO can support MTA-dependent anaerobic photosynthetic growth via its
ability to catalyze the enolase reaction of the sulfur salvage pathway, much like
*B. subtilis*. 
FIGURE 1.4: Chemical structures of the substrates for RubisCO

The above illustrations demonstrate the structural similarities between RuBP (top) and DKMTP (bottom), suggesting they interact in a similar manner with RubisCO. These structural models were obtained from http://pubchem.ncbi.nlm.nih.gov/

However, these earlier studies did not consider whether RubisCO functions concurrently as both a required carboxylase and enolase (i.e., the same enzyme simultaneously acts as a focal point for distinct carbon and sulfur metabolic pathways). *B. subtilis* RLP catalyses a reaction analogous to photosynthetic RubisCO, whose substrate, resembles the structure of RuBP (Figure 1.4), consistent with the evolutionary linkage between RLPs and RubisCO.
In the reaction mechanism for the enolization of RuBP by RubisCO (Figure 1.5), the carbamate group attached to Lys201/Lys191 is the charged residue for proton abstraction from C3, while the amino group of Lys175/Lys166 is involved in the deprotonation step preceded by the stabilization of the enolate. Although in the proposed reaction catalyzed by RLP (Imker et al., 2007), Lys 98 is the residue for de-protonation at C1 despite the two residues Lys 201 and Lys 175 being
conserved in *Geobacillus kaustophilus*, *B. subtilis* and *M. aeruginosa* RLPs. What specific residues participate in the DKMTP enolization remains to be established.

It has also been reported that *R. rubrum* and *Rps. palustris* have a functional methionine salvage pathway as these organism can grow using MTA as the sole source of sulfur (Tabita et al., 2007). This observation suggests that in *R. rubrum* and *Rps. palustris*, RLPs function as the DKMTP enolase. However, the critical importance of this residue and studies done by mutation analysis at Lys 98 in *G. kaustophilus* RLP suggest that the RLPs from *R. rubrum* and *Rps. palustris* are not DKMTP enolases, because of the lack of conservation of Lys at 123. The ε-amino group of Lys123 is thought to be required for the abstraction of the 1-proS proton in the *G. kaustophilus* RLP, which belongs to group α1, together with the *B. subtilis* RLP (Saito et al., 2009). It is plausible that in *R. rubrum* and *Rps. palustris*, the reaction step of DKMTP enolase is catalyzed by a photosynthetic form II RubisCO (Singh and Tabita, 2010) and not by RLPs. In support of this hypothesis, evidence suggests that *R. rubrum* RubisCO can catalyze the DKMTP enolase reaction, although at a very low rate (Ashida et al., 2003). However, there
is a paucity of evidence on the ability of RubisCO to function as a DKMTP enolase apart from acting as a carboxylase/oxygenase.

The enolase reaction mechanism for the RLP catalysis seems to be similar to the enolase part of the carboxylation reaction catalyzed by RubisCO (Figure 1.5). There is a step of proton abstraction followed by formation of an enol-intermediate. In *B. subtilis*, both its endogenous RLP, as well as complemented *R. rubrum* RubisCO, catalyze the enolization/tautomerization of DKMTP,

reminiscent of the enolization reaction performed by RubisCO. Interestingly, RLP in *R. rubrum* is also involved in sulfur salvage, but catalyzes a completely novel reaction (Figure 1.6) (Imker et al., 2008).

The study by Imker et al. (2008) reports that in *R. rubrum*, RLP catalyzes distinct reactions and the work of Singh and Tabita (2010) suggests that RubisCO catalyzes a reaction of a potentially novel sulfur salvage pathways under aerobic or anaerobic growth conditions, respectively in the reaction catalyzed by *R. rubrum*. This RLP catalyzes an unusual 1,3 – proton transfer reaction by means of two successive 1,2-proton transfers. This reaction happens only under aerobic growth conditions. Two different products are formed in the ratio of 3:1 namely, 1-thiomethyl-D-xylulose-5-phosphate and 1-thiomethyl-D-ribulose-5-phosphate.

Consequent studies performed showed that RLP from *R. rubrum* would be able to support the MTA metabolism under anaerobic conditions (Singh and Tabita,
Since the expression of RLP was down-regulated under anaerobic conditions, how the cell thrives under anoxygenic conditions was the query in consideration. Preliminary genetic studies in *R. rubrum* initially by Singh and Tabita (2010) and subsequently confirmed and greatly extended here have defined a whole new role for RubisCO in sulfur metabolism (Dey and Tabita, *unpublished results*). This dual role hypothesis was validated in Chapter 2 of this dissertation.

Furthermore, structure-function studies (refer to Chapter 3) provided insight into how the same enzyme reorients itself and offers two different active sites for the two substrates. Results from Chapter 4 indicate form I and form II RubisCOs, RLP-1, and RLP-2 have comparable roles in sulfur metabolism, the mechanism being unknown yet. The final chapter of this dissertation ties in the results from other chapters to provide a composite view on the mechanism and function of RubisCO as a dual role metabolic enzyme.

In summary, using genetic and molecular tools, this dissertation advances our
knowledge on the mechanism and regulation of novel, wide-spread sulfur
salvage roles of the well characterized enzyme RubisCO. Important insight was
obtained into the structure-function relationships of the enzyme in its two
different metabolic roles. The discovery of RLP and the elucidation of its
function, gives an entirely new dimension to the function and evolution of
RubisCO, i.e., there is one enzyme for two simultaneous functions in two
different metabolic pathways.
Chapter Two

One Enzyme Catalyzes Two Obligatorily Required Reactions for Distinct Carbon and Sulfur Metabolic Pathways in the Cell
Chapter 2

Introduction

Ribulose 1,5- bisphosphate (RuBP) carboxylase/oxygenase (RubisCO) is one of the most abundant proteins on earth. RubisCO is responsible for the bulk of biologically produced organic carbon and is the key enzyme in the Calvin-Benson-Bassham (CBB) reductive pentose phosphate pathway. Extensive research over the past sixty years have revealed much about the structure and function of RubisCO, including catalytic mechanism, physiological relevance in both CO₂ and O₂ fixation (Andersson and Backlund, 2008; Kreel and Tabita, 2007; Pierce and Reddy, 1986; Spreitzer and Salvucci, 2002). As such, it has been used as a model system to study various aspects of protein folding and assembly.

Based on amino acid sequence homologies, three different forms of the enzyme have been described, each capable of catalyzing the typical carboxylase reaction.
required for CO$_2$ fixation via the CBB pathway (Tabita et al., 2008b). In addition to these three bona fide forms of RubisCO, a new member of the RubisCO family was discovered about 15 years ago, i.e., the RubisCO-Like Protein (RLP) or form IV RubisCO (Ashida et al., 2008; Hanson and Tabita, 2001; Imker et al., 2007; Imker et al., 2008). RLP is a close homolog of forms I, II, and III, but it lacks the capacity to catalyze the typical carboxylation reaction. Since then, RLPs have been identified in proteobacteria, cyanobacteria, archaea and algae (Tabita et al., 2007; Tabita et al., 2008a).

Although there is significant structural homology between RubisCO and RLP, initially no functional similarity was noted between these proteins based on the substitution of some key active site residues. Earlier studies with the RLP from *Chlorobaculum tepidum* (formerly *Chlorobium*) suggested it was involved in some aspects of sulfur metabolism (Hanson and Tabita, 2001; Hanson and Tabita, 2003). Subsequent studies performed with RLP from *Bacillus subtilis* (Ashida et al., 2003; Tamura et al., 2009a), *Microcystis aeruginosa* (Carre-Mlouka et al., 2006), and *Geobacillus kaustophilus* (Imker et al., 2007) indicated that these RLPs catalyze
a key reaction of a sulfur (methionine) salvage pathway, thereby allowing these organisms to metabolize 5-methylthioadenosine (MTA). This class of RLPs catalyze an enolase reaction of a methionine salvage pathway (MSP) in which the substrate 2,3-diketo-5-methylthiopentyl-1-phosphate (DKMTP) is converted to 2-hydroxy-3 keto-5 thiomethylpent-ene-1-phosphate (HK-MTP-1P), a key step in MTA metabolism. Mechanistically, this reaction is quite analogous to the enolization of RuBP catalyzed by RubisCO.

However, not all classes of RLP catalyze the same reaction. For example, the RLP from *R. rubrum* catalyzes a unique isomerization reaction using a different substrate (Imker et al., 2008) such that this RLP appears to be part of a novel and hitherto, undescribed sulfur salvage pathway under aerobic growth conditions in this organism.

Despite the obvious functional differences between RubisCO and RLP, the conservation of structural features along with the identity of several key active site residues and motifs, raises the possibility that RubisCO and RLP share an
unknown function. In support of this idea, the form II RubisCO from *Rhodospirillum rubrum* was shown to substitute for RLP in MTA-grown *Bacillus subtilis* and catalyze the typical RLP enolase reaction, both *in vivo* and *in vitro* (Ashida et al., 2008).

*R. rubrum* RubisCO was also shown to support anaerobic MTA-dependent photosynthetic growth, but not aerobic (nonphotosynthetic) MTA-dependent growth, which required RLP (Singh and Tabita, 2010). Presumably, *R. rubrum* RubisCO can support MTA-dependent anaerobic photosynthetic growth via its ability to catalyze the enolase reaction of the sulfur salvage pathway, much like *B. subtilis*. A clear evolutional linkage between RubisCO and RLP is certainly suggested by these findings, despite their different functions under aerobic or anaerobic growth conditions in *R. rubrum*. Moreover, these earlier studies did not consider whether RubisCO might function concurrently as both a required carboxylase and enolase in the cell such that the same enzyme would simultaneously act as a focal point for distinct carbon and sulfur metabolic pathways.
The work discussed in the current chapter demonstrates that RubisCO can
catalyze two physiologically significant reactions concurrently; each reaction is
obligatorily required for distinct and unrelated central carbon and sulfur
pathways in the cell required for growth. *In vivo* studies indicate that all forms of
RubisCO were able to catalyze the two essential reactions.

**Materials and methods**

*Bacterial strains and growth conditions*

*R. rubrum* strain Str-2 is the wild type strain of *R. rubrum* used in this study; it has
a spontaneous mutation which imparts streptomycin resistance. Str-2 is a
derivative of strain S1 (ATCC 11170). Strain I-19 is the RubisCO (*cbbM*)-
disruption strain (Falcone and Tabita, 1993). Strain IR is a RubisCO (*cbbM*) and
RLP knockout strain (Singh and Tabita, 2010). All other strains used in this study
are listed in Table 2.1.
Table 2.1. List of strains used in the current chapter

<table>
<thead>
<tr>
<th>Rhodospirillum rubrum strains</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Wt (str) Wild type strain</td>
<td>(Falcone and Tabita, 1993)</td>
</tr>
<tr>
<td>WR</td>
<td>(Singh and Tabita, 2010)</td>
</tr>
<tr>
<td>I19</td>
<td>(Falcone and Tabita, 1993)</td>
</tr>
<tr>
<td>IR</td>
<td>(Singh and Tabita, 2010)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>E. coli</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>DH5-α</td>
<td>(Dower et al., 1988)</td>
</tr>
<tr>
<td>SM-10</td>
<td>(Simon et al., 1983)</td>
</tr>
<tr>
<td>One Shot® TOP10 E</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>DH5a (pRK2013)</td>
<td>DH5a strain contains the helper plasmid pRK2013</td>
</tr>
<tr>
<td>Stellar™ Competent Cells</td>
<td>Clontech</td>
</tr>
</tbody>
</table>

*R. rubrum* strains were grown under photoheterotrophic conditions in Ormerod’s minimal media containing malate as the carbon source. For photoautotrophic growth, strains were bubbled with 1.5% CO₂, 98.5% H₂ in Ormerod’s minimal media devoid of malate. MTA-dependent growth was achieved by preparing sulfur depleted media and adding equimolar amounts of chloride salts.

Growth was monitored by measuring the absorbance at 660 nm of samples with
a Beckman spectrophotometer. Photoautotrophic and photoheterotrophic cultures were incubated at 28 to 30°C behind banks of incandescent lights as described (Ormerod et al., 1961). Cells were first grown in peptone yeast extract (PYE) media in Erlenmeyer flasks under aerobic conditions at 30°C with shaking at 200 RPM to mid-exponential phase ($A_{660}$: 0.6 - 0.8). These cells were harvested by centrifugation and then washed with sulfur-free minimal media three times. Cells were then resuspended in sulfur-free media and used to inoculate sulfur-depleted media supplemented with MTA as sole sulfur source.

**Bacterial conjugation and complementation studies**

Bacterial conjugation was achieved by biparental mating. *R. rubrum* strain IR was grown to the late exponential phase (optical density $A_{660}$~ 1.2 - 1.5); the cells were then diluted 1:10 and incubated until they reached mid-to late exponential phase ($A_{660}$~0.9 - 1.2). *E. coli* strain SM-10 was used as the donor strain. *E. coli* cells were grown overnight in LB media containing 5 mg/ml tetracycline at 37°C with shaking at 220 rpm. Mating was set up by combining 1.0 ml donor cells (*E. coli*
strain top10 with the desired plasmid in it) with 1.0 ml recipient cells (R. rubrum strain IR) in an Eppendorf tube; the mixture was then centrifuged for 4 min at 13,600 x g in a microcentrifuge. Cell pellets were then resuspended in 30 μl of PYE medium and the resuspension was spotted onto a PYE medium plate.

Control cells contained recipient cells and donor cells with empty plasmid (pRPS-MCS3) without any insert and were mated in the same way as mentioned above. Table 2.2 lists all the plasmids tested and used in the current chapter. The PYE mating plates were incubated in the dark at 30°C overnight.

Following mating, cells were resuspended in 1 ml of PYE medium. Dilutions of 10^{-1} to 10^{-4} were plated on PYE medium plates containing appropriate antibiotics. The R. rubrum wild type strain is resistant to streptomycin. Streptomycin, kanamycin and gentamycin were used for counter-selection when the RLP/RubisCO double-disruption strain was used as the recipient.
Table 2.2. List of plasmids used in the current chapter

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Relevant Characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRPS-MCS3</td>
<td>Broad host range plasmid containing <em>R. rubrum</em> cbbM promoter and cbbR gene</td>
<td>(Smith and Tabita, 2003)</td>
</tr>
<tr>
<td>pRPS-Mcs3-cbbM</td>
<td><em>R. rubrum</em> cbbM gene cloned into pRPSMCS3</td>
<td>This study</td>
</tr>
<tr>
<td>Topo-RrcbbM</td>
<td><em>R. rubrum</em> cbbM gene cloned into pCRTOPoblunt</td>
<td>This study</td>
</tr>
<tr>
<td>pCRTOPoblunt</td>
<td>Common cloning vector</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pRPS-RpcbbM-I165T</td>
<td><em>Rps. palustris</em> cbbM gene with mutation I165T cloned into pRPS-MCS3</td>
<td>(Satagopan et al., 2009)</td>
</tr>
<tr>
<td>pRPS-RpcbbM-I165V</td>
<td><em>Rps. palustris</em> cbbM gene with mutation I165T cloned into pRPS-MCS3</td>
<td>Satagopan et al., 2009</td>
</tr>
<tr>
<td>pRPS-6301-D103V</td>
<td><em>Synechococcus</em> 6301 RubisCO mutant D103V cloned into pRPS-MCS3</td>
<td>Satagopan et al., 2009</td>
</tr>
<tr>
<td>pRPS-RpcbbM-F97L</td>
<td><em>Synechococcus</em> 6301 RubisCO mutant F97L cloned into pRPS-mcs3</td>
<td>Satagopan et al., 2009</td>
</tr>
<tr>
<td>pRPS-MBR</td>
<td><em>Methanococcoides burtonii</em> RubisCO cloned into pRPS-MCS3</td>
<td>Brian Witte</td>
</tr>
<tr>
<td>pJG336</td>
<td>Tcr, pVK102 with 24-kb insert of <em>Rb. sphaeroides</em> DNA encoding CO₂ fixation gene cluster A</td>
<td>(Gibson and Tabita, 1986; Gibson and Tabita, 1987)</td>
</tr>
<tr>
<td>pJG106</td>
<td>Tcr, pVK102 with 26-kb insert of <em>Rb. sphaeroides</em> DNA encoding CO₂ fixation gene cluster B</td>
<td>Gibson and Tabita, 1986; Gibson and Tabita, 1987</td>
</tr>
</tbody>
</table>
Transconjugants were grown in Ormerod’s media with either CO₂ or malate as a source of carbon. MTA (1 mM) was used as sole source of sulfur for complementation studies. For positive controls, ammonium sulfate or methionine (1 mM) was used as sulfur source, while negative growth control cultures did not receive exogenous sulfur. The cultures were grown in sealed crimped test tubes under strict anaerobic conditions.

*In vitro enzyme assays and immunoblot analysis*

*R. rubrum cells* were harvested in the late logarithmic to early stationary phase, centrifuged (12,000g for 5 min at 4°C) and washed once with TEM buffer (50 mM Tris-Cl, pH 7.5, 1 mM EDTA, 1 mM ß-mercaptoethanol). The cell pellets were then resuspended in 500 μl of TEM buffer. The cells were disrupted by sonication (Sonicator, Heat system-ultrasonication, NY). The soluble protein extract obtained after centrifugation at 12,000 RPM for 10 min at 4°C was assayed for RubisCO activity as previously described (Chen et al. 1991). The extract was stored at -80°C. The Bradford method was used to determine protein
concentrations with bovine serum albumin as standard. Soluble proteins were resolved after SDS-PAGE with 12% acrylamide in the separating gels (Laemmli, 1970). Western blot analysis was performed. Species-specific primary antiserum was raised against the form II *R. rubrum* RubisCO (*cbbM*) holoenzyme and was used at a dilution of 1:3,000. Alkaline phosphatase-labeled goat anti-rabbit immunoglobulin G (Bio-Rad Laboratories, Hercules, CA) was used as the secondary antibody. Proteins were transferred to nitrocellulose membrane (Immobilon-P; Millipore, Bedford, MA) using a semidry electro-blotting apparatus.

*Analysis of growth curves*

In this dissertation, the majority of the conclusions were based on analysis and interpretation of growth curves of microbial cultures. Therefore, it is important to discuss how these data were analyzed and interpreted, as well as to identify potential caveats and compare related methodology.

Microbial cells incubated with a single batch of medium grow as a result of cell
division processes (e.g., binary fission). Under ideal conditions, these cells would grow in an exponential manner. Although a theoretical possibility and with supporting evidence from some bacterial colonies, a purely exponential population growth is rarely observed. Because the medium is not changed during incubation, the rate of decline in nutrient concentration and rate of increase in waste concentration are the key determinants of growth i.e. limit microbial populations from undergoing uncontrolled exponential growth. As a result, these populations adhere more closely to a sigmoidal growth curve.

By plotting the number of viable cells (ordinate) as a function of incubation time (abscissa), the resulting growth curve typically demonstrates four distinct phases: lag; exponential; stationary; and death. Whereas the latter is not a focus of study in this dissertation, the lag, exponential and stationary phases were quantified and compared under different conditions using a logistic sigmoidal model (Becker et al., 2007; Bohlin et al., 2012; Cao et al., 2010; Fujikawa, 2010; Fujikawa and Morozumi, 2002; Fujikawa and Morozumi, 2005; Keklik et al., 2012; Nakai et al., 2000; Peleg, 2003; Peleg and Corradini, 2011; Russell et al., 1979;
Schaechter, 2006; Stein et al., 2012).

The **lag phase** represents the time period of no immediate increase in cell number when the microbes are introduced into fresh culture medium. During this time, the cells synthesize the components necessary for growth. The availability of nutrients, energy sources, essential cofactors, and ribosomes affect the duration of this period. Moreover, if the new culture medium is different from the one the microbe was growing in previously, synthesis of new enzymes is often necessary to utilize the different nutrients before the cells replicate their DNA, increase in mass, and divide. For example, if a culture were to be transferred from a nutritionally poor medium to a richer one, most of the lag would account for the time it takes for the cells to construct new ribosomes to enhance their capacity for protein synthesis. Conversely, if a culture was to be transferred from a rich medium to a poor one, the lag would represent the time it takes for the cells to make the enzymes necessary for biosynthesis of unavailable nutrients.
During the **exponential phase**, microbial division and growth occur at the maximal possible rate based on their genetic potential, the nature of the medium and environmental conditions. During this phase, the population has the most homogeneous chemical and physiological properties, and its growth rate is mostly constant (i.e., cellular constituents are manufactured at constant rates relative to each other and the cells complete their cycle and double in number at regular intervals). If the system is perturbed (i.e. by a change in nutrient level or other environmental conditions), the rates of synthesis of cell components would vary until the system reaches a new steady-state (i.e., balanced growth). The rate of growth is affected in a hyperbolic manner by nutrient concentration or the microbe’s potential for utilizing nutrients (i.e., the rate of nutrient uptake by microbial transport proteins). The experiments reported herein were calibrated in advance to ensure sufficient nutrient levels to saturate transport systems at the onset of the experiments.

The **stationary phase** represents the period of no further net growth (i.e., number of viable microbes remains constant). During this time, the population either has
reached a new steady-state such that the rates of cell division and death are equal, or remains metabolically active but has stopped dividing. The maximum population size can vary with the availability of nutrients or the potential to utilize nutrients. Aerobic microbes are often limited by availability of oxygen, which is not very soluble and thus, can be depleted in a gradient such that cells beneath the surface are not able to grow (unless the culture is shaken or aerated in another way). On the other hand, growth of anaerobic microbes is more affected by accumulation of toxic waste products. For example, despite an abundance of sugar, the growth of streptococci can be limited by a reduction in pH secondary to the production of lactic acid and organic acids. Nevertheless, both nutrient utilization and waste accumulation are highly coupled processes and thus, are important determinants of entrance into the stationary phase.

Another important determinant of entrance into stationary phase is the type of microbe. During starvation, many bacteria can alter their gene expression and physiology and produce a variety of starvation proteins that make them much more resistant to damage (e.g., increase peptidoglycan crosslinking and cell wall
strength, DNA-binding protein protects DNA, chaperones prevent protein
denaturation and renature damaged proteins). Some bacteria can even become
more virulent when starved (e.g., *Salmonella enterica serovar typhimurium*).

The rate of growth during the exponential phase in a batch culture can be
expressed in terms of the **mean growth rate** ($k$), which represents the number of
generations per unit time and is equal to the reciprocal of the **mean generation
time** ($g$). Mathematically expressed

$$k = \frac{\log_{10} N_t - \log_{10} N_0}{\log_{10} 2 \times t} \quad \text{(Equation 1)}$$

where $N_0$ is the initial population number and $N_t$ is the population number at a
certain time $t$. However, this function does not account for all three phases and
instead, reflects only the exponential phase, which is highly coupled to (i.e., is a
function of) the other phases. Moreover, identifying the fiducial marker (i.e.,
inflection point of the linear component) on a logarithmic scale and using the
population count on opposing sides of this inflection point poses a practical
challenge and thus, may lead to an inaccurate calculation of the doubling time. More importantly, however, fitting the model on a semi-logarithmic scale does not satisfy the assumptions of linear regression, i.e., scatter of points around the line always follows a Gaussian distribution and that the standard deviation is the same at every value of the independent variable. Finally, the doubling time (a linear estimate) is not a true representation of the growth curve.

Although the stochastic nature of large microbial populations allow the study of their growth without knowledge of their cellular mechanisms, mathematical descriptions and models can provide mechanistic insight (e.g., effect of a mutation) and quantitatively distinguish one species from another. To find the values of the model’s parameters (fit coefficients) that yield the curve closest to the data points, one must define a function that measures the closeness between the data and the model. This function depends on the method used to do the fitting, and is typically chosen as the sum of the squares of the vertical deviations from each data point to the growth curve. This method assumes that the
measurement errors are independent and normally distributed with constant standard deviation.

A logistic sigmoidal growth curve, although empirically derived, has been widely used for quantitative assessment of all phases of the growth curve (Becker et al., 2007; Fujikawa, 2010; Fujikawa and Morozumi, 2002; Fujikawa and Morozumi, 2005; Keklik et al., 2012; Peleg, 2003; Peleg and Corradini, 2011; Schaechter, 2006; Stein et al., 2012). The sigmoidal curve for microbial growth is commonly described by four parameters:

1. Initial microbial concentration (base), which is generally a random variable specific to the type of available nutrients at that starting point, and generally, is fixed or normalized to a baseline value.

2. The estimated time to reach 50% of the maximum microbial concentration (ET50), which primarily reflects the length of the lag phase as well as the rate of death (or cessation of growth) and, to a much lesser degree, the growth rate constant. Whereas the latter is an intrinsic characteristic of the
bacteria, the lag phase is primarily a function of current environmental conditions and the physiological history of the cells. As discussed above, cells that are damaged or introduced into a new environment may require more time to synthesize macromolecules and/or repair damage before they can divide (i.e., compared to undamaged cells in a similar environment).

3. The generation time (TAU) is exactly the reciprocal of the growth rate constant, which is a more accurate quantitative measure of growth than the doubling time or mean generation time. The growth rate constant is considered to be a specific and intrinsic characteristic of the microbe under the current conditions. Unlike the lag phase, for example, the growth rate constant is independent of the microbe’s history. Moreover, the population doubling time (or growth at any point in time) can be precisely calculated from the growth rate constant.

4. The maximum population size (MAX) is determined by the entrance into
stationary phase and as discussed above, provides important mechanistic insight (e.g., rate of accumulation of waste or toxic byproducts relative to the population’s capacity to metabolize waste or toxic byproducts, response to starvation, steady-state reached between the rates of death and growth).

The logistic sigmoid model is more effective than the one described by equation 1 because the relationship between measured values and measurement variables of microbial growth is nonlinear. Nonlinear fitting seeks to find those parameter values that minimize deviations between observed and expected ordinate values. However, the equation for the parameters cannot be solved and instead, iterative procedures are used to fit the data with an initial guess of parameter values.

The general shape of the sigmoid curve was compared with the shape of the data points with use of confidence intervals (CI), which are easier to interpret than standard error values. The CI is always centered at the best fit value and extends the same distance above and below it, and this distance depends on the degree of
confidence and the number of degrees of freedom, which equals the number of
data points minus the number of parameters fit by nonlinear regression. The CI
represents the chance that the true value of the variable lies within the interval.
Another (more precise) interpretation of the CI is that if the nonlinear regression
fitting routine were to be repeated many times on different data sets, then the
interval would include the true value the CI percent of times. Thus, in many
ways, the CI is analogous to the P value. Moreover, the CI is an essential
component for quantifying and comparing bacterial growth curves (Bohlin et al.,
2012; Cao et al., 2010; Fujikawa and Morozumi, 2002; Fujikawa and Morozumi,
2005; Nakai et al., 2000; Russell et al., 1979).

In this dissertation, the optical density data (representing microbial population
number) were first normalized to the maximum optical density value of the
positive control, and then, were fit using an iterative, nonlinear, least-squares
method to the following logistic sigmoidal equation

\[ N_t = base + \frac{max}{1 + e^{\frac{ET50-t}{TAU}}} \]  

(Equation 2)
where the coefficient \( base \) sets the \( N_t \) value at initial time \( t_0 \) and the value of \( base + max \) sets the \( N_t \) value at later time \( t \), \( ET50 \) sets the estimated time at which \( N_t \) is at half the value of \( base + max \), and the time constant \( TAU \) is the generation time and also equal to the reciprocal of the \textit{growth rate constant} (e.g., a smaller value of \( TAU \) represents a higher growth rate constant). Compared to equation 1, equation 2 more accurately quantifies the process of microbial growth for all time points (Becker et al., 2007; Fujikawa, 2010; Fujikawa and Morozumi, 2002; Fujikawa and Morozumi, 2005; Keklik et al., 2012; Peleg, 2003; Peleg and Corradini, 2011; Schaechter, 2006; Stein et al., 2012).

Groups were compared based on their fit coefficients ± 95% CI, including MAX, ET50, and TAU. Graphs were plotted in Igor Pro version 6.22 (WaveMetrics, Inc., Lake Oswego, Oregon). Statistical comparisons were performed using Graphpad Prism version 5.0 (GraphPad Software, Inc., La Jolla, California). Comparisons were performed between 2 groups by an unpaired, two-tailed, t-test, and between ≥ 3 groups by ANOVA. A P value of <0.01 was considered to be statistically significant.
Results

*Involvement of RubisCO in MTA-dependent and CO$_2$-dependent growth*

The generic methionine salvage pathway (MSP) includes a requirement for an enolase reaction whereby MTA is converted to methionine (Figure 1.3). The ability of *R. rubrum* RubisCO to catalyze an enolase reaction, using DKMTP as substrate was previously noted in *B. subtilis* (Ashida et al., 2003) and *R. rubrum* itself since RubisCO was shown to be required for anaerobic MTA-dependent growth (Singh and Tabita, 2010). However, these studies did not address whether RubisCO might support both CO$_2$-dependent growth and MTA-dependent growth simultaneously. In other words, could RubisCO catalyze both the CO$_2$ fixation reaction required for the CBB pathway and the enolase reaction required for the MTA pathway?
FIGURE 2.1: *R. rubrum* strains grown in OM medium with different sources of sulfur under photoautotrophic conditions

PA growth of *R. rubrum* strains (from left-to-right): (1) Wild type with MTA; (2) WT with ammonium sulfate; (3) WR with MTA; (4) WR with ammonium sulfate; (5) IR with MTA; (6) IR with ammonium sulfate. The tube were bubbled with CO₂. The degree of transparency and lack of pigmentation indicates lack of growth, whereas the converse correlates with the degree of growth.

Clearly, in *R. rubrum* strains that possess a functional endogenous RubisCO (*cbbM*) gene, photoautotrophic growth was attained when MTA was used as sole sulfur source and CO₂ was employed as sole carbon source (*Figure 2.2, 2.3 and 2.4*). Moreover, as expected, CO₂-dependent growth required *cbbM* expression.
when either sulfate or methionine was provided as the source of sulfur. This suggests that RubisCO under the given conditions is simultaneously catalyzing both the carboxylation and enolase reaction.

Furthermore, MTA dependent growth was also tested under photoheterotrophic conditions. Figure 2.5, 2.6 and 2.7 show the growth plots of the different strains of *R. rubrum* under photoheterotrophic conditions. This raised the question as to whether other forms of *bona fide* RubisCO could also enable MTA-dependent growth. Using the *R. rubrum* complementation system, in which the RubisCO/RLP double knockout strain (strain IR) could achieve MTA-dependent growth with *cbbM* added *in trans*, we sought to determine whether other RubisCO genes, encoding form I and form III RubisCO, might also enable MTA-dependent growth.
Microbial population data [mean ± SE] are plotted (ordinate) in normalized (top) & absolute (right) values vs. time (abscissa) for photoautotrophic growth in (NH₄)₂SO₄ for *R. rubrum* (N=3/group): 1. WT (squares); 2. WR [ΔRLP] (circles); 3. IR [ΔcbbM/ΔRLP] (triangles); 4. I19 [ΔcbbM] (diamonds). Growth curves (lines) with 95% CI were determined by fitting a sigmoidal function and compared by fit coefficients, i.e., stationary phase population (MAX), time to reach 50% MAX (ET50), time constant (TAU). Data beyond stationary phase were excluded from the fit. Groups 1 and 2 demonstrated significant growth with similar TAU and MAX values, but group 2 had a higher ET50 (p=0.0022). Groups 3 and 4 failed to demonstrate significant growth on (NH₄)₂SO₄.
FIGURE 2.3: Photoautotrophic growth of *R. rubrum* strains with methionine

Microbial population data [mean ± SE] are plotted (ordinate) in normalized (top) & absolute (right) values vs. time (abscissa) for photoautotrophic growth in methionine for *R. rubrum* (N=3/group): 1. WT (squares); 2. WR [ΔRLP] (circles); 3. IR [Δ*cbbM*/ΔRLP] (triangles); 4. I19 [Δ*cbbM*] (diamonds). Growth curves (lines) were determined by fitting a sigmoidal function and compared by fit coefficients, i.e., stationary phase population (MAX), time to reach 50% MAX (ET50), time constant (TAU). Data beyond stationary phase were excluded from the fit. Groups 1 and 2 demonstrated significant growth and had similar fit coefficients. However, groups 3 and 4 failed to demonstrate significant growth on methionine.

Microbial population [maximum OD = 2.15]
FIGURE 2.4: Photoautotrophic growth of *R. rubrum* strains with MTA

Microbial population data [mean ± SE] are plotted (ordinate) in normalized (top) & absolute (right) values vs. time (abscissa) for photoautotrophic growth in MTA for *R. rubrum* (N=3/group): 1. WT (squares); 2. WR [∆RLP] (circles); 3. IR [∆*cbbM*/*ΔRLP*] (triangles); 4. I19 [∆*cbbM*] (diamonds).

Growth curves (lines) were determined by fitting a sigmoidal function and compared by fit coefficients, i.e., stationary phase population (MAX), time to reach 50% MAX (ET50), time constant (TAU). Data beyond stationary phase were excluded from the fit. Groups 1 and 2 demonstrated significant growth and had similar TAU and MAX values, but group 2 had a smaller ET50 (p=0.0001). Groups 3 and 4 failed to demonstrate significant growth on MTA.

maximum OD of WT (squares) = 1.62
FIGURE 2.5: Photoheterotrophic growth of *R. rubrum* strains with ammonium sulfate

Microbial population data [mean ± SE] are plotted (ordinate) in normalized (*top panel*) & absolute (*right*) values vs. time (abscissa) for photoheterotrophic growth in (NH$_4$)$_2$SO$_4$ for *R. rubrum* (N=3/group): 1. WT (squares); 2. WR [ΔRLP] (circles); 3. IR [ΔcbbM/ΔRLP] (triangles); 4. I19 [ΔcbbM] (diamonds). Growth curves (lines) with 95% confidence limits were determined by fitting a logistic sigmoidal function and compared by fit coefficients, i.e., population at stationary phase (MAX), time to reach 50% MAX (ET50), time constant (TAU; generation time). Data beyond stationary phase were excluded from the fit. The MAX and TAU were similar for all groups (ANOVA; p>0.5). However, the ET50 of group 2 was significantly higher (p=0.010) than ET50 of the other groups.

maximum OD of WT (squares) = 2.17
Microbial population data [mean ± SE] are plotted (ordinate) in normalized (top panel) & absolute (right) values vs. time (abscissa) for photoheterotrophic growth in methionine for *R. rubrum* strains: 1. WT (squares); 2. WR [ΔRLP] (circles); 3. IR [ΔcbbM/ΔRLP] (triangles); 4. I19 [ΔcbbM] (diamonds). Growth curves (lines) with 95% confidence limits were determined by fitting these data (N=3/group) to a logistic sigmoidal function and compared by fit coefficients, i.e., population at stationary phase (MAX), time to reach 50% MAX (ET50), time constant (TAU; generation time). Data beyond stationary phase were excluded from the fit. All groups had the same MAX, ET50 and TAU (ANOVA; p>0.5).
Microbial population data [mean ± SE] are plotted (ordinate) in normalized (top panel) & absolute (right) values vs. time (abscissa) for photoheterotrophic growth in MTA for *R. rubrum* strains: 1. WT (squares); 2. WR [ΔRLP] (circles); 3. IR [Δ*cbbM*ΔRLP] (triangles); 4. I19 [Δ*cbbM*] (diamonds). Growth curves (lines) with 95% confidence limits were determined by fitting these data (N=3/group) to a logistic sigmoidal function and compared by fit coefficients, i.e., population at stationary phase (MAX), time to reach 50% MAX (ET50), time constant (TAU; generation time). Groups 1 and 2 demonstrated significant growth with similar fit coefficients. In contrast, groups 3 and 4 failed to show any significant growth.
Further complementation studies show the involvement of all forms of RubisCO in MTA-dependent growth

RubisCO knockout strains of *R. rubrum* are capable of photoheterotrophic growth using substrates such as malate as electron donor and carbon source. With such strains the role of RubisCO and the CBB pathway in balancing the redox potential of the cell is taken over by other physiological processes, such as nitrogenase-dependent hydrogen evolution (Dubbs and Tabita, 2004; Tabita et al., 2008b). However, in order for such strains to grow photoheterotrophically using MTA as sole sulfur source, a functional RubisCO gene is required. Using strain I-19, it was thus feasible to determine whether single RubisCO genes offered on plasmids genes could complement to photoheterotrophic MTA-dependent growth in trans.

A broad host complementation vector (pRPS-MCS3) was used to express the form I RubisCO (*rbcLS*) genes from the cyanobacterium *Synechococcus* sp. strain PCC 6301. A number of *Synechococcus* RubisCO gene constructs containing
mutations that alter the properties of this enzyme are available (Satagopan et al., 2009). A prior study reported that a change of residue Asp-103 to a Val on the large subunit (D103V mutant) negatively affected activity and resulted in the inability of the D103V enzyme to support CO2-dependent growth of a *Rhodobacter capsulatus* RubisCO knockout strain SBI/II- (Satagopan et al., 2009).

It is thought that this mutation leads to a negative growth phenotype by virtue of a disruption of interactions between large subunit dimers, instigating conformational changes that lead to diminished functionality. In addition, a substitution at position 97 of the large subunit, resulting in the formation of a F97L enzyme, conferred a negative phenotype in that the F97L enzyme, with greatly diminished *in vitro* activity, also did not allow for CO2-dependent growth of *Rb. capsulatus* strain SBI/II- (Satagopan et al., 2009).

Constructs encoding the mutant cyanobacterial RubisCOs, D103V and F97L, were prepared in complementation vector pRPS-MCS3) and were tested for their ability to complement *R. rubrum* strain IR under anaerobic photoheterotrophic
growth conditions with MTA as sole sulfur source. It was apparent that though these mutations in the large subunit of cyanobacterial RubisCO were not capable of the carboxylase/oxygenase function and thus were incapable of supporting CO₂-dependent growth in *Rb. capsulatus*, both mutant enzymes enabled growth of *R. rubrum* strain IR on MTA and were presumably able to catalyze the enolase reaction of the sulfur salvage pathway (*Figure 2.8 and 2.9*). Clearly, at least residues which affect the carboxylase function of RubisCO are not essential for MTA-dependent growth.

Photoautotrophic growth complemented with single RubisCO genes is not feasible in *R. rubrum* RubisCO knockout strains because of a requirement for additional upstream sequences (Falcone and Tabita, 1993). However, such sequences could be provided by *Rhodobacter sphaeroides* plasmids containing form I (*cbbLS*) and form II (*cbbM*) genes. As shown, plasmids containing both the form I and form II RubisCO genes, plus required additional sequences, did support and complement CO₂- and MTA-dependent photoautotrophic growth of *R. rubrum* RubisCO/RLP deletion strain (strain IR) (*Figure 2.10 and 2.11*).
Microbial population data [mean ± SE] are plotted (ordinate) in normalized (top) & absolute (right) values vs. time (abscissa) for photoautotrophic growth of *Rb. capsulatus* complemented with *Synechococcus 6301* RubisCO genes: 1. **WT** (squares); 2. **F97L** (circles); 3. **D103V** (triangles); 4. **Empty plasmid** (diamonds). Growth curves (lines) with 95% confidence limits were determined by fitting these data (N=3/group) to a logistic sigmoidal function and compared by fit coefficients, i.e., population at stationary phase (**MAX**), time to reach 50% **MAX** (**ET50**), time constant (**TAU**; generation time). Data beyond stationary phase were excluded from the fit. Groups 2, 3 and 4 failed to demonstrate significant growth.

**FIGURE 2.8: PA growth of Rb. capsulatus SBI’/II’ with Synechococcus 6301 rbcLS**

Maximum OD of WT (squares) = 1.75
FIGURE 2.9: MTA dependent growth of *R. rubrum* IR with *Synechococcus rbcLS*

Microbial population data [mean ± SE] are plotted (ordinate) in normalized (top) & absolute (right) values vs. time (abscissa) for MTA dependent photoheterotrophic growth of *R. rubrum* strain IR (ΔcbbM/ΔRLP) with *Synechococcus rbcL*: 1. WT (squares); 2. F97L (circles); 3. D103V (triangles); 4. Empty plasmid (diamonds). Growth curves (lines) were determined by fitting a sigmoidal function and compared by fit coefficients, i.e., stationary phase population (MAX), time to reach 50% MAX (ET50), time constant (TAU). Data beyond stationary phase were excluded from the fit. Groups 1, 2 and 3 demonstrated significant growth. Except for ET50 (ANOVA; p=0.006), groups 1, 2 and 3 had similar fit coefficients. Group 4 failed to demonstrate significant growth.

Maximum OD of WT (squares) = 1.44
A growth study was done to see if form I and form II RubisCO from *Rb. sphaeroides* can complement the RubisCO knockout strains of *R. rubrum* with CO\(_2\) as sole carbon source and MTA as sole sulfur source. **Figure 2.10 and 2.11** shows the growth curve of RubisCO/RLP deletion strain of *R. rubrum* complemented with *Rb. sphaeroides* RubisCO genes along with required upstream sequences provided by plasmid pJG106 and pJG336. Both the form I and form II RubisCO genes from *Rb. sphaeroides* were able to complement the growth of *R. rubrum* RubisCO knockout strains. The growth plot shows that the *R. rubrum* RubisCO/RLP knockout strain was not able to grow with MTA as a sole sulfur source by itself. Though *Rb. sphaeroides* is unable to grow with MTA as a sole sulfur source, form I and form II RubisCO from *Rb. sphaeroides* still show the ability to catalyze the predicted enolase reaction of the sulfur salvage pathway. Hence we can conclude that both form I and form II RubisCO from *Rb. sphaeroides* are capable of enolase activity.

The RubisCO form I from *R. sphaeroides* was encoded in a cosmid pJG336 and demonstrated substantial growth in MTA (i.e., a maximum OD of 1.2). However,
this represented only 50% of the growth in sulfate (i.e., the positive control) (p<0.0001), albeit with the same growth rate constant, as demonstrated in the top panel of Figure 2.2. Interestingly, RubisCO form I entered stationary phase more quickly in MTA (p<0.01). It is possible that RubisCO form II is the primarily responsible for metabolism of the toxic MTA under anaerobic conditions.

Since exogenous form I and form II RubisCO genes allowed MTA-dependent growth of *R. rubrum* strain IR, it was of interest to determine if the other distinct form of RubisCO, from archaea, might also support MTA-dependent growth. *Methanococcoides burtonii* is an obligate anaerobe methanogenic archaeon that contains a RubisCO gene that phylogenetically appears between forms II and III (Tabita et al., 2008b).
Microbial population data [mean ± SE] are plotted (ordinate) in normalized (top panel) & absolute (right) values vs. time (abscissa) for photoheterotrophic growth of *R. rubrum* IR (ΔcbbM/ΔRLP) strain with *R. sphaeroides* cbbLS with either: 1. Sulfate (squares); 2. MTA (circles); 3. IR with MTA (diamonds). Growth curves (lines) were compared by fit coefficients, i.e., population at stationary phase (MAX), time to reach 50% MAX (ET50), time constant (TAU). Data beyond stationary phase were excluded from the fit. Although group 2 reached an OD of 1.2 (right), its growth was only 50% of group 1, resulting in a shorter ET50 (p<0.01); these distinctions were more apparent in the normalized plot. Group 4 failed to demonstrate significant growth.
FIGURE 2.11: PH growth of *R. rubrum* IR (Δ*cbbM*/ΔRLP) with *Rb. sphaeroides* cbbM

Microbial population data [mean ± SE] are plotted (ordinate) in normalized (top panel) & absolute (right) values vs. time (abscissa) for photoheterotrophic growth of *R. rubrum* IR (Δ*cbbM*/ΔRLP) strain with *Rb. sphaeroides* cbbM with media containing either (N=3/group): 1. Sulfate (squares); 2. MTA (circles); 3. IR with MTA (diamonds). Growth curves (lines) with 95% confidence limits were determined by fitting a sigmoidal function and compared by fit coefficients, i.e., population at stationary phase (MAX), time to reach 50% MAX (ET50), time constant (TAU). Data beyond stationary phase were excluded from the fit. The fit coefficients were similar for groups 1 and 2. However, the large uncertainty late in the time course precluded an accurate comparison of MAX1 and MAX2. Group 4 failed to demonstrate significant growth.
The *M. burtonii rbcL* gene was also found to complement strain IR under photoheterotrophic growth conditions when MTA was used as sole sulfur source (Figure 2.12), indicating that this archaeal form III RubisCO is capable of the enolase reaction *in vivo*. Thus, it is apparent that all forms (I, II, III) of bonafide RubisCO will enable MTA-dependent growth to occur.

The complementation studies with the different forms of RubisCO indicated that these enzymes were catalyzing a *B. subtilis* enolase like reaction of the methionine salvage pathway under anaerobic growth conditions. The *in vivo* studies suggested that various forms of RubisCO enabled *R. rubrum* to grow phototrophically in a defined growth medium containing MTA as sole sulfur source, much like *B. subtilis* RLP (provided *in trans* as the *ykrW/mtnW* gene) (Jaya Sriram, unpublished results).
FIGURE 2.12: PH growth of *R. rubrum* strain IR (Δ*cbbM*/ΔRLP) with *M. burtonii* *rbcL*

Microbial population data [mean ± SE] are plotted (ordinate) in normalized (*top panel*) & absolute (*right*) values vs. time (abscissa) for photoheterotrophic growth of *R. rubrum* strain IR (Δ*cbbM*/ΔRLP) with *M. burtonii* RubisCO with media containing either: 1. Sulfate (squares); 2. MTA (circles); 3. Empty plasmid with MTA (diamonds). Growth curves (lines) with 95% confidence limits were determined by fitting these data (N=3/group) to a logistic sigmoidal function and compared by fit coefficients, i.e., population at stationary phase (MAX), time to reach 50% MAX (ET50), time constant (TAU; generation time). Data beyond stationary phase were excluded from the fit. Groups 1 and 2 had similar values of MAX and TAU, but Group 2 had a higher ET50 (p=0.0025). Group 4 failed to demonstrate significant growth.

**Fit Coefficients ± SD**

- MAX<sub>1</sub> (%) = 100 ± 6
- ET50<sub>1</sub> (hrs) = 100 ± 2
- TAU<sub>1</sub> (hrs) = 15 ± 2
- MAX<sub>2</sub> = 94 ± 5
- ET50<sub>2</sub> = 114 ± 3
- TAU<sub>2</sub> = 18 ± 2

*maximum OD of WT (squares) = 1.50*
Figure 2.13 shows Western immunoblots indicating that the proteins were synthesized in these cells. These results strongly suggested that RubisCO catalyzed an enolase reaction typical of MTA metabolism and the methionine salvage pathway under anaerobic phototrophic growth conditions.

FIGURE 2.13: Western blot of RubisCO mutants in *R. rubrum*

Western immunoblot using *R. rubrum* RubisCO antibody showing the presence of RubisCO protein in extracts from the *R. rubrum* Wild type and RLP strain grown under anaerobic conditions (PH or PA) with MTA. From left to right lanes: (1) WT *R. rubrum*, PA with MTA; (2) WT *R. rubrum*, PH with MTA; (3) WR, PH with MTA; (4) WR, PA with MTA; (5) WR PA with methionine.
### TABLE 2.3: Quantitative comparison of growth parameters between strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>MAX (%)</th>
<th>ET50 (hours)</th>
<th>TAU (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>R. rubrum</em> IR (ΔcbbM/ΔRLP) with <em>M. burtonii</em> RubisCO with Sulfate</td>
<td>100 ± 6</td>
<td>100 ± 2</td>
<td>15 ± 2</td>
</tr>
<tr>
<td><em>R. rubrum</em> IR (ΔcbbM/ΔRLP) with <em>M. burtonii</em> RubisCO with MTA</td>
<td>94 ± 5</td>
<td>114 ± 3</td>
<td>18 ± 2</td>
</tr>
<tr>
<td>PH growth for <em>R. rubrum</em> with MTA: WT</td>
<td>91 ± 2.4</td>
<td>43 ± 3.3</td>
<td>8.7 ± 2.7</td>
</tr>
<tr>
<td>WR [ΔRLP]</td>
<td>85 ± 2.5</td>
<td>38 ± 5.3</td>
<td>7.4 ± 3.3</td>
</tr>
<tr>
<td>IR [ΔcbbM/ΔRLP]</td>
<td>NG</td>
<td>NG</td>
<td>NG</td>
</tr>
<tr>
<td>I19 [ΔRLP] with MTA</td>
<td>NG</td>
<td>NG</td>
<td>NG</td>
</tr>
<tr>
<td>PH growth for <em>R. rubrum</em> with ammonium sulfate: WT</td>
<td>100 ± 3.4</td>
<td>99 ± 11</td>
<td>19 ± 6.5</td>
</tr>
<tr>
<td>WR [ΔRLP]</td>
<td>98 ± 6.7</td>
<td>117 ± 3.3</td>
<td>26 ± 3.7</td>
</tr>
<tr>
<td>IR [ΔcbbM/ΔRLP]</td>
<td>93 ± 16</td>
<td>81 ± 12</td>
<td>25 ± 10</td>
</tr>
<tr>
<td>I19 [ΔcbbM]</td>
<td>99 ± 8.5</td>
<td>98 ± 8.3</td>
<td>22 ± 5.5</td>
</tr>
<tr>
<td>PA growth for <em>R. rubrum</em> with MTA: WT</td>
<td>100 ± 4.4</td>
<td>106 ± 2.9</td>
<td>23 ± 2.8</td>
</tr>
<tr>
<td>WR [ΔRLP]</td>
<td>88 ± 2.7</td>
<td>70 ± 1.6</td>
<td>20 ± 1.5</td>
</tr>
<tr>
<td>IR [ΔcbbM/ΔRLP]</td>
<td>NG</td>
<td>NG</td>
<td>NG</td>
</tr>
<tr>
<td>Strain</td>
<td>MAX (%)</td>
<td>ET50 (hours)</td>
<td>TAU (hours)</td>
</tr>
<tr>
<td>--------</td>
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<td>-------------</td>
</tr>
<tr>
<td>I19 [ΔcbbM]</td>
<td>NG</td>
<td>NG</td>
<td>NG</td>
</tr>
<tr>
<td>PA growth for <em>R. rubrum</em> with ammonium sulfate: WT</td>
<td>100 ± 6</td>
<td>180 ± 5</td>
<td>38 ± 4</td>
</tr>
<tr>
<td>WR [ΔRLP]</td>
<td>90 ± 8</td>
<td>218 ± 8</td>
<td>46 ± 6</td>
</tr>
<tr>
<td>IR [ΔcbbM/ΔRLP]</td>
<td>NG</td>
<td>NG</td>
<td>NG</td>
</tr>
<tr>
<td>I19 [ΔcbbM]</td>
<td>NG</td>
<td>NG</td>
<td>NG</td>
</tr>
<tr>
<td>PH growth of <em>R. rubrum</em> strain IR (ΔcbbM/ΔRLP) with <em>Synechococcus 6301-rbcL</em> with MTA:</td>
<td>93 ± 9</td>
<td>187 ± 17</td>
<td>52 ± 14</td>
</tr>
<tr>
<td>F97L</td>
<td>95 ± 3</td>
<td>118 ± 5</td>
<td>25 ± 6</td>
</tr>
<tr>
<td>D103V</td>
<td>74 ± 9</td>
<td>223 ± 39</td>
<td>52 ± 21</td>
</tr>
<tr>
<td>empty plasmid</td>
<td>NG</td>
<td>NG</td>
<td>NG</td>
</tr>
<tr>
<td>PA growth of <em>Rb. capsulatus</em> with <em>Synechococcus 6301 RubisCO</em> with MTA: WT</td>
<td>100 ± 4</td>
<td>132 ± 9</td>
<td>26 ± 7</td>
</tr>
<tr>
<td>F97L</td>
<td>NG</td>
<td>NG</td>
<td>NG</td>
</tr>
<tr>
<td>D103V</td>
<td>NG</td>
<td>NG</td>
<td>NG</td>
</tr>
<tr>
<td>empty plasmid</td>
<td>NG</td>
<td>NG</td>
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</tbody>
</table>
Table 2.3 summarizes some of the key results from the comparison of growth curves in the current chapter. Of note, in the *R. rubrum* strains lacking Rubisco gene, the IR (ΔcbbM/ΔRLP) and I19 (ΔcbbM) groups failed to demonstrate significant growth on MTA dependent media. The WT and WR (ΔRLP) strains demonstrated growth under both PA and PH based MTA dependent growth.
Growth on MTA was demonstrated by *R. rubrum* strain IR (ΔcbbM/ΔRLP) when complemented with any other form of RubisCO.

**Discussion**

Previous studies with both *B. subtilis* and *R. rubrum* (Singh and Tabita, 2010) had indicated that the form II RubisCO from *R. rubrum* could catalyze an enolase reaction typical of the RLP of *B. subtilis*, such that each organism could grow on MTA as sole source of sulfur when the RubisCO gene (*cbbM*) was provided *in trans* to RLP knockout strains of each organism. In *R. rubrum* RubisCO involvement appears limited to anaerobic phototrophic growth.

In the current study, we show that RubisCO functions and plays a key role for both carbon fixation and sulfur salvage pathways of the cell. Moreover, the enzyme appears to catalyze both unrelated functions concurrently *in vivo* in order to allow for both CO₂-dependent and MTA-dependent anaerobic phototrophic growth. Clearly, a functional RubisCO is required and plays a significant role in these two unrelated but essential metabolic pathways of the
cell. Moreover, it appears that these discrete functions may be partially separated as certain mutant RubisCO proteins show a differential effect on either carboxylation or enolase functions, as exemplified by the ability of the D103V and F97L mutant cyanobacterial enzymes to support MTA-dependent growth but not CO$_2$-dependent growth. These results thus suggest that the active site of RubisCO may be somewhat different for the carboxylation and enolase reactions catalyzed by the enzyme.

It appears, however, that the active site of all three distinct forms (I, II, and III) of bona fide RubisCO has been similarly modified by evolution, as all three forms appear to be utilized for both the carboxylation and enolase functions. These results further suggest that the two functions have distinct physiological relevance in many if not all organisms that contain RubisCO, as clearly exemplified by strains of _R. rubrum_ where growth is impossible unless both carboxylation and enolase reactions take place.

The results in this chapter have also raised several interesting questions. First, it is not clear what the fate of the RubisCO-catalyzed enolase reaction product
might be in vivo since there does not appear to be recognizable genes in the genome that encode proteins for its subsequent metabolism. Moreover, a distinct and novel sulfur salvage pathway that involves R. rubrum RLP participating in an isomerase reaction occurs under aerobic growth conditions; this isomerase reaction appears to be linked to isoprenoid biosynthesis (Erb et al, 2012; in press).

Clearly, it will be of interest to determine how these diverse sulfur salvage pathways, involving both RubisCO and RLP, are differentially controlled in the cell.

One approach to look at the relatedness of the RLP and the RubisCO would be to scrutinize the reaction details. There is a structural similarity between the substrates of the two proteins, namely RuBP and DKMTP. Further there are some similarities in the reaction mechanism as well. One of the partial reactions catalyzed by RubisCO [as shown in Figure 1.5] (Tabita et al. 2007; Myers, 1993) is the enolization of the C2-C3 carbons of the five carbon substrate RuBP. The reaction mechanism of RLP as characterized is also an enolase reaction. The structural similarities between DKMTP and RuBP indicate that RubisCO may
potentially use DKMTP as a substrate but the pathway might be slightly different
from the MSP because of the presence of an oxygen based reaction in the MSP.

Also *R. rubrum* lacks a few genes required for a RLP based methionine salvage
pathway. Form II Rubisco from *R. rubrum* was able to rescue the growth of a
RLP negative strain of *B. subtilis* showing the potential of Rubisco to be able to
catalyze the RLP typical reaction.

To determine the evolutionary relationship of Rubisco and RLP, *R. rubrum* has
turned out to be a good model system. Previous results from our lab have shown
that an RLP disruption strain of *R. rubrum* was incapable of using MTA as sole
sulfur source only under aerobic growth conditions, indicating that RLP is
required for metabolizing MTA (Singh and Tabita, 2010). In contrast, the form II
Rubisco disruption strain (*cbbM*) was able to grow using MTA as the sole sulfur
source under anaerobic conditions. As anticipated, the Rubisco/RLP double-
disruption strain was unable to carry out aerobic or anaerobic MTA-dependent
growth. It was also observed that a *R. rubrum* cbbM strain and a Rubisco/RLP
double disruption strain (*cbbM -/RLP -*), both of which still had a functional RLP,
were unable to grow on MTA media under anaerobic conditions, indicating the potential involvement of RubisCO (instead of RLP) in MTA metabolism under these growth conditions. Thus there is physiological role of both RLP and RubisCO for sulfur salvage. Clearly these studies show that this organism uses both aerobic and anaerobic MTA (sulfur salvage) pathways and that these pathways are differentially controlled.

In conclusion, the findings in this chapter demonstrate RubisCO can support the \textit{in vivo} reactions required for sulfur salvage with MTA as a sole sulfur source. RubisCO is required for both the CO$_2$ dependent and MTA dependent anaerobic photoautotrophic growth. From the findings in this chapter, it can be inferred that the enzyme has the ability to catalyze both carboxylase and enolase reaction by of the using DKMTP as substrate \textit{in vivo} as well as \textit{in vitro}. 
Chapter Three

Structure-function studies to better understand the ability of RubisCO to catalyze two different reactions
Chapter 3

Introduction

The evolutionary relationship and the structural identity between RubisCO and RLP have raised many intriguing questions. Previous studies have provided new dimensions to the field of RubisCO-RLP research. For example, a study of the *Bacillus subtilis* RLP has suggested that the protein might be involved in a methionine salvage pathway. The RLP from *B. subtilis* demonstrated the catalysis of an enolase reaction with a reaction mechanism similar to that of the enolase step in the carboxylase reaction catalyzed by RubisCO.

In *B. subtilis*, both its endogenous RLP, as well as complemented *R. rubrum* RubisCO, catalyze the enolization/tautomerization of 2, 3 – diketo – 5 – methylthiopentany1 – 1- phosphate (DKMTP), reminiscent of the enolization reaction performed by RubisCO (Ashida et al., 2008; Saito et al., 2009). This
reaction is a key component of the methionine salvage pathway of *B. subtilis* (Saito et al., 2009; Tamura et al., 2009a; Tamura et al., 2009b). DKMTP, an intermediate in the methionine salvage pathway (MSP), is a subsequent product of 5-methylthioadenosine (MTA) metabolism. MTA is a byproduct of spermidine biosynthesis, acyl homoserine lactone and ethylene biosynthesis.

In most organisms including plants and humans, MTA is converted back to methionine by a methionine salvage pathway (MSP) (Ashida et al., 2003; Sekowska et al., 2004). It has been reported that form II RubisCO from *R. rubrum* possesses *in vitro* DKMTP 1-P enolase activity and the RubisCO gene (*cbbM*) is able to complement the loss of RLP function in *B. subtilis* (Ashida et al., 2003).

RLP from the cyanobacterium *Microcystis aeruginosa*, which has all the genes of MSP present in its genome (Frangeul et al., 2008) also has the ability to catalyze the enolization of DKMTP. Moreover, this enzyme is also able to support MTA-dependent growth in the RLP disruption strain of *B. subtilis* (Carre-Mlouka et al., 2006).
In order to study how the active sites of RubisCO and RLP have evolved for specific functions, it is important to study the structure, function and physiological role of these proteins in various systems. *R. rubrum* presents an especially interesting system to study RLP function because both RLP and RubisCO have been well characterized. Unlike *B. subtilis* RLP, *R. rubrum* RLP does not catalyze the enolization of 2,3-diketo-5-methylthiopentyl-1-phosphate (Singh and Tabita, 2010). Instead, under aerobic growth conditions, *R. rubrum* RLP uses a different substrate (5-methylythioribulose-1-phosphate), and catalyzes a unique 1,3 – proton transfer reaction by two successive 1,2-proton transfers only under aerobic growth conditions, leading to a 3:1 ratio of two products, 1-thiomethyl- D- xylulose-5-phosphate and 1-thiomethyl-D-ribulose-5-phosphate (Imker et al., 2008).

The presence of functional, yet separate, MSPs in *R. rubrum* under both aerobic (*chemoheterotrophic*) and anaerobic (*photoheterotrophic*) growth conditions has not been previously reported for any organism. Moreover, the aerobic and anaerobic sulfur salvage pathways appear to be differentially controlled via a
novel reaction that had remained unexplored until only recently (Singh and Tabita, 2010)

*Sulfur salvage pathways*

Sulfur metabolism in cells is energetically costly and has been a topic of extensive study in both eukaryotic and prokaryotic systems (Chattopadhyay et al., 2003; Thomas et al., 2000; Thomas and Surdin-Kerjan, 1997). However, little is known about sulfur salvage pathway and its enzymes. Sulfur-based compounds play an essential role for cell survival and homeostasis. Sulfur is supplied in the form of amino acids (i.e., cysteine, methionine) and S-adenosylmethionine (Vashisht et al.), the adenosylated form of methionine.

SAM has many different functions in the cell. SAM is the starting point for the synthesis of polyamines such as putrescine, spermine and spermidine.

Methylases are involved in the metabolism of DNA and RNA and also in protein posttranslational modifications (Lu, 2000; Thomas et al., 2000). Furthermore, SAM dependent methylases play an important role in the synthesis of small
metabolites such as various coenzymes or prosthetic groups of proteins (Marzluf, 1997) responsible for the synthesis of S-adenosyl homocysteine (SAH). SAH is then recycled or further metabolized into adenosine and homocysteine. When the aminopropyl moiety is transferred to putrescine to form spermidine, MTA is produced. MTA is a strong inhibitor of polyamine biosynthesis and transmethylolation reactions, and its concentration is tightly regulated in the cell. This control is achieved through the methionine salvage pathway, where MTA is recycled through a series of reactions that convert its 5-methylthio-D-ribose moiety to methionine.

As the assimilation of sulfur is strongly energy consuming in the form of redox equivalents, most organisms from bacteria to mammals and plants have evolved recycling pathways to reuse sulfur and regenerate methionine: for example the ‘methionine salvage pathway’ or MSP (Thomas and Surdin-Kerjan, 1997). In this pathway, growth with MTA as the sole sulfur source involves a sequential series of reactions that leads to the formation of 5-methylthioribulose-1-phosphate (MTRu-1P) and then to 2,3-diketo-5-methylthiopentyl-P (DKMTP). DKMTP is
then acted upon by a bifunctional enolase-phosphatase to produce an intermediate, 2-keto-4-methylthiobutyrate, the direct ketoacid precursor to methionine, which is formed after transamination via a tyrosine aminotransferase.

Rationale and approach

It has been established that RubisCO has a dual role in *R. rubrum*. The structural similarity of substrates of the two reactions and the reaction mechanism, in combination with results from the previous chapters of this dissertation, indicate that RubisCO catalyzes both reactions. An interesting question is whether the substrates share the same active site. Because the structures of the substrate are similar, it is plausible that they share a common active site. In this study, genetic modifications were made in the active site of the enzyme to study the structure function relationship of the enzyme with respect to both the substrates and the reactions.

Genetic manipulations were used to identify residues that play an important role
in enzyme function. For example, a substitution or mutation of an amino acid in the critical binding region of the active site could result in the inhibition of the reaction. At the same time, an important caveat to keep in mind for this interpretation is that the alteration of a residue not in close proximity of the binding site could also have an effect at the binding site, albeit indirectly via an electrostatic mechanism. Therefore, it is necessary to pay close attention to the type of substitutions that are being made for enzyme alteration studies to account for changes in charge, polarity, hydrophobicity, energetics, and steric.

In the experiments reported in this chapter, apart from making site specific changes in RubisCO, the growth medium was also manipulated. The site directed mutations were directed by structural models and were made in the enzyme to identify critical residues involved in the enolase, or carboxylase reactions; or both reactions.

Hypothetical models of the protein-substrate were constructed using molecular docking software (http://www.dockingserver.com). Protein–ligand and protein–protein molecular docking simulations were used to predict binding models,
substrate interactions with amino acid side chains, and energetics of ligand binding to the proteins. For an accurate and complex geometry and estimation of binding energetics, an appropriate method for calculating partial charges is essential. AutoDockTools software, the interface for preparing input files for one of the most widely used docking programs Auto Dock 4, utilizes the Gasteiger partial charge calculation method for both protein and ligand charge calculation (Bikadi and Hazai, 2009; Huey et al., 2007). Ligand structure (http://pubchem.ncbi.nlm.nih.gov/) was inserted into the protein model structure of the different forms of RubisCO (PDBID: 5RUB, 9RUB, 2 RUB), though *R. rubrum* RubisCO was considered as the reference model. For each simulation run, 255 runs of docking calculations were performed and 20 hypothetical models were generated that were closely examined. Interactions predicted by the software are illustrated in Figure 3.1. These predictions were based on:

1. Carbon-carbon bonds

2. Hydrogen bonds
3. Any other ionic bonds

FIGURE 3.1: Example of the interactions predicted by the Docking software

<table>
<thead>
<tr>
<th>hydrogen bonds</th>
<th>polar</th>
<th>hydrophobic</th>
<th>other</th>
</tr>
</thead>
<tbody>
<tr>
<td>O$_5$ [3.37]</td>
<td>ARG288 (N9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O$_1$ [3.27]</td>
<td>HIS321 (NE9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C$_2$ [3.32]</td>
<td>HIS291 (NE9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O$_6$ [3.31]</td>
<td>HIS291 (NE9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C$_6$ [3.87]</td>
<td>HIS291 (NE9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O$_3$ [3.19]</td>
<td>HIS321 (C22, C9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O$_2$ [3.08]</td>
<td>HIS321 (C22)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O$_1$ [3.84]</td>
<td>HIS321 (C22, CEF)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C$_4$ [3.19]</td>
<td>HIS321 (C22)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

This table shows an example of the various protein-ligand interactions (e.g. polar, hydrogen, hydrophobic bonds) predicted by the molecular docking software (http://www.dockingserver.com)

The hydrogen bond (HB) plot is a novel approach that is used for studying hydrogen bond networks (Figure 3.2). Results from HB plots reveal the possible
interactions of the amino acid side chain with the neighboring amino acids and provide insight into relationships between structure and dynamic molecular behavior. Secondary interactions in the protein play a key role in the tertiary structure and protein flexibility.
FIGURE 3.2: Example of the interactions predicted by the Docking software

The hydrogen bond (HB) plot predicts hydrogen bond networks within the protein. Results from HB plots reveal the possible interactions of the amino acid side chain with the neighboring amino acids and provide insight into relationships between structure and dynamic molecular behavior.

The secondary structure of the protein is stabilized by the network of hydrogen bonds. The scheme suggests a hydrogen bond network, such that for the
purposes of this dissertation chapter, the substitution mutations do not perturb the structure significantly by the introduction of a different amino acid side-chain (refer to Figure 3.2 for an example illustrated in a hydrogen bond (HB) plot.)

In the hypothetical model, each of the amino acids predicted by the HB plot were closely examined in the following aspects:

- distance between molecules (4Å)
- possibility of forming polar bonds with the ligand
- possibility of forming hydrophobic bonds with the ligand
- possibility of forming hydrogen bonds with the ligand
- HB plots for residue-residue interactions
- Free energy
- $\Delta G^\circ$ for each substitution/rotamer calculated ($\Delta G^\circ$ of the substitution were calculated on the Swiss-Pdb Viewer)

The docking Protein-Ligand models are illustrated in Figures 3.3 and 3.4.
FIGURE 3.2: Molecular models showing substrates bound into RubisCO

pYMOL was used to dock RuBP (left) and DKMTP (right) in the RubisCO hydrophobic pocket. The figure illustrates the substrate and the surrounding residues within the 4Å distance.
FIGURE 3.3: Molecular model showing substrate-RubisCO interaction

pYMOL was used to create the molecular models as predicted by the docking software. The figures illustrate the orientation of RuBP (blue) and DKMTP (red) in the RubisCO hydrophobic pocket. Residues in proximity of the substrates (4Å) are shown in yellow. The dotted lines represent the distance between atoms of the residues and the substrate.
pYMOL was used to create this schematic representation of the *G. kaustophilus* liganded with Mg$^{2+}$ and 2,3-diketohexane 1-phosphate. The pdb file (pdb id 2OEM) was obtained from Protein data bank (http://www.pdb.org).
pYMOLO was used to superimpose the *R. rubrum* RubisCO docked with DKMTP (red) on *G. kaustophilus* RLP (2OEM) liganded with Mg2+ and 2,3-diketohexane 1-phosphate (pink). This model superimposed the two proteins with respect to ligand orientation. The model was used to examine surrounding residues and select candidates for site-directed mutagenesis. The Pdb file of 2OEM was obtained from the Protein data bank.
pYMOL was used to create the molecular models as predicted by the docking software. The figures illustrate the orientation of DKMTP (green) in the RubisCO hydrophobic pocket. Residues in proximity of the substrates (4Å) are shown in red.
Materials and Methods

Bacterial strains and growth conditions

*R. rubrum* strain Str-2 is the wild type strain of *R. rubrum* used in this study; it has a spontaneous mutation which imparts streptomycin resistance. Str-2 is a derivative of strain S1 (ATCC 11170). Strain I-19 is the RubisCO (*cbbM*)-disruption strain (Falcone and Tabita, 1993). Strain IR is a double RubisCO, RLP knockout strain (Singh and Tabita, 2010). All other strains used in this study are listed in Table 3.1.

*R. rubrum* strains were grown under photoheterotrophic conditions in Ormerod’s minimal media (Ormerod et al., 1961) containing malate as the carbon source. For photoautotrophic growth, strains were bubbled with 1.5% CO₂, 98.5% H₂ in Ormerod’s minimal media devoid of malate. MTA-dependent growth was achieved by preparing sulfur depleted media and adding equimolar amounts of chloride salts. Growth was monitored by measuring the absorbance at 660 nm of samples with a Beckman spectrophotometer.
### Table 3.1. Strains used in the study and references

<table>
<thead>
<tr>
<th>Rhodospirillum rubrum strains</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Wt (str) Wild type strain</td>
<td>(Falcone and Tabita, 1993)</td>
</tr>
<tr>
<td>WR (ΔRLP)</td>
<td>(Singh and Tabita, 2010)</td>
</tr>
<tr>
<td>I19(ΔcbbM)</td>
<td>(Falcone and Tabita, 1993)</td>
</tr>
<tr>
<td>IR(ΔcbbM/ΔRLP)</td>
<td>(Singh and Tabita, 2010)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>E. coli strains</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>DH5-α</td>
<td>(Dower et al., 1988)</td>
</tr>
<tr>
<td>SM-10</td>
<td>(Simon et al., 1983)</td>
</tr>
<tr>
<td>One Shot TOP10 E</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>DH5a (pRK2013)</td>
<td>DH5a strain contains the helper plasmid pRK2013</td>
</tr>
<tr>
<td>Stellar competent cells</td>
<td>Clontech</td>
</tr>
</tbody>
</table>

Photoautotrophic and photoheterotrophic cultures were incubated at 28 to 30°C behind banks of incandescent lights as previously described (Ormerod et al., 1961). Cells were first grown in peptone yeast extract (PYE) media in Erlenmeyer flasks under aerobic conditions at 30°C with shaking at 200 RPM to mid-exponential phase (A_{660} 0.6 - 0.8). These cells were harvested by centrifugation and then washed with sulfur-free minimal media three times. Cells were then resuspended in sulfur-free media and used to inoculate sulfur-depleted media.
supplemented with MTA as sole sulfur source. Table 3.2 lists all the plasmids used in the current chapter.

**Table 3.2. List of plasmids used in the current chapter**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pFL370E48Q</td>
<td>Derived from pFL200/pFL245 ampicillin-resistant</td>
<td>(Larimer et al., 1990)</td>
</tr>
<tr>
<td>pFL247N111S</td>
<td>Derived from pFL200/pFL245 ampicillin-resistant</td>
<td>(Larimer et al., 1990)</td>
</tr>
<tr>
<td>pFL348K166G</td>
<td>Derived from pFL200/pFL245 ampicillin-resistant</td>
<td>(Larimer et al., 1990)</td>
</tr>
<tr>
<td>pFL431 K191C</td>
<td>Derived from pFL200/pFL245 ampicillin-resistant</td>
<td>(Larimer et al., 1990)</td>
</tr>
<tr>
<td>pFL259H321Q</td>
<td>Derived from pFL200/pFL245 ampicillin-resistant</td>
<td>(Larimer et al., 1990)</td>
</tr>
<tr>
<td>pFL322S368A</td>
<td>Derived from pFL200/pFL245 ampicillin-resistant</td>
<td>(Larimer et al., 1990)</td>
</tr>
<tr>
<td>pRPS-MCS3</td>
<td>Cloning Vector</td>
<td></td>
</tr>
<tr>
<td>pRPS-E48Q</td>
<td><em>R. rubrum</em> mutant E48Q cloned into pRPS-MCS3</td>
<td>This study</td>
</tr>
<tr>
<td>pRPS-N111S</td>
<td><em>R. rubrum</em> mutant N111S cloned into pRPS-MCS3</td>
<td>This study</td>
</tr>
<tr>
<td>pRPS-K166G</td>
<td><em>R. rubrum</em> mutant K166G cloned into pRPS-MCS3</td>
<td>This study</td>
</tr>
<tr>
<td>pRPS-K191C</td>
<td><em>R. rubrum</em> mutant K191C cloned into pRPS-MCS3</td>
<td>This study</td>
</tr>
<tr>
<td>pRPS-H321</td>
<td><em>R. rubrum</em> mutant H321Q cloned into pRPS-MCS3</td>
<td>This study</td>
</tr>
<tr>
<td>Plasmid</td>
<td>Relevant characteristics</td>
<td>Reference</td>
</tr>
<tr>
<td>-----------------</td>
<td>---------------------------------------------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>pPRS-S368Q</td>
<td><em>R. rubrum</em> mutant S368Q cloned into pRPS-MCS3</td>
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<tr>
<td>TOPOH287Q</td>
<td><em>R. rubrum</em> mutant H287Q cloned into TOPO</td>
<td>This study</td>
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<td>TOPOR288H</td>
<td><em>R. rubrum</em> mutant H287Q cloned into TOPO</td>
<td>This study</td>
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<tr>
<td>TOPOH291Q</td>
<td><em>R. rubrum</em> mutant R288H cloned into TOPO</td>
<td>This study</td>
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<tr>
<td>TOPOT295S</td>
<td><em>R. rubrum</em> mutant 295S cloned into TOPO</td>
<td>This study</td>
</tr>
<tr>
<td>TOPOA305V</td>
<td><em>R. rubrum</em> mutant A305V cloned into TOPO</td>
<td>This study</td>
</tr>
<tr>
<td>TOPOT391S</td>
<td><em>R. rubrum</em> mutant T391S cloned into TOPO</td>
<td>This study</td>
</tr>
<tr>
<td>pPRS-H287Q</td>
<td><em>R. rubrum</em> mutant H287Q cloned into pRPS-mcs3</td>
<td>This study</td>
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<tr>
<td>pPRS-R288H</td>
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<td>This study</td>
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<tr>
<td>pPRS-H291Q</td>
<td><em>R. rubrum</em> mutant -H291Q cloned into pRPS-mcs3</td>
<td>This study</td>
</tr>
<tr>
<td>pPRS-T295S</td>
<td><em>R. rubrum</em> mutant T295S cloned into pRPS-mcs3</td>
<td>This study</td>
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<td>pPRS-A305V</td>
<td><em>R. rubrum</em> mutant A305V cloned into pRPS-mcs3</td>
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<tr>
<td>pPRS-T391S</td>
<td><em>R. rubrum</em> mutant T391S cloned into pRPS-mcs3</td>
<td>This study</td>
</tr>
</tbody>
</table>
**Site-directed mutagenesis**

Site-directed mutations in the *R. rubrum* RubisCO (*cbbM*) gene were made to study protein structure-function relationship. The Quick change site-directed mutagenesis kit from Strategene was used to make the site directed mutations. Primers targeting substitutions in the amino acid sequence were made using the online software Primer x (http://www.bioinformatics.org/primerx/). Table 3.3 lists the primers used for making the site directed mutations.

Oligonucleotide primers each complementary to opposite strands of the vector containing the substituted base pairs were used for the site-directed mutations. primers (ordered from Sigma-Aldrich) Rru-pst F

CACCCTGCAGTCCGGTCTCCTACATCGCG; Rru-xho R

CTCGAGGCCTGAAGGGAGGGTGCG were used to PCR amplify the *cbbM* gene from the whole genome of *R. rubrum*. The amplified gene was then cloned into the pCR2.1TOPO cloning vector. The primers were extended during the PCR reaction by using the PfuTurbo DNA polymerase. Since the primers
contained the modified base pairs, the resulting plasmids after amplification generated a mutated plasmid with staggered nicks. After the PCR amplification, the plasmids were treated with DpnI endonuclease for about an hour. The DpnI endonuclease (target sequence: 5’-Gm6ATC-3’) is specific for methylated and hemimethylated DNA and is used to digest the parental DNA template and to select for plasmids containing the mutation.

DNA isolated from *E. coli* strain JM109 is dam methylated and therefore subject to DpnI digestion. The plasmid after the DpnI digestion was transformed into *Epicurian coli* XL1-Blue supercompetent cells. The transformants were plated on LB plates with kanamycin (25μg/ml). Colonies growing on kanamycin plates were selected and plasmids were extracted. The RubisCO gene containing the mutation was then PCR amplified by primers MCS3cbbMinfusionpst1: TATAGG GCGAATTGGA GCTCATGGA CCAGTCAT CTCGTTA CGTCAA; MCS3cbbminfusionsac1: TTGATAT CGAATT CCGCAGAG GGCGCTG CGGGTGTCCT incorporating a PstI and SacI restriction site on the amplified gene template. The amplified DNA template was then cloned into pRPS-MCS3
into the PstI- SacI site using the In-Fusion HD Cloning Kit (Clontech).

Many RubisCO active site residue mutants were kindly donated by Dr. Frank Larimer, ORNL, TN. The following active sites mutants in the \textit{R. rubrum} cbb\textit{M} gene were given in the pFL200/pFL245 plasmid as described (Larimer et al., 1990).

- pFL247N111S
- pFL259H321Q
- pFL322S368A
- pFL348K166G
- pFL370E48Q
- pFL431 K191C
### Table 3.3. List of primers

<table>
<thead>
<tr>
<th>Primer</th>
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<td>infusion_Rrc_bbmfr</td>
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<tr>
<td></td>
<td>CCGGCCCCC CCCTCGAGCGCCGCTATCATGACGAGCTCTGA CCGTCTCCG</td>
</tr>
<tr>
<td>Rru-pst F</td>
<td>CACCCTGCAGTCCCGGTCTCCTTACATCGCG</td>
</tr>
<tr>
<td>Rru-xho R</td>
<td>CTCGAGGCCCTGAAGGGAGGGGTGCG</td>
</tr>
<tr>
<td>Mcs3cbbminfusionpst1</td>
<td>TATAGG GCAGATTGGAGCTCATGGACCAGTCAT CTCGTTACGTCA</td>
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<tr>
<td>Mcs3cbbminfusionSac1</td>
<td>TTGATATCGAATTCCGCAGAGGGCGCTGCGGTTGCTTCTT</td>
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<td>H287QF</td>
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<td>H291QR</td>
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<td>T391F</td>
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<td>T391R</td>
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<td>A305VF</td>
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<tr>
<td>A305VR</td>
<td>CATCTTGCAATTGGACGATACGGGTATAGCCGCAGTTTGG</td>
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The mutant \textit{cbbM} from the pFL245 plasmid gene was PCR amplified with
oligonucleotide primers infusion\textsubscript{Rrcbbmf}: TGGCGGC CGCTCTAGACGG
CTCGTATCATATG TGGAATTG TGAGCGG; infusion\textsubscript{Rrcbbmr}: CGGGCCCC
CCCTCGAGCC GCTTACAGAC GAGCTCTGA CCGTCTCCG using the
infusion cloning kit (In-Fusion HD Cloning System, Clontech) incorporated at
the 5’ and 3’ end respectively. The PCR amplified template was then cloned into
the pRPS-MCS3 vector at the PstI-XhoI sites. The vector was transformed into
\textit{E.coli} top10 supercompetent cells by electroporation. Colonies were selected by a
blue white selection (on tetracycline, IPTG, X-gal LB plates). The white colonies
were selected, the plasmid DNA was extracted and sequenced.

\textit{Bacterial conjugation and transconjugants}

Conjugation was performed by tri-parental matings. \textit{R. rubrum} recipient strains (IR, I19) were grown 3-4 days in PYE (complex) medium to the late exponential or
early stationary phase (optical density (OD) at 660 to 1.2-1.5); the cells were then
diluted 1:10 and grown for 1 - 2 days till the mid to late exponential phase
\( \text{OD}_{660} \sim 0.9-1.2 \). *E. coli* strain top 10 was used as the donor strain for the matings.

The *E. coli* DH5\( \alpha \) (pRK2013) strain contains the helper plasmid pRK2013, which provides \textit{tra} and \textit{mob} genes required to transfer the DNA cloned in pUTmini-Tn5 plasmids into the recipient strain, from donor strains, lacking these genes.

Overnight cultures of *E. coli* grown in LB medium with appropriate antibiotics were diluted 1:10 in LB (without antibiotic) and incubated at 37\( ^\circ \) C with shaking at 220 RPM for 2 hrs. Matings were set up by combining recipient cells, donor cells and helper cells in the ration of 2:1:1 in an Eppendorf tube and centrifuging the cells for 2 min at 13,600 \( \times \) g in a microcentrifuge. This mating mixture pellet was resuspended in 20 \( \mu \)l of PYE medium and the resuspension was spotted onto a PYE medium plate. Control plates containing either recipient cells only or donor cells with empty plasmid (pRPS-MCS3) without any insert were prepared as described above and included in each conjugation experiment. The mating PYE plates were incubated in the dark at 30\( ^\circ \) C overnight. Following mating, cells from each plate were resuspended in 1 ml of PYE medium.
Dilutions of $10^{-1}$ to $10^{-4}$ were plated on PYE medium plates containing appropriate antibiotics. The *R. rubrum* wild type strain is resistant to streptomycin. Strain IR is resistant to gentamycin and kanamycin. Streptomycin, gentamycin and kanamycin were used as a counter selection for *E. coli*. Since the pPRS-MCS3 vector has the tetracycline gene as a marker, hence tetracycline was also used as a selection for the IR strains containing the desired vector in this case. Selection was accomplished in all experiments by incubating plates in the dark at $30^\circ$ C until colonies appeared (6 to 10 days). Colonies were grown in PYE or OM broth supplemented with appropriate antibiotics and used for further experimentations. Single colonies were isolated and used for complementation growth studies.

*Complementation studies - MTA dependent photoheterotrophic growth of R. rubrum.*

Single colonies were used to inoculate culture tubes containing Ormerod’s liquid media under aerobic conditions. Cells were pelleted by centrifuging at 12,000 X g
for 3 min; pellets were washed three times with the sulfur depleted medium and then resuspended in the same medium. Washed cells were inoculated in sulfur-depleted medium supplemented with MTA. As a negative control in all experiments, cells were also inoculated in sulfur-depleted medium lacking any sulfur source.

Anaerobic phototrophic MTA-dependent growth was done by performing the same procedure described above using cells grown chemoheterotrophically and then manipulated inside an anaerobic chamber (Coy labs, Grass Lake, MI), maintained at an atmosphere of 2.5 - 3% hydrogen and balance nitrogen.

Anaerobic media was prepared under a 100% nitrogen atmosphere and dispensed (10 ml per tube) in 25 ml tubes fitted with butyl rubber stoppers with an aluminum seal crimped over the stopper (Bellco Glass Inc. Vineland, NJ). Anaerobic cultures were grown in the light at 30° C in a growth chamber (Environment Growth Chambers, Chagrin Falls, OH).

In the experiments testing for MTA-dependent growth, all the strains were
grown in medium without any sulfur source as a negative control and medium containing ammonium sulfate (sole sulfur source) as positive control. The concentrations of MTA and ammonium sulfate used in the media were 1 mM. Kanamycin and gentamycin antibiotics which are commercially available as sulfate salts were not used for MTA dependent growth as they can be a potential source of sulfate in the medium. All the growth experiments were performed using three independent isolates and results are representative of at least two independent experiments.

**Western blots**

Proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). Western blot analysis was done by protein transfer to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA) according to the manufacturer’s instructions using a BioRad Transblot semi-dry transfer cell (BioRad, Hercules, CA). Species-specific primary antiserum was raised against the form II *R. rubrum* RubisCO (CbbM)
holoenzyme and was used at a dilution of 1:3,000. Alkaline phosphatase-labeled goat anti-rabbit immunoglobulin G (Bio-Rad Laboratories, Hercules, CA) was used as the secondary antibody. Proteins were transferred to nitrocellulose membranes (Immobilon-P; Millipore, Bedford, MA) using a semidry electroblotting apparatus.

**Results**

Site directed mutant proteins used in this study were made in residues that were previously shown to be involved in the carboxylase reaction and are located in the active site. In addition, novel mutations were made in residues that were selected based on the hypothetical model. These mutants were complemented into the IR strain and the results for each mutant are detailed in the following sections.

**Glutamic Acid 48**

The active site of RubisCO is constituted from domains of adjacent subunits and
includes an intersubunit electrostatic interaction between Lys (K)-166 and Glu (E)-48, which has been recently identified by x-ray crystallography (Andersson and Backlund, 2008; Lundqvist and Schneider, 1989a; Lundqvist and Schneider, 1989b). This residue is not involved in any kind of subunit association or in the processes of reaction mechanism. In vitro studies show that substitution in E to D or Q resulted in 5% of the normal level of carboxylation (Mural et al., 1990).

The mutated E48Q gene when complemented into Rb. capsulatus. SBI/II-, could not support growth under photoautotrophic conditions (Figure 3.7). The in vivo results were consistent with the in vitro observations as reported by Mural et al. (1989). E48Q was complemented into the IR strain of R. rubrum for growth under photoheterotrophic conditions. This mutant enzyme could only partially support the growth with MTA as a sole sulfur source under photoheterotrophic conditions (Figure 3.8). Although the OD went to a max of 0.5, the mutant could grow only up to 22% of that of the wild type RubisCO on a normalized scale.
FIGURE 3.7: PA growth in *Rb. capsulatus* SBI’II’ with *R. rubrum* cbbM (K166G and E48Q)

Microbial population data [mean ± SE] are plotted (ordinate) in normalized (top panel) & absolute (right) values vs. time (abscissa) for photoautotrophic growth of *Rb. capsulatus* strain SBI’II’ complemented with *R. rubrum* RubisCO genes:

1. WT (squares); 2. K166G (circles); 3. E48Q (triangles); 4. Empty plasmid (diamonds). Growth curves (lines) with 95% confidence limits were determined by fitting these data (N=3/group) to a logistic sigmoidal function and compared by fit coefficients, i.e., population at stationary phase (MAX), time to reach 50% MAX (ET50), time constant (TAU). Data beyond stationary phase were excluded from the fit. In contrast to Group 1, the other groups failed to demonstrate any significant growth, as evidenced by the failure of fit convergence after 200 iterations.

Maximum OD of WT (squares) = 2.29
FIGURE 3.8: PH growth of *R. rubrum* strain IR complemented with mutant E48Q

Microbial population data [mean ± SE] are plotted (ordinate) in normalized (top) & absolute (right) values vs. time (abscissa) for photoheterotrophic growth of *R. rubrum* IR (∆cbbM/∆RLP) in media containing different sulfur sources complemented with *R. rubrum* genes (N=3/group): 1. WT *cbbM* with MTA (squares); 2. E48Q with MTA (circles); 3. E48Q with sulfate (triangles); 4. Empty plasmid with MTA (diamonds). Growth curves (lines) were determined by fitting a logistic sigmoidal function. Compared to groups 1 and 3, group 2 grew much less and reached a markedly lower MAX. Group 3 had a lower ET50 than groups 1 and 3 (ANOVA; p<0.001). The TAU values were similar between groups 1, 2 and 3. Group 4 failed to demonstrate significant growth.

maximum OD of WT (squares) = 2.25
Lysine 166

K166 has been established as a key catalytic residue. It is exquisitely positioned to act as the general acid that stereospecifically protonates the terminal aci-acid intermediate and thereby form upper 3-PGA at the terminal step of carboxylation (Mural et al., 1990). The K166G *R. rubrum* Rubisco is severely impaired as a carboxylase, although it catalyzes forward processing (hydration and cleavage) of exogenously supplied carboxylated reaction intermediate 2’-cardoxyarabinitol bisphosphate (CABP). CABP is a 6-carbon transition state analog of substrate RuBP used for crystal structure studies. The product diisopropyl methyl phosphonate (DiMP) arises from â-elimination of phosphate from the terminal aci-acid, presumably due to the absence of the normal proton donor.

Three different position-166 mutants (K166G, K166C, and K166S) were examined to be sure that any novel properties observed reflected the absence of the lysyl side chain and were not unique to the particular side chain introduced. Each of these mutants is able to process RuBP, but the major product is DiMP derived
from $\alpha$ elimination of phosphate from the enediol. Hence it was assumed that it is indirectly involved in the initial enolization as a secondary acceptor of the C3 proton (Harpel and Hartman, 1996; Harpel et al., 2002). *R. rubrum* strain IR transconjugants with the same mutant (mutant gene cloned into the complementation vector, pRPS-MCS3), were tested for growth with different sulfur sources.

With MTA as a sole sulfur source, photoheterotrophic growth was observed. The mutant enzyme did not seem to have much effect on the enolase reaction and was able to rescue the growth of strain IR with MTA as a sole sulfur source *(Figure 3.9)*. On the normalized plot, it demonstrates growth of about 90% of that of the wild type enzyme. The protein-ligand model indicates that Lys-166, which is in the active site of the enzyme, is not well positioned with respect to its proximity to the new substrate DKMTP. This is consistent with the growth data which shows little effect on the strain IR in the presence of MTA. Our model *(Figure 3.6)* is also consistent with these experimental observations.
FIGURE 3.9: PH growth of \textit{R. rubrum} strain IR complemented with mutant K166G

Microbial population data [mean ± SE] are plotted (ordinate) in normalized (\textit{top panel}) & absolute (\textit{right}) values vs. time (abscissa) for photoheterotrophic growth of \textit{R. rubrum} strain IR (\textit{∆cbbM/∆RLP}) in media containing different sources of sulfur complemented with \textit{R. rubrum} genes: 1. \textbf{WT cbbM with MTA} (squares); 2. \textbf{K166G with MTA} (circles); 3. \textbf{K166G with sulfate} (triangles); 4. \textbf{Empty plasmid with MTA} (diamonds). Growth curves (lines) with 95\% confidence limits were determined by fitting these data (N=3/group) to a logistic sigmoidal function and compared by fit coefficients. Groups 1, 2 and 3 had similar MAX and TAU values. However, the ET50 for group 2 was significantly higher than those of groups 1 and 3 (ANOVA; p<0.001). Group 4 failed to demonstrate significant growth.

Microbial population data [mean ± SE] are plotted (ordinate) in normalized (\textit{top panel}) & absolute (\textit{right}) values vs. time (abscissa) for photoheterotrophic growth of \textit{R. rubrum} strain IR (\textit{∆cbbM/∆RLP}) in media containing different sources of sulfur complemented with \textit{R. rubrum} genes: 1. \textbf{WT cbbM with MTA} (squares); 2. \textbf{K166G with MTA} (circles); 3. \textbf{K166G with sulfate} (triangles); 4. \textbf{Empty plasmid with MTA} (diamonds). Growth curves (lines) with 95\% confidence limits were determined by fitting these data (N=3/group) to a logistic sigmoidal function and compared by fit coefficients. Groups 1, 2 and 3 had similar MAX and TAU values. However, the ET50 for group 2 was significantly higher than those of groups 1 and 3 (ANOVA; p<0.001). Group 4 failed to demonstrate significant growth.

Maximum OD of WT (squares) = 2.25
Asparagine 111

The importance of Asn (N) 111 is indicated by its high degree of conservation in all sequenced RubisCO genes and by its position at the active site. The mutant N111S, is well characterized and studied. Substitutions were made to serine (polar with no additional bulk), glutamine (amide functionality), aspartate (introduce a negative charge) and lysine (introduce a positive charge) (Chene et al., 1992; Soper et al., 1992). Loss of measurable carboxylase activity (in vitro assays) showed the high stringency of this residue.

This residue is considered to be associated with subunit-subunit association, activation, substrate binding and initial steps of catalysis. It is presumed that N111 is perhaps involved in stabilization of the transition state that occurs subsequent to enediol (ate) formation. The mutant proteins retain enolization activity, as measured by exchange of the C3 proton of ribulose bisphosphate with solvent thereby demonstrating a preferential role of N111 in some later step of overall catalysis. The crystal structure indicates that when the enzyme is
activated, the N111 is complexed to the magnesium ion. And when substrate is bound, the residue moves away from the metal and is hydrogen bonded to C-4 oxygen of RuBP (Knight et al., 1990). The mutant protein is activated by binding carbon dioxide and magnesium and it also can bind CABP. This suggests that the active site is not grossly disturbed. However, when the \textit{in vivo} effect of the mutant N111S was tested, interesting results were observed. This mutant was incapable of supporting the growth of \textit{R. rubrum} strain IR (ΔcbbM/ΔRLP) under photoheterotrophic conditions with MTA as a sole sulfur source in the media (Figure 3.10).

The positive controls for the photoautotrophic growth experiment was \textit{Rb. capsulatus} SBI/II- with the \textit{R. rubrum} wild type \textit{cbbM} gene cloned into pRPS-MCS3 and the negative control was strain SBI/II- with an empty vector (pRPS-MCS3). Positive and negative controls for the photoheterotrophic growth experiment of \textit{R. rubrum} strain IR included MTA as a sole sulfur source with the wild type \textit{R. rubrum} \textit{cbbM} gene cloned into pRPS-MCS3 and an empty plasmid vector, respectively.
Microbial population data [mean ± SE] are plotted (ordinate) in normalized (top panel) & absolute (right) values vs. time (abscissa) for photoheterotrophic growth of *R. rubrum* strain IR (∆*cbbM/∆RLP*) in media containing different sources of sulfur complemented with *R. rubrum* genes: 1. WT *cbbM* with MTA (squares); 2. N111S with MTA (circles); 3. N111S with sulfate (triangles); 4. Empty plasmid with MTA (diamonds). Growth curves (lines) with 95% CI were determined by fitting these data (N=3/group) to a logistic sigmoidal function and compared by fit coefficients. Groups 1 and 3 had similar fit coefficients except for the ET50, which was significantly higher in group 1. Groups 2 and 4 failed to demonstrate significant growth.
Prior studies of the carboxylation reaction have demonstrated that the N111S mutation compromises enzymatic activity under *in vitro* conditions (Soper et al., 1992). These results were confirmed by the *in vitro* results reported in the current chapter. However, under *in vivo* conditions, the N111S mutation was associated with a lesser degree of compromised enzymatic activity (i.e., 60% of wild type activity; **Figure 3.11**) than that predicted (10% activity) from the enzymatic activity measured from *in vitro* experiments alone. The presence of another counter mutation is highly unlikely based on reextraction of the plasmid after the growth experiments and confirmation of the gene sequence. Western blot experiments confirmed that the mutated gene was indeed expressed.

These results suggest that the *in vivo* and *in vitro* catalytic activities of RubisCO are different, at least in relation to the N111 residue. There are several possible explanations for this phenomenon. First, the *in vivo* milieu may include the compensatory mechanisms such additional residue(s) overlap in the role of N111 for the function of this important metabolic enzyme. This is plausible because random mutations are not uncommon in microbes, and these organisms protect
important enzymatic functions by evolving redundant mechanisms, including cofactors, accessory subunits, alternate conformations, and flexible active site that may enhance function under certain conditions. Second, although in vitro enzymatic activity was compromised (i.e., to 10% compared to the wild type enzyme), perhaps this reduced level of efficacy is sufficient to eschew an organism’s inability to grow. Moreover, this residue does not seem to be involved in the initial steps of enolization (of the carboxylation reaction) and instead, is involved in the non-rate-limiting steps in the later stages of catalysis. One may speculate that these later steps play a lesser role or are even bypassed under in vivo conditions. For example, unlike the activity measured under equilibrium conditions in vitro, hardly any physiological process exists at a steady state in vivo and these in vitro results do not fully reflect the in vivo condition.

In summary, the N111 residue plays an important role for in vivo growth, albeit not as much as predicted from in vitro studies. Nevertheless, the results indicate N111 is an important component of the catalytic domain of the carboxylase
reaction (photoautotrophic growth) with CO₂ as a sole source of carbon. More importantly, this residue plays a prominent role in the enolase reaction with photoheterotrophic conditions with MTA as a sole sulfur source (Figure 3.10).

**Lysine 191**

In all forms of RubisCO, K 191 (or K-201/191/179) is highly conserved and plays an important role in the active site, including enzymatic activation and forming a complex with Mg²⁺. K191 becomes carbamylated when RubisCO is "activated" by CO₂ in the presence of a divalent metal prior to the actual catalytic event when the RubisCO-CO₂-Mg²⁺ ternary complex is acted on by a second molecule of CO₂. The K 191 carbamate nitrogen abstracts a proton from C3 of RuBP (Cleland et al., 1998b; Tabita et al., 2008b). Previous studies (Smith et al., 1990) showed, that when this K residue was substituted by a cysteine, the mutant protein was catalytically inept under standard assay conditions compared to fully activated WT enzyme. The specific activity was 3 x 10⁻⁵ units/mg in comparison to 3 units/mg for the wild-type enzyme (Smith et al., 1990).
FIGURE 3.11: PA growth in *Rb. capsulatus* with *R. rubrum cbbM* (K191C and N111S)

Microbial population data [mean ± SE] are plotted (ordinate) in normalized (top panel) & absolute (right) values vs. time (abscissa) for photoautotrophic growth of *Rb. capsulatus* strain SBI’II’ complemented with *R. rubrum* RubisCO genes: 1. **WT cbbM** (squares); 2. **N111S** (circles); 3. **K191C** (triangles); 4. **Empty plasmid** (diamonds). Growth curves (lines) with 95% confidence limits were determined by fitting these data (N=3/group) to a logistic sigmoidal function and compared by fit coefficients. Compared to Group 1, Group 2 had a lower MAX and higher ET50 (p<0.001), but had the same TAU. Although group 2 demonstrated significant growth and reached an OD of 1.2 (right), it only grew 60% as much as group 1. In contrast, Groups 3 and 4 failed to demonstrate significant growth.

**Fit Coefficients ± SD**
- MAX₁ (%) = 98 ± 3
- ET50₁ (hrs) = 182 ± 4
- TAU₁ (hrs) = 22 ± 2
- MAX₂ = 60 ± 2
- ET50₂ = 207 ± 6
- TAU₂ = 23 ± 2
- MAX₃ = 2.3 ± 0.1
- ET50₃ = 153 ± 8
- TAU₃ = 6 ± 3

*maximum OD of WT (squares) = 2.29*
Rubisco is activated for both carboxylation and oxygenation by formation of a lysyl carbamate in the active site (Badger et al., 1980; Badger and Lorimer, 1976; Lorimer et al., 1976; Lorimer and Miziorko, 1980; Pierce, 1986; Pierce et al., 1986). The K191C mutant protein, in the presence of CO₂ and Mg²⁺, exhibits tight binding of the reaction intermediate analogue 2-carboxyarabinitol bisphosphate. Cysteine is a polar neutral amino acid, whereas lysine is a polar positive residue. Thus, a cysteine residue at this position would make residue 191 incapable of donating protons.

When the K191C mutant was used to complement photoautotrophically grown *Rb. capsulatus* SBI/II, very little growth was observed (Figure 3.11), in keeping with the low in vitro catalytic activity of this mutant enzyme (Smith et al., 1990). In parallel to this experiment, the K191C mutant gene was complemented in *R. rubrum* strain IR grown under anaerobic conditions MTA as the sole sulfur source. MTA is an intermediate in the MSP and growth with this substrate as the sole sulfur source is tantamount to the presence of an intact sulfur salvage pathway. It was seen that K191C could partially complement the growth of the
IR, double deletion strain of *R. rubrum* (*ΔcbbM/ΔRLP*). (Figure.3.12); whereas K191 is absolutely critical for the carboxylation reaction. The growth of mutants K191C was highly reduced on MTA and it grew only to 30% of that of the wild type. These data suggest K191 is not involved in the initial protonation and deprotonation in the enolase reaction.

**Histidine 321**

This residue is an evolutionarily conserved residue between both the form I and the form II RubisCOs. This histidine residue assists in stabilizing the transition state. When the H residue was substituted with a glutamine, the *K_m* for RuBP was insignificantly altered. It is assumed that this residue is involved in the preferential binding of the transition state relative to substrate (Harpel et al., 1991).
FIGURE 3.12: PH growth of *R. rubrum* strain IR complemented with mutant K191C

Microbial population data [mean ± SE] are plotted (ordinate) in normalized (*top panel*) & absolute (*right*) values vs. time (abscissa) for photoheterotrophic growth of *R. rubrum* strain IR (Δ*cbbM/ΔRLP) in media containing different sources of sulfur complemented with *R. rubrum* genes: 1. WT *cbbM* with MTA (squares); 2. K191C with MTA (circles); 3. K191C with sulfate (triangles); 4. Empty plasmid with MTA (diamonds). Growth curves (lines) with 95% confidence limits were determined by fitting these data (N=3/group) to a logistic sigmoidal function and compared by fit coefficients. Groups 1 and 3 had similar fit coefficients. Although group 2 reached an OD of 0.7 (*right*), it had a markedly lower MAX in comparison to groups 1 and 2 (P<0.001). Group 4 failed to demonstrate significant growth.

Microbial Population (% normalized to max OD$^{660}$) vs. Time (hours)

Fit Coefficients ± SD
- MAX$_1$ (%) = 96 ± 7
- ET50$_1$ (hrs) = 85 ± 6
- TAU$_1$ (hrs) = 24 ± 6
- MAX$_2$ = 29 ± 1
- ET50$_2$ = 85 ± 7
- TAU$_2$ = 19 ± 4
- MAX$_3$ = 95 ± 2
- ET50$_3$ = 77 ± 1
- TAU$_3$ = 16 ± 1

maximum OD of WT (squares) = 2.25
Various substitutions for this residue were tested (with different side chain functionality), e.g. H321A, H321S and H321Q. It was determined that all mutants retain only 5-10% of the wild-type activities in both the carboxylation and enolization/carboxylation reactions. The mutant enzyme which was used in this study was H321Q. A side chain substitution to glutamine was made resulting in a slight loss in the positive charge. It was observed that the mutant was not able to support photoautotrophic growth in *Rb. capsulatus* strain SBI-/II- i.e., no carboxylation reaction took place (Figure 3.13).

Interestingly, this mutant was capable of supporting the growth of *R. rubrum* strain IR using MTA as sole sulfur source (Figure 3.14). Mutant H321Q seems to be an important residue in the ligand-protein interaction when either RuBP or DKMTP were used as a substrate. However since the substitution was a conservative substitution, further studies might be pursued by making substitutions which alter the charge or bulk or polarity of the residue at this position.
FIGURE 3.13: PA growth in SBI’II’ complemented with R. rubrum cbbM (H321 and S368)

Microbial population data [mean ± SE] are plotted (ordinate) in normalized (top panel) & absolute (right) values vs. time (abscissa) for photoautotrophic growth of Rb. capsulatus strain SBI’II’ complemented with R. rubrum RubisCO genes:

1. WT cbbM (squares); 2. H321Q (circles); 3. S368Q (triangles); 4. Empty plasmid (diamonds). Growth curves (lines) with 95% CI were determined by fitting these data (N=3/group) to a logistic sigmoidal function and compared by fit coefficients. Data beyond stationary phase were excluded from the fit. Whereas group 1 demonstrated significant growth, the rest of the groups failed to demonstrate significant growth.

maximum OD of WT (squares) = 2.29
Microbial population data [mean ± SE] are plotted (ordinate) in normalized (top panel) & absolute (right) values vs. time (abscissa) for photoheterotrophic growth of *R. rubrum* strain IR (ΔcbbM/ΔRLP) in media containing different sources of sulfur complemented with *R. rubrum* genes: 1. WT *cbbM* with MTA (squares); 2. H321Q with MTA (circles); 3. H321Q with sulfate (triangles); 4. Empty plasmid with MTA (diamonds). Growth curves (lines) with 95% confidence limits were determined by fitting these data (N=3/group) to a logistic sigmoidal function and compared by fit coefficients. Groups 1, 2 and 3 had the same MAX and TAU (ANOVA; p>0.45), but their ET50 values were significantly different (ANOVA; p<0.001). Group 4 failed to demonstrate significant growth.
Serine 368

Substitutions for residue Ser-368 show five-fold enhanced enolization activity (when substituted with C or A) (Harpel et al., 1991). It is presumed that since this residue does not seem to be involved directly in the reaction mechanism of enolization, it might be a possibility that the absence of a seryl residue in this position could allow alternate binding conformations.

Alternatively, this residue plays a potential role in assisting the addition of H₂O to enediol(ate) (Harpel et al., 1991; Spreitzer and Salvucci, 2002). The enzyme lost its ability to do the carboxylation reaction when S368 was substituted by a glutamine. The mutant gene, when complemented into *Rb. capsulatus* strain SBI /II, for photoautotrophic growth, it failed to support any growth (Figure 3.13). When complemented into *R. rubrum* strain IR to test for the ability to do the enolase reaction for MTA metabolism *in vivo*, mutant S368Q was not able to support growth. These results indicate that this residue might be critical for the enolase based MTA metabolism (Figure 3.15).
FIGURE 3.15: PH growth of *R. rubrum* strain IR complemented with mutant S368Q

Microbial population data [mean ± SE] are plotted (ordinate) in normalized (top panel) & absolute (right) values vs. time (abscissa) for photoheterotrophic growth of *R. rubrum* strain IR (∆cbbM/∆RLP) in media containing different sources of sulfur complemented with *R. rubrum* genes: 1. WT *cbbM* with MTA (squares); 2. S368Q with MTA (circles); 3. S368Q with sulfate (triangles); 4. Empty plasmid with MTA (diamonds). Growth curves (lines) with 95% confidence limits were determined by fitting these data (N=3/group) to a logistic sigmoidal function and compared by fit coefficients. Groups 1 and 3 demonstrated significant growth and had similar fit coefficients. Groups 2 and 4 failed to demonstrate significant growth.

**Fit Coefficients ± SD**
- MAX₁ (%) = 98 ± 7
- ET50₁ (hrs) = 85 ± 6
- TAU₁ (hrs) = 24 ± 6
- MAX₂ = 3.4 ± 1.4
- ET50₂ = n/a
- TAU₂ = n/a
- MAX₃ = 84 ± 9
- ET50₃ = 73 ± 6
- TAU₃ = 22 ± 6

*maximum OD of WT (squares) = 2.29*
Arginine 288

The Arg at position 288 was the next residue targeted to test for its role in the interaction with the substrate DKMTP. This residue was chosen from the docking model, which predicted its role in DKMTP binding. It was replaced by a histidine, maintaining the polar positive nature of the residue but slightly changing the charge on the side chain. Arg has a higher pKa value hence is more basic than His.

The photoautotrophic growth of *Rb. capsulatus* strain SBI/II was not affected by the R288H mutation(Figure 3.16) The R288H mutated gene, when complemented in *R. rubrum strain* IR and grown under PH conditions with MTA as sole sulfur source, demonstrate 60% growth of the positive control change (Figure 3.17). These results suggest that either R 288 is not critical for the carboxylase or the enolase reactions *in vivo* or a more nonconservative mutation has to be made to test the role of the mutant.
FIGURE 3.16: PA growth of SBI'II' with *R. rubrum cbbM* mutants A305V and R288H

Microbial population data [mean ± SE] are plotted (ordinate) in normalized (top panel) & absolute (right) values vs. time (abscissa) for photoautotrophic growth of *Rb. capsulatus* strain SBI'II' complemented with *R. rubrum* RubisCO genes:

1. **WT** (squares); 2. **A305V** (circles); 3. **R288H** (triangles); 4. **Empty plasmid** (diamonds). Growth curves (lines) with 95% confidence limits were determined by fitting these data (N=3/group) to a logistic sigmoidal function and compared by fit coefficients. Group 1 had the highest MAX and ET50 (ANOVA; p<0.001). Groups 1, 2 and 3 had the same TAU (p=0.4). Group 4 failed to demonstrate significant growth.

*maximum OD of WT (squares) = 2.29*
FIGURE 3.17: PH growth of *R. rubrum* strain IR complemented with mutant R288H

Microbial population data [mean ± SE] are plotted (ordinate) in normalized (top panel) & absolute (right) values vs. time (abscissa) for photoheterotrophic growth of *R. rubrum* strain IR (ΔcbbM/ΔRLP) in media containing different sources of sulfur complemented with *R. rubrum* genes: 1. WT *cbbM* with MTA (squares); 2. R288H with MTA (circles); 3. R288H with sulfate. (triangles); 4. Empty plasmid with MTA (diamonds). Although group 2 reached an OD of 1.0 (right), it only grew 50% as much as group 1. Compared to group 2, groups 1 and 3 had similar values of TAU but significantly higher MAX and ET50 (ANOVA; p<0.01). Group 4 failed to demonstrate significant growth.

Maximum OD of WT (squares) = 2.13
Alanine 305

The alanine at position 305 was substituted by a valine, changing the polarity of the side chain. The number of alkyl groups influences the polarity. More the alkyl groups present, the more non-polar the amino acid will be. This effect makes valine more non-polar than alanine.

The mutant gene when complemented in *Rb. capsulatus* SBI/II was shown to support the growth under PA conditions (**Figure 3.16**). The mutant enzyme when tested for growth with MTA as a sole sulfur source under PH conditions in *R. rubrum* strain IR, was seen to not to support growth (**Figure 3.18**).

These results suggested that the Ala residue at position 305 is differentially involved in the two reactions performed by the enzyme. On one hand a substitution in this residue affected growth under PH conditions with MTA as sole sulfur source, whereas the ability of the enzyme to perform its normal carboxylation function was not disturbed by the same mutation at this residue.
Microbial population data [mean ± SE] are plotted (ordinate) in normalized (top panel) & absolute (right) values vs. time (abscissa) for photoheterotrophic growth of *R. rubrum* strain IR (∆cbbM/∆RLP) in media containing different sources of sulfur complemented with *R. rubrum* genes: 1. **WT cbbM** with MTA (squares); 2. **A305V** with MTA (circles); 3. **A305V** with sulfate (triangles); 4. **Empty plasmid** with MTA (diamonds). Growth curves (lines) with 95% confidence limits were determined by fitting these data (N=3/group) to a logistic sigmoidal function and compared by fit coefficients. Compared to group 1, group 3 had a higher ET50 and TAU (p<0.001) but they had similar values of MAX (p=0.1). Groups 2 and 4 failed to demonstrate significant growth.
Histidine-291

The histidine residue at position 291 was mutated and substituted by a glutamine, based on the hypothetical protein-ligand model (Figure 3.6). The polar, positively charged histidine was replaced by a glutamine, which is also polar but neutral, thereby producing only a slight change in the side chain charge. Glutamine was chosen to minimize unfavorable steric contacts.

Moreover, glutamine can form hydrogen bonds which might take over this role from the two nitrogen atoms in the bifunctional his residue. The ε1-nitrogen can be mimicked by the amide nitrogen atom of a glutamine residue.

This mutant gene failed to complement the growth of *R. rubrum* strain IR with MTA. A growth of 7% of that of the WT *cbbM* was observed. The maximum OD as seen is growth of the wild type was 2.13 (Figure 3.19).
FIGURE 3.19: PH growth of *R. rubrum* strain IR complemented with mutant H291Q

Microbial population data [mean ± SE] are plotted (ordinate) in normalized (top panel) & absolute (right) values vs. time for photoheterotrophic growth of *R. rubrum* strain IR (Δ*cbbM/ΔRLP) in media containing different sources of sulfur complemented with *R. rubrum* genes: 1. WT *cbbM* with MTA (squares); 2. H291Q with MTA (circles); 3. H291Q with sulfate (triangles); 4. Empty plasmid with MTA (diamonds). Growth curves (lines) with 95% confidence limits were determined by fitting these data (N=3/group): to a logistic sigmoidal function and compared by fit coefficients. Groups 1 and 3 had similar fit coefficients for MAX and TAU which was higher than group 3. The ET50 value for group 3 was higher than group 1 (p<0.001). Groups 2 and 4 failed to demonstrate significant growth.

Fit Coefficients ± SD
- MAX<sub>1</sub> (%) = 100 ± 10
- ET50<sub>1</sub> (hrs) = 119 ± 9
- TAU<sub>1</sub> (hrs) = 41 ± 9
- MAX<sub>2</sub> = 7.5 ± 1
- ET50<sub>2</sub> = 58 ± 10
- TAU<sub>2</sub> = 9 ± 5
- MAX<sub>3</sub> = 100 ± 8
- ET50<sub>3</sub> = 160 ± 8
- TAU<sub>3</sub> = 35 ± 3

maximum OD of WT (squares) = 2.13
It was also determined whether this substitution would affect the ability of the enzyme to perform the carboxylation reaction. In *Rb. capsulatus* strain SBI/II, the H291Q gene demonstrated a reduced growth (**Figure 3.20**). Although the growth of the mutant reached an OD of 0.5, it represented only 22% of the growth on sulfate (i.e., the positive control (p<0.01). This phenotypic effect suggests that this residue was involved in the interaction with substrate (RuBP), or interaction with any other residue which could disrupt the active site for RuBP.

**Threonine 391**

Threonine (T) 391 was mutated based on the hypothetical protein-ligand docking model (**Figure 3.4**). T was substituted by a serine (S), which has a shorter side chain (i.e., one less methyl group) and consequently, forms a more polar residue. The effect of this mutation was tested for photoautotrophic (PA) growth of *Rb. capsulatus* strain SBI/II (carboxylase reaction) and photoheterotrophic growth of *R. rubrum* strain IR with MTA as a sole source of sulfur (to test the enolase activity *in vivo*).
FIGURE 3.20: PA growth of SBI’/II’ with *R. rubrum cbbM* mutants H291Q and T391V

Microbial population data [mean ± SE] are plotted (ordinate) in normalized *(top panel)* & absolute *(right)* values vs. time (abscissa) for photoautotrophic growth of *Rb. capsulatus* strain SBI’/II’ complemented with *R. rubrum* RubisCO genes: 1. WT (squares); 2. H291Q (circles); 3. T391V (triangles); 4. Empty plasmid (diamonds). Growth curves (lines) with 95% confidence limits were determined by fitting these data (N=3/group) to a logistic sigmoidal function and compared by fit coefficients. Data beyond stationary phase were excluded from the fit. Only group 1 demonstrates significant growth. Although groups 2 and 3 reached an OD of 0.5 *(right panel)*, its growth was only ~20% of that of group 1. Groups 1, 2 and 3 had similar TAU (p=0.1). Group 4 failed to demonstrate significant growth.

Maximum OD of WT (squares) = 2.13
In strain SBI/II- under PA conditions, the mutant was barely able to support the
growth of SBI/II-. The mutant demonstrate growth of only 16% of that of the WT
$cbbM$, the OD of the culture reached a high of only 0.4 (Figure 3.20)

With MTA as a sulfur source under photoheterotrophic (PH) growth conditions,
in $R. rubrum$ strain IR the mutant failed to demonstrate any significant growth
(Figure 3.21). Hence, determining the role of this residue either in the reaction
mechanism or the protein ligand binding, though it is still not clear how this
residue may be involved in the enolase reaction

To assess gene expression of all mutants, Western blots were performed (Figures
3.22 and 3.23). These results confirm that mutant proteins were indeed
synthesized in the cells.
FIGURE 3.21: PH growth of *R. rubrum* strain IR complemented with mutant T391V

Microbial population data [mean ± SE] are plotted (ordinate) in normalized (top panel) & absolute (right) values vs. time (abscissa) for photoheterotrophic growth of *R. rubrum* strain IR (ΔcbbM/ΔRLP) in media containing different sources of sulfur complemented with *R. rubrum* genes: 1. WT *cbbM* with MTA (squares); 2. T391V with MTA (circles); 3. T391V with sulfate (triangles); 4. Empty plasmid with MTA (diamonds). Growth curves (lines) with 95% confidence limits were determined by fitting these data (N=3/group) to a logistic sigmoidal function and compared by fit coefficients. Compared to group 1, group 3 had a mildly higher ET50 and MAX with borderline significance (0.01<p<0.05). Groups 2 and 4 failed to demonstrate significant growth.
FIGURE 3.22: Western blot showing expression of RubisCO mutants genes in MTA dependent growth

Western immunoblot using *R. rubrum* RubisCO antibody showing the expression of RubisCO under MTA dependent PH growth conditions was confirmed with western immunoblots. Figure shows protein bands (from left to right) of RubisCO WT, mutants H321Q, K191C, K166G, E48Q

FIGURE 3.23: Western blot showing expression of mutant RubisCO genes in *Rb. capsulatus* strain SBI'/II'

Western immunoblot using *R. rubrum* RubisCO antibody showing expression of mutant RubisCO genes proteins under PA growth condition (in *Rb. capsulatus* SBI- /II-). Protein bands from left to right show R288H, T391S, A305V, *R. rubrum* WT, *R. rubrum cbbM* in IR, N111S, WR
### TABLE 3.4: Quantitative comparison of growth parameters between strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>MAX (%)</th>
<th>ET50 (hours)</th>
<th>TAU (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PH growth of <em>R. rubrum</em> IR (ΔcbbM/ΔRLP) mutant Rubisco E48Q with ammonium sulfate</td>
<td>96 ± 6</td>
<td>68 ± 3</td>
<td>17 ± 3</td>
</tr>
<tr>
<td>E48Q with MTA</td>
<td>21 ± 2</td>
<td>88 ± 9</td>
<td>20 ± 8</td>
</tr>
<tr>
<td>K166G with ammonium sulfate</td>
<td>95 ± 2</td>
<td>77 ± 1</td>
<td>16 ± 1</td>
</tr>
<tr>
<td>K166G with MTA</td>
<td>89 ± 4</td>
<td>113 ± 3</td>
<td>25 ± 2</td>
</tr>
<tr>
<td>K191C with ammonium sulfate</td>
<td>98 ± 7</td>
<td>85 ± 6</td>
<td>24 ± 6</td>
</tr>
<tr>
<td>K191C with MTA</td>
<td>29 ± 1</td>
<td>85 ± 7</td>
<td>19 ± 4</td>
</tr>
<tr>
<td>N111S with ammonium sulfate</td>
<td>98 ± 7</td>
<td>85 ± 6</td>
<td>24 ± 6</td>
</tr>
<tr>
<td>N111S with MTA</td>
<td>NG</td>
<td>NG</td>
<td>NG</td>
</tr>
<tr>
<td>H321Q with ammonium sulfate</td>
<td>99 ± 8</td>
<td>74 ± 4</td>
<td>21 ± 4</td>
</tr>
<tr>
<td>H231Q with MTA</td>
<td>93 ± 3</td>
<td>108 ± 4</td>
<td>19 ± 2</td>
</tr>
<tr>
<td>S368Q with ammonium sulfate</td>
<td>84 ± 9</td>
<td>73 ± 6</td>
<td>22 ± 6</td>
</tr>
<tr>
<td>S368Q with MTA</td>
<td>NG</td>
<td>NG</td>
<td>NG</td>
</tr>
<tr>
<td><em>R. rubrum</em> IR (ΔcbbM/ΔRLP) with mutant Rubisco R288H with ammonium sulfate</td>
<td>87 ± 1</td>
<td>90 ± 3</td>
<td>21 ± 3</td>
</tr>
<tr>
<td>R288H with MTA</td>
<td>54 ± 1</td>
<td>87 ± 3</td>
<td>16 ± 1</td>
</tr>
<tr>
<td>A305V with ammonium sulfate</td>
<td>87 ± 2</td>
<td>73 ± 4</td>
<td>15 ± 4</td>
</tr>
<tr>
<td>A305V with MTA</td>
<td>NG</td>
<td>NG</td>
<td>NG</td>
</tr>
<tr>
<td>H291Q with ammonium sulfate</td>
<td>100 ± 8</td>
<td>160 ± 8</td>
<td>35 ± 3</td>
</tr>
</tbody>
</table>
TABLE 3.4: Quantitative comparison of growth parameters between strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>MAX (%)</th>
<th>ET50 (hours)</th>
<th>TAU (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H291Q with MTA</td>
<td>NG</td>
<td>NG</td>
<td>NG</td>
</tr>
<tr>
<td>T391V with ammonium sulfate</td>
<td>122 ± 4</td>
<td>145 ± 6</td>
<td>30 ± 6</td>
</tr>
<tr>
<td>T391V with MTA</td>
<td>NG</td>
<td>NG</td>
<td>NG</td>
</tr>
<tr>
<td>PA growth of <em>Rb. capsulatus</em> strain SBI/II with <em>R. rubrum</em> RubisCO gene: WT</td>
<td>98 ± 3</td>
<td>182 ± 4</td>
<td>22 ± 2</td>
</tr>
<tr>
<td>E48Q</td>
<td>NG</td>
<td>NG</td>
<td>Ng</td>
</tr>
<tr>
<td>K166G</td>
<td>NG</td>
<td>NG</td>
<td>NG</td>
</tr>
<tr>
<td>K191C</td>
<td>NG</td>
<td>NG</td>
<td>NG</td>
</tr>
<tr>
<td>N111S</td>
<td>60 ± 2</td>
<td>207 ± 6</td>
<td>23 ± 2</td>
</tr>
<tr>
<td>H321Q</td>
<td>NG</td>
<td>NG</td>
<td>NG</td>
</tr>
<tr>
<td>S368Q</td>
<td>NG</td>
<td>NG</td>
<td>NG</td>
</tr>
<tr>
<td>R288H</td>
<td>16 ± 2</td>
<td>92 ± 8</td>
<td>27 ± 10</td>
</tr>
<tr>
<td>A305V</td>
<td>70 ± 8</td>
<td>135 ± 12</td>
<td>39 ± 10</td>
</tr>
<tr>
<td>H291Q</td>
<td>22 ± 2</td>
<td>82 ± 11</td>
<td>22 ± 12</td>
</tr>
<tr>
<td>T391V</td>
<td>16 ± 2</td>
<td>141 ± 8</td>
<td>43 ± 10</td>
</tr>
</tbody>
</table>

Microbial population growth data were fit to a logistic sigmoidal function using an iterative, nonlinear, least-squares method, and compared by fit coefficients (mean ± SD), i.e., population size at stationary phase (MAX), estimated time to reach 50% MAX (ET50), time constant (TAU).
Table 3.4 summarizes some of the key results from the sigmoidal fits of the normalized growth data in the current chapter. Of note, MAX was significantly different between the sulfate and MTA groups for mutants E48Q, K166G, K191C (reduced growth) and for mutants N111S, S368Q, A305V, H291Q and T391V (no growth). Interestingly the TAU was similar for sulfate or MTA. Although some of the mutants (e.g., E48Q, K166G and K191C) demonstrated significant growth (i.e., an absolute OD value >0.5), these growths were relatively minor when normalized to their respective positive controls. Some mutants, including N111S, A305V, H291Q and T391V revealed differentiation in enolase and carboxylation activities. Table 3.5 lists a summary of all statistical analyses. RubisCO specific activity, for wild type and mutants were calculated from in vitro assays. These values have been listed in table 3.5
Table 3.5. Mutants used in the current chapter

<table>
<thead>
<tr>
<th>R. rubrum cbbM gene/mutant</th>
<th>Carboxylation reaction</th>
<th>Enolase reaction</th>
<th>RubisCO specific activity (nmol/mg/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>+</td>
<td>+</td>
<td>267 ± 16</td>
</tr>
<tr>
<td>E48Q</td>
<td>-</td>
<td>+</td>
<td>NA</td>
</tr>
<tr>
<td>N111S</td>
<td>+</td>
<td>-</td>
<td>45 ± 2</td>
</tr>
<tr>
<td>K166G</td>
<td>-</td>
<td>+</td>
<td>NA</td>
</tr>
<tr>
<td>K191C</td>
<td>-</td>
<td>+</td>
<td>NA</td>
</tr>
<tr>
<td>H321Q</td>
<td>-</td>
<td>+</td>
<td>NA</td>
</tr>
<tr>
<td>S368Q</td>
<td>-</td>
<td>-</td>
<td>NA</td>
</tr>
<tr>
<td>R288H</td>
<td>+</td>
<td>+</td>
<td>123 ± 10</td>
</tr>
<tr>
<td>H291Q</td>
<td>+</td>
<td>-</td>
<td>80 ± 7</td>
</tr>
<tr>
<td>A305V</td>
<td>+</td>
<td>-</td>
<td>210 ± 22</td>
</tr>
<tr>
<td>T391V</td>
<td>+</td>
<td>-</td>
<td>80 ± 12</td>
</tr>
</tbody>
</table>

The above listed table summarize the properties of RubisCO mutants. Column 2 and 3 shows the catalytic characteristics and column 2 enlists the RubisCO specific activity assay from cell extracts.
Discussion

The carboxylation reaction catalyzed by RubisCO has been an area of investigation for many years, and consequently, has been well characterized (Andersson and Backlund, 2008; Falcone and Tabita, 1993; Miziorko and Lorimer, 1983; Spreitzer and Salvucci, 2002; Tabita, 2007). By virtue of crystallographic studies to date, the active site, critical residues and the hydrophobic pocket of the enzyme are established. A large body of evidence is available on how the bona fide reaction works, the reaction mechanism, how each active site residue contributes to the stability of the enzyme, and how some of the residues push the reaction forward (Cleland et al., 1998a). What is not known, however, is how the same enzyme reshuffles its active site to dock a new substrate and perform a new function.

In B. subtilis and Geobacillus RLP, proton abstraction by Lys 98 is required for the enolase reaction. In contrast, proton abstraction in the RubisCO carboxylase reaction requires Lys-191 of the R. rubrum enzyme (equivalent to Lys-201 of the
plant form I enzyme). Because Lys-98 is not conserved in RubisCO, the residue that serves as the required base for the enolase reaction with DKMTP remains to be identified. Moreover, there is a paucity of information on the amino acid residue involved in the reaction mechanism of protonation and de-protonation. It is important to determine how the residues interact with the novel substrate. The results reported in the previously dissertation chapter suggest that the carboxylation and enolase reactions occur concurrently, thereby raising the possibility that either there is a single active site of the enzyme with similar specificities for the substrates, the active sites overlap or that there are distinct active sites for each substrate. Moreover, the results of this dissertation chapter provide insight into how the enzyme docks with the two substrates.

Hypothetical molecular models were made docking the ligand into the enzyme active site. Using the docking software, simulations were made and possible bond and interactions were predicted by the model. The crystal structure of a RubisCO-like protein from *Geobacillus kaustophilus* liganded with Mg$^{2+}$ and 2,3-diketohexane 1-phosphate (Imker et al., 2007) is available (pdb id 2OEM) (Figure
3.4). The hypothetical protein model of *R. rubrum* RubisCO (5RUB) was superimposed on the above mentioned 2OEM. Based on this molecular model superimposition, and the molecular docking models, candidate amino acids were selected (Figure 3.5). These residues presumably form interactions with the ligand and hence any mutations in the residues would restrain the enolase reaction with the disruption in interaction.

The proposed model is supported by the results reported in this chapter. Known RubisCO active site mutants (with respect to the carboxylase reaction) were tested for their role in the enolase reaction. At the same time, residues based on the models were tested for enolase and the ability to support *in vivo* carboxylation. The results indicate that there are amino acid residues which are involved in both enolase and carboxylase reactions. Some residues were found to be differentially involved in either of the reactions. The results suggest that there is an overlap in the binding sites for the substrates within the active site. It was seen that residues more towards the N terminal of the protein seems to be interacting with DKMTP (substrate for enolase reaction). In the model, mutant
S368Q was seen to be in close proximity of the substrates DKMTP and RuBP.

From previous studies (and confirmed in this study), we know that S368Q affects the carboxylation reaction. As predicted by the model, we observed that S368Q also has an effect on the enolase reaction. Other residues which affect the enolase and carboxylase reaction, suggest that the model is a good fit predicting the orientation of the substrates in the active site.

Whereas some of the RubisCO active site residues (e.g., lysine at 191) partially affect the enolase activity, residues such as asparagine 111 and serine 368 affected the enolase reaction, i.e., a mutation in these residues could not support the growth of *R. rubrum* strain IR on MTA. On the other hand, residues such Lys 166 or His 321 had no effect on the enolase activity. Of the site directed mutations made, His at 288, Ala at 305, His at 291, and Thr at 391 affected the enolase reactions, either partially or fully. Interestingly, some of these residues seem to be involved with the carboxylase reaction too. The alteration of His 291 to Glu also support CO₂ dependent growth, albeit partially. Whereas residues such as Ala-305, changed to Val and His 391 to Glu only affected the enolase reaction;
they had no effect on the carboxylase reaction.

In conclusion, these findings support the hypothetical models to a large extent. The results indicate that there are residue changes that differentially affect each activity or affect both activities. Results from both *in vivo* studies established the significance of residues required for each reaction and provided insight into underlying mechanisms that will need to be verified by *in vitro* studies.
Chapter Four

*Elucidating the Role of Two RubisCOs and Two RLPs in Rhodopseudomonas palustris*
Chapter 4

Introduction

*Rhodopseudomonas palustris* is a photosynthetic nonsulfur purple bacterium. Like other photosynthetic bacteria, *Rps. palustris* is metabolically versatile, exhibits and even grown under conditions where other photosynthetic bacteria cannot (Larimer et al., 2004). This organism is also versatile about the growth conditions. It is a facultative anaerobe capable of growing autotrophically and heterotrophically under anaerobic conditions using light as the energy source. It can also grow under aerobic heterotrophic conditions using oxygen as the terminal electron acceptor. Analysis of the genome of *Rps. palustris* indicates that it possesses form I and form II RubisCOs and two RLPs, one each of the IV-Photo (RLP2) and IV-Deep YkrW group (RLP1) (Larimer et al., 2004; Tabita et al., 2007). Little is known about the function of the two RLP’s in this organism. The goal of the work in this chapter was to elucidate the role of two RLPs and two RubisCOs
in this organism, with special reference to sulfur metabolism.

The structure of the RLP2 protein from *Rps. palustris* was elucidated (Tabita et al., 2007) and like other RLPs the overall secondary structure of RLP is similar to the secondary structure of RubisCO. Like RubisCO, RLP polypeptides like RubisCO, are composed of two domains, an N-terminal $\alpha+\beta$ domain and a C-terminal $(\beta/\alpha)_8$-barrel domain. The functional unit is a homo-dimer, with the active site, like RubisCO, at the interface of two subunits. There are two active sites per dimer (Imker et al., 2007; Li et al., 1999). The two important questions to be addressed in this study are:

1. Is either form I and form II RubisCO, or both involved in sulfur metabolism under anaerobic conditions [as form II in *R. rubrum* (Singh and Tabita, 2010)]?

2. How does each RLP participates in sulfur metabolism?

These questions are addressed in this chapter by examining growth phenotypes.
of different strains when using MTA as a sulfur source. The redundancy of the RLP and RubisCO genes in this organism make it a good model to study functional relationships and the contribution of different genes during different physiological conditions.

**Materials and methods**

**Bacterial strains and growth conditions**

The *Rps. palustris* strains used in the current study is strain CGA010 (wild type). Apart from the wild type, a series of knockout strains were used in this study. A table of list of all the gene knockout mutant strains is shown in (Table 4.1). PYE complex medium consisting of 0.3% peptone, 0.3% yeast extract, in Ormerod’s basal salts and 15 μg of biotin per liter, was used for chemoheterotrophic growth of *Rps. palustris* for conjugation experiments. Ormerod medium (OM) (Ormerod et al., 1961) containing malate as the carbon source, with 2-mg/ml of para-aminobenzoic acid was used for photoheterotrophic (PH) growth. MTA-dependent growth was achieved with sulfur depleted Ormerod’s medium,
prepared by replacing the sulfate salts with equimolar amounts of chloride salts. Antibiotics used for selection of *Rps. palustris* mutants and transconjugants were kanamycin (50 μg/ml, gentamycin (10 μg/ml), tetracycline (36 μg/ml) and streptomycin (50 μg/ml). *E. coli* strain DH5α was used for cloning all the plasmid constructs; *E. coli* strain top-10 was used as the donor strain in the conjugation experiments. Antibiotics used for plasmid selection in *E. coli* were, kanamycin at 20 μg/ml, gentamycin at 15 μg/ml and spectinomycin at 25 μg/ml. Antibiotics and media components were purchased from Sigma or Fisher. Table 4.1 lists all the strains used in the study.
<table>
<thead>
<tr>
<th>Table 4.1. List of strains used in the current chapter</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rps. palustris strains</strong></td>
</tr>
<tr>
<td><strong>CGA010</strong></td>
</tr>
<tr>
<td>WT</td>
</tr>
<tr>
<td>C. S. Harwood</td>
</tr>
<tr>
<td><strong>CGA2028</strong></td>
</tr>
<tr>
<td>ΔcbbLS</td>
</tr>
<tr>
<td>(Romagnoli and Tabita, 2006)</td>
</tr>
<tr>
<td><strong>CGA2040</strong></td>
</tr>
<tr>
<td>ΔcbbLS/ΔcbbM</td>
</tr>
<tr>
<td>(Romagnoli and Tabita, 2006)</td>
</tr>
<tr>
<td><strong>CGA2046</strong></td>
</tr>
<tr>
<td>ΔRLP2</td>
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<tr>
<td>S. Romagnoli</td>
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<tr>
<td><strong>CGA2053</strong></td>
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<tr>
<td>ΔcbbLS/ΔcbbM/ΔRLP2</td>
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<td>S. Romagnoli</td>
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<tr>
<td>ΔcbbM</td>
</tr>
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<td>S. Romagnoli</td>
</tr>
<tr>
<td><strong>CGA2087</strong></td>
</tr>
<tr>
<td>ΔRLP1/ΔRLP2</td>
</tr>
<tr>
<td>S. Romagnoli</td>
</tr>
<tr>
<td><strong>CGA2096</strong></td>
</tr>
<tr>
<td>ΔRLP1</td>
</tr>
<tr>
<td>S. Romagnoli</td>
</tr>
<tr>
<td><strong>CGA2099</strong></td>
</tr>
<tr>
<td>ΔcbbLS/ΔcbbM/ΔRLP2/ΔRLP1</td>
</tr>
<tr>
<td>This study</td>
</tr>
<tr>
<td><strong>E. Coli strains</strong></td>
</tr>
<tr>
<td><strong>Stellar™ Cells</strong></td>
</tr>
<tr>
<td>Common cloning strain</td>
</tr>
<tr>
<td>Clontech</td>
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<tr>
<td><strong>Top10 cells</strong></td>
</tr>
<tr>
<td>Common cloning cells</td>
</tr>
<tr>
<td>Invitrogen</td>
</tr>
<tr>
<td><strong>DH5a (pRK2013)</strong></td>
</tr>
<tr>
<td>Helper cells for mating</td>
</tr>
<tr>
<td>DH5a containing helper plasmid pRK2013</td>
</tr>
</tbody>
</table>
MTA (5’-Methylthioadenosine) dependent growth of Rps. palustris

Single colonies were used to inoculate culture tubes containing Ormerod’s liquid media under aerobic conditions at 30°C with shaking at 200 RPM until the mid exponential phase of growth (OD$_{660}$~0.6-0.8). Cells were pelleted by centrifuging at 12,000 X g for 3 min; cell pellets were washed three times with sulfur free medium and then resuspended in the same medium. Washed cells were inoculated in sulfur-free medium supplemented with MTA as a sole sulfur source. As a negative control in all experiments, cells were also inoculated in sulfur-depleted medium lacking any sulfur source. Anaerobic phototrophic MTA-dependent growth was done by performing the same procedure described above using cells grown chemoheterotrophically and then prepared inside an anaerobic chamber (Coy labs, Grass Lake, Michigan), maintained at an atmosphere of 2.5 - 3% hydrogen and balance nitrogen. Anaerobic media was prepared under a 100% nitrogen atmosphere and dispensed (10 ml per tube) in 25 ml tubes fitted with butyl rubber stoppers with an aluminum seal crimped over the stopper (Bellco Glass Inc. Vineland, NJ, USA).
Anaerobic cultures were grown in the light at 27° C in a growth chamber (Environment Growth Chambers, Chagrin Falls, OH, USA). In the experiments testing for MTA-dependent growth, all the strains were grown in medium without any sulfur source as a negative control and medium containing ammonium sulfate (sole sulfur source) as positive control. The concentrations of MTA and ammonium sulfate used in the media were 1 mM. Kanamycin and gentamycin antibiotics, which are commercially available as sulfate salts were not used for MTA dependent growth as they can be a potential source of sulfate in the medium. All the growth experiments were performed using three independent isolates and results are representation of at least two independent experiments.

*Construction of a quadruple knockout strain in Rps. palustris*

\((\Delta cbbLS/\Delta cbbM/\Delta RLP1/\Delta RLP2)\)

*Rps. palustris* mutant strains were made by allelic exchange by double recombination mediated by a suicide vector containing the conditionally lethal
sacB gene (Gay et al., 1983). The triple knockout strain was obtained from Simona Romagnoli (Lab of F. R. Tabita). This strain has the cbbLS gene knocked out with a gentamycin cassette and cbbM knocked out with a kanamycin cassette.

Convenient restriction sites were present in the gene of interest (cbbM/cbbLS), and mutant strains were constructed as in-frame deletions of the target gene.

Primers with appropriate restriction sites (rlp1pal1F: GGATCCT CGCGATA TTGGGTG GACATG; rlp1pal1R: GGATCC CGGCGC TGCTGC GTTGTC) were used for PCR amplification of the regions flanking the gene of interest (RLP1) from the chromosome of the wild type Rps. palustris. The PCR-amplified RLP region was cloned into the pCR-TOPOBLUNT vector resulting in the plasmid TrpsRLP1. The gene was disrupted by deleting a 100 bp (∆Stu) region. This resulted in the plasmid TrpsRLP1∆Stu. An aadA gene (impacting spectinomycin resistance) from the plasmid pHP45Ω was amplified using primers (sacbtopoF: ACATGATTAC GAATTCTGTA AAACGAC GGCCAG sacbtopoR: TACCGAGC TCGAATTCAG GAAACAGCT ATGACC). This spectinomycin cassette was cloned at StuI site in the disrupted RLP1 gene in the
plasmid TrpsRLP1ΔStu using the In-Fusion® HD Cloning Kits (Clontech).

This plasmid was then transformed into Stellar supercomptetent cells (Genotype- 
F–, endA1, supE44, thi-1, recA1, relA1, gyrA96, phoA, Φ80d lacZΔ M15, Δ (lacZYA - 
argF) U169, Δ (mrr - hsdRMS - mcrBC), ΔmcrA, λ). This strain was named as

St_flk18RLP1spec. Matings were set up by combining recipient cell St-

flk18RLP1spec, using the helper strain transferred by filter mating into Rps. 
palustris mating. E. coli DH5α contains the helper plasmid pRK2013, which

provides tra and mob genes required to transfer the DNA cloned in pUTmini-Tn5 
plasmids into the recipient strain, from donor strains, lacking these genes.

Overnight cultures of E. coli grown in LB medium with appropriate antibiotics

were diluted 1:10 in LB (without antibiotic) and incubated at 37° C with shaking

at 220 RPM for 2 hrs. Donor cells and helper cells in the ration of 2:1:1 were

placed in an Eppendorf tube and the cells were centrifuged for 2 min at 13,600 X

g in a micro-centrifuge. This mating mixture pellet was resuspended in 20 μl of

PYE medium and the resuspension was spotted onto a PYE medium plate.
Exconjugants harboring a chromosomal insertion of the knockout plasmid were
selected for spectinomycin resistance and sucrose sensitivity to confirm the
occurrence of the first recombination event.

Positive colonies were grown for two additional days in 1 ml of nonselective
minimal liquid medium (OM media) to favor the second recombination event.
Afterwards, dilution of $10^3$ and $10^2$ of culture was plated on 10% sucrose-
succinate-PM medium plates. The plates were grown under PH conditions in gas
pack jars. Sucrose-resistant colonies were patched on antibiotic (spectinomycin)
plates to eliminate false positives. The resulting colonies were screened for the
presence of the desired mutations by colony PCR amplification. The quadruple
gene deletion strain was named as strain 2099.
<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>RLP2F</td>
<td>GATCCGCTTCAGATTGTTCC</td>
</tr>
<tr>
<td>RLP2R</td>
<td>CTGCAAGCCCCAAAAAGTCTCT</td>
</tr>
<tr>
<td>Cbbmpalf</td>
<td>TTCGAATTTCCGGAGTGTC</td>
</tr>
<tr>
<td>Cbbmpalr</td>
<td>TTCTCGGACATTGGGCTC</td>
</tr>
<tr>
<td>CbbIspalf</td>
<td>CCGCTGACGTAGTTGGATTC</td>
</tr>
<tr>
<td>CbbIspalr</td>
<td>GAAGATGACCGACGACGCAAT</td>
</tr>
<tr>
<td>rlp1palseq F</td>
<td>TCTAGAGAGGCATGCTGCGCAATA</td>
</tr>
<tr>
<td>rlp1pal seq R</td>
<td>TCTAGAGCTTGCGGTGACTTTTTCTTGGA</td>
</tr>
<tr>
<td>rlp1pal 1 R</td>
<td>GGATCCTCGCGATATTGGGGGTGACATG</td>
</tr>
<tr>
<td>rlp1pal 1 R</td>
<td>GGATCCCGCGCGCTGCGTTGTC</td>
</tr>
<tr>
<td>sacbtopoF</td>
<td>ACATGATTACGAATTCTGAAAAACGACGCGCA</td>
</tr>
<tr>
<td>sacbtopoR</td>
<td>TACCGAGCGTGAATTCCAGGAAACGATGTACG</td>
</tr>
<tr>
<td>rlp1knocko utf</td>
<td>ATGGTTCGAAAGAAACACCG</td>
</tr>
<tr>
<td>rlp1knockoutr</td>
<td>TTACATCACTCAGCGCAACGAC</td>
</tr>
<tr>
<td>Spec_rlp1infusionF</td>
<td>GCGCGATGCGTGGCCTCAGGCTCAAGTACGAGATGTGCTTGGCGA</td>
</tr>
<tr>
<td>Spec_rlp1infusionR</td>
<td>CGCGGACGCGCCAGGCTCAAGTACGAGATGTGCTTGGCGA</td>
</tr>
</tbody>
</table>
Tables 4.2 and 4.3 lists all the plasmids and primers used in this study respectively.

Table 4.3. List of plasmids used in the current chapter

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Relevant Characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCR2.1-TOPO</td>
<td>Common cloning strain</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pH45Ω</td>
<td>aadA gene containing vector</td>
<td></td>
</tr>
<tr>
<td>TOPO RLPL1</td>
<td>RLP1 gene from <em>Rps. palustris</em> cloned into TOPO</td>
<td>This study</td>
</tr>
<tr>
<td>TOPO RLPL2</td>
<td>RLP2 gene from <em>Rps. palustris</em> cloned into TOPO</td>
<td>This study</td>
</tr>
<tr>
<td>TOPO RLPLΔstu</td>
<td>100bp deleted TOPO RLPL</td>
<td>This study</td>
</tr>
<tr>
<td>pK18mobsacB</td>
<td>cloning vector with sacB gene, Kan and Neo resistance, derived from the <em>E. coli</em> plasmids pK18 and pK19</td>
<td>This study</td>
</tr>
<tr>
<td>TRLP1Δstu-spec</td>
<td><em>aadA</em> gene cloned stu site in TOPO RLPLΔstu</td>
<td>This study</td>
</tr>
<tr>
<td>PsacB-RLP1-spec</td>
<td>RLP1 gene from TRLP1Δstu-spec cloned into pK18mobsacB</td>
<td>This study</td>
</tr>
</tbody>
</table>

Results

The strains of *Rps. palustris* were grown under chemoheterotrophic and photoheterotrophic conditions with MTA as a sole sulfur source. Experiments were designed to study which genes (*cbbLS/cbbM/RLP1/RLP2*) were particularly
involved in sulfur metabolism for specific growth conditions. The aim was to study the involvement of each gene in the metabolism of MTA. This was investigated by testing *Rps. palustris* mutant strains that contain disruptions of various genes encoding for these proteins (e.g., ∆RLP1, ∆RLP2, ∆formI, ∆formII (Romagnoli and Tabita, unpublished results) and their ability to metabolize MTA.

Our results indicated that any of the four genes from the RubisCO family are capable of rescuing the growth on medium with MTA as the sole sulfur source, both under aerobic and anaerobic conditions.

**Strain CGA2028 (∆cbbLS)**

This is a *cbbLS* deletion strain (Romagnoli and Tabita, 2006). The strain has an 865 base pair segment of sequence removed from *cbbL* and *cbbS*. When grown under PH condition, this strain was capable of growth with MTA as a sole sulfur source and malate as a carbon source. The fit coefficients of the sigmoidal growth curve were the same for growth of MTA and on sulfate (Figure 4.1). Since the strain
still has the \textit{cbbM} gene intact, the growth under PA conditions was also possible.

Under aerobic CH conditions, using OM media with malate as a carbon source and MTA as a sulfur source, the lack of \textit{cbbLS} genes did not have an appreciable effect on the ability of the \textit{Rps. palustris} strain to grow on MTA as a sole sulfur source. Compared to sulfate, the MTA media yielded the same growth rate constant suggesting the efficacy of metabolic enzymes are similar under both conditions (Figure 4.2). Interestingly, growth on MTA led to a slightly earlier entrance into the stationary phase, thereby raising the possibility of a subtle effect of the mutation. Regardless, these results indicate that either form I is minimally involved in sulfur metabolism under aerobic conditions, or that the absence of form I RubisCO is fulfilled by the other three genes, consistent with the concept of redundancy that is an ubiquitous feature of complex systems.
FIGURE 4.1: Photoheterotrophic (anaerobic) growth of \textit{Rps. palustris} strain CGA2028

Microbial population data [mean ± SE] are plotted (ordinate) in normalized \textit{(top panel)} & absolute \textit{(right)} values vs. time (abscissa) for anaerobic photoheterotrophic growth of \textit{Rps. palustris} strain CGA2028 ($\Delta$cbbLS) in media containing either: 1. Sulfate (squares); 2. MTA (circles); 3. No sulfur (diamonds). Growth curves (lines) with 95% confidence limits were determined by fitting these data (N=3/group) to a logistic sigmoidal function and compared by fit coefficients, i.e., population at stationary phase (MAX), time to reach 50% MAX (ET50), time constant (TAU). Data beyond stationary phase were excluded from the fit. Groups 1 and 2 demonstrated significant growth and had similar fit coefficients. Group 3 failed to demonstrate significant growth.

Maximum OD of WT (squares) = 2.29
FIGURE 4.2: Chemoheterotrophic (aerobic) growth of Rps. palustris strain CGA2028

Microbial population data [mean ± SE] are plotted (ordinate) in normalized (top) & absolute (right) values vs. time (abscissa) for aerobic chemoheterotrophic growth of Rps. palustris strain CGA2028 (∆cbbLS) in media containing either: 1. Sulfate (squares); 2. MTA (circles); 3. No sulfur (diamonds). Growth curves (lines) with 95% confidence limits were determined by fitting these data (N=3/group) to a logistic sigmoidal function and compared by fit coefficients. Data beyond stationary phase were excluded from the fit. Groups 1 and 2 demonstrated significant growth. Group 1 had significantly higher values for MAX and ET50 (p<0.005) than group 2, but their TAU values were the same. Group 3 failed to demonstrate significant growth.

Maximum OD of WT (squares) = 0.66

Fit Coefficients ± SD

- MAX₁ (%) = 100 ± 3
- ET50₁ (hrs) = 116 ± 3
- TAU₁ (hrs) = 26 ± 3
- MAX₂ = 79 ± 2
- ET50₂ = 93 ± 3
- TAU₂ = 22 ± 3
CGA2040 ($\Delta cbbLS/\Delta cbbM$)

This strain was made by inserting a kanamycin cassette into the $cbbM$ gene of
*Rps. palustris* strain CGA010 (Romagnoli and Tabita, 2006), and a gentamycin
cassette in the $cbbL$-$cbbS$ genes.

When CGA2040 was grown under PH conditions, as expected, deletion of both
structural RubisCO genes was lethal under both photoheterotrophic and
photolithoautotrophic conditions. Thus, it cannot be determined whether either
of the two RubisCO proteins was involved in sulfur metabolism under these
growth conditions (figure 4.3).

However, under aerobic conditions, with MTA as sulfur source in OM malate
media, the strain was able to grow, albeit at a reduced extent compared to
growth on sulfate. We can infer that either the RLP1 or RLP2 or both might be
involved in sulfur metabolism as the cells were capable of using a toxic substrate
like MTA as a sulfur source (Figure 4.4).
Microbial population data [mean ± SE] are plotted (ordinate) in absolute values vs. time (abscissa) for anaerobic photoheterotrophic growth of *Rps. palustris* strain CGA2040 (ΔcbbLS/ΔcbbM) in media containing either (N=3/group): 1. Sulfate (squares); 2. MTA (circles); 3. No sulfur (diamonds). All groups failed to demonstrate any significant growth.

*maximum OD of WT (squares) = 0.023*
Microbial population data [mean ± SE] are plotted (ordinate) in normalized (top panel) & absolute (right) values vs. time (abscissa) for aerobic chemoheterotrophic growth of *Rps. palustris* strain CGA2040 (Δ*cbbLS*/Δ*cbbM*) in media containing either: 1. Sulfate (squares); 2. MTA (circles); 3. No sulfur (diamonds). Growth curves (lines) with 95% confidence limits were determined by fitting these data (N=3/group) to a logistic sigmoidal function and compared by fit coefficients. Data beyond stationary phase were excluded from the fit. Groups 1 and 2 had similar fit coefficient except for ET50 which was significantly higher for group 2 (p=0.0002). Group 3 failed to demonstrate significant growth.

*maximum OD of WT(squares) = 0.712*
CGA2046 (ΔRLP2)

The strain CGA2046 has the RLP2 gene knocked out from the chromosome via direct double recombination mediated by a suicide vector containing the conditionally lethal sacB gene (Simona Romagnoli). Compared to growth on sulfate, this strain was capable of growing with the same growth rate constant (measure of an intrinsic property) under anaerobic PH conditions with MTA as a sole sulfur source (Figure 4.5). The growth on MTA led to stationary phase a little sooner, which was also reflected by the slightly smaller ET50.

When grown under aerobic CH conditions (i.e., on MTA), there was an obvious lag in the growth curve (Figure 4.6), probably reflecting the time required to synthesize proteins. Even though the organism grew markedly less well on MTA compared to sulfate, the growth rate constant was the same as that on sulfate, thereby suggesting that there was little effect on the efficacy of metabolic enzymes (even if they were saturated and probably fewer in number). More importantly, the fact that the ET50 was the same despite a markedly lower MAX
on MTA indicates that the organisms were dying at a faster rate (because otherwise, the ET50 would have decreased with a decrease in the MAX). By about 200 hours, the growth rate apparently reached a steady state with the death rate (i.e., as bacteria that grew earlier were dying, they were represented in the growth curve by those that grew later after synthesizing the necessary proteins). A separate study aimed at quantifying the death rate may yield additional insight.

In summary, the lag in the growth response probably represents the adaptive process (e.g., upregulation of other RubisCO family genes) to support growth on MTA and a faster death rate. The efficacy of the metabolic enzyme is probably not affected by this mutation. It is also possible that either of the RubisCO family genes complements growth in absence of RLP2.
FIGURE 4.5: Photoheterotrophic (anaerobic) growth of *Rps. palustris* strain CGA2046

Microbial population data [mean ± SE] are plotted (ordinate) in normalized (top panel) & absolute (right) values vs. time (abscissa) for anaerobic photoheterotrophic growth of *Rps. palustris* strain CGA2046 (∆RLP2) in media containing either:

1. **Sulfate** (squares);
2. **MTA** (circles);
3. **No sulfur** (diamonds).

Growth curves (lines) with 95% confidence limits were determined by fitting these data (N=3/group) to a logistic sigmoidal function and compared by fit coefficients. Data beyond stationary phase were excluded from the fit. Group 1 had a significantly different MAX (p=0.006) and ET50 (0.0034) than group 2, but similar TAU. Group 3 failed to demonstrate significant growth.

**Fit Coefficients ± SD**

- MAX₁ (%) = 95 ± 8
- ET50₁ (hrs) = 179 ± 6
- TAU₁ (hrs) = 38 ± 4
- MAX₂ = 68 ± 4
- ET50₂ = 155 ± 3
- TAU₂ = 33 ± 4

*maximum OD of WT(squares) = 1.98*
Microbial population data [mean ± SE] are plotted (ordinate) in normalized (top panel) & absolute (right) values vs. time (abscissa) for aerobic chemoheterotrophic growth of *Rps. palustris* strain CGA2046 (∆RLP2) in media containing either: 1. Sulfate (squares); 2. MTA (circles); 3. No sulfur (diamonds). Growth curves (lines) with 95% confidence limits were determined by fitting these data (N=3/group) to a logistic sigmoidal function and compared by fit coefficients. Group 1 had a significantly higher MAX (p<0.0001) than group 2 but similar ET50 and TAU values. Group 3 failed to demonstrate significant growth.

maximum OD of WT (squares) = 0.711
Strain CGA 2053 ($\Delta$RLP2, $\Delta$cbbLS, $\Delta$cbbM)

The only functional gene from the RubisCO family in strain CGA 2053 this mutant was that which encodes RLP1. This mutant, like the other mutants, was grown under PH (anaerobic) and CH (aerobic) conditions with MTA as a sole sulfur source (figures 4.7 and 4.8). The same strain was grown with ammonium sulfate as a positive control.

The results indicate that RLP1 could be actively involved in the MSP under aerobic growth conditions. This strain lacks three out of the four genes of the RubisCO family, the anticipated proteins involved in the MSP. However the presence of the RLP1 gene had little effect on the growth of the organism under anaerobic PH conditions with MTA as a sole sulfur source.
Microbial population data [mean ± SE] are plotted (ordinate) in normalized (top panel) & absolute (right) values vs. time (abscissa) for anaerobic photoheterotrophic growth of *Rps. palustris* strain CGA2053 (Δ*cbbLS/ΔcbbM/ΔRLP2) in media containing either: 1. Sulfate (squares); 2. MTA (circles); 3. No sulfur (diamonds). Growth curves (lines) with 95% confidence limits were determined by fitting these data (N=3/group) to a logistic sigmoidal function and compared by fit coefficients, i.e., population at stationary phase (MAX), time to reach 50% MAX (ET50), time constant (TAU). Data beyond stationary phase were excluded from the fit. All groups failed to demonstrate significant growth.

maximum OD of WT(squares) = 0.10
FIGURE 4.8: Aerobic growth of *Rps. palustris* strain CGA2053

Microbial population data [mean ± SE] are plotted (ordinate) in normalized (top panel) & absolute (right) values vs. time (abscissa) for aerobic chemoheterotrophic growth of *Rps. palustris* strain CGA2053 (*ΔcbbLS/ΔcbbM/ΔRLP2*) in media containing either: 1. Sulfate (squares); 2. MTA (circles); 3. No sulfur (diamonds). Growth curves (lines) with 95% confidence limits were determined by fitting these data (N=3/group) to a logistic sigmoidal function and compared by fit coefficients. Data beyond stationary phase were excluded from the fit. As compared to group 2, group 1 had significantly higher MAX (p=0.001), but their ET50 and TAU values were similar. Group 3 failed to demonstrate significant growth.

**Fit Coefficients ± SD**
- **MAX** (squares): 100 ± 3
- **ET50** (squares): 126 ± 3
- **TAU** (squares): 25 ± 3
- MAX (circles): 75 ± 9
- ET50 (circles): 136 ± 8
- TAU (circles): 21 ± 3

*maximum OD of WT (squares) = 0.760*
CGA2071 (ΔcbbM)

The strain was made by disrupting the cbbM gene by inserting a kanamycin cassette. It was obvious from previous studies that the absence of one set of RubisCO genes (cbbLS, strain CGA2028; or cbbM, strain CGA2071) allowed normal growth under the conditions tested (Romagnoli and Tabita, 2006).

The strain could grow under the PH (anaerobic) and CH (aerobic) conditions with MTA as a sole source of sulfur, thereby illustrating that the absence of this gene can be overcome by the presence of other genes of the RubisCO family (Figures 4.9 and 4.10).
FIGURE 4.9: Photoheterotrophic (anaerobic) growth of *Rps. palustris* strain CGA2071

Microbial population data [mean ± SE] are plotted (ordinate) in normalized (top) & absolute (right) values vs. time (abscissa) for anaerobic photoheterotrophic growth of *Rps. palustris* strain CGA2071 (∆cbbM) in media containing either:

1. *Sulfate* (squares);
2. *MTA* (circles);
3. *No sulfur* (diamonds).

Growth curves (lines) with 95% confidence limits were determined by fitting these data (N=3/group) to a logistic sigmoidal function and compared by fit coefficients. Data beyond stationary phase were excluded from the fit. Group 2 grew 60% as much as group 1. Compared to group 2, group 1 had significantly higher MAX (p=0.0008), but similar TAU and ET50. Group 3 failed to demonstrate significant growth.

Maximum OD of WT (squares) = 2.2
FIGURE 4.10: Aerobic growth of *Rps. palustris* strain CGA2071

Microbial population data [mean ± SE] are plotted (ordinate) in normalized (top panel) & absolute (right) values vs. time (abscissa) for aerobic chemoheterotrophic growth of *Rps. palustris* strain CGA2071 (Δ*cbbM*) in media containing either:

1. Sulfate (squares); 2. MTA (circles); 3. No sulfur (diamonds).

Growth curves (lines) with 95% confidence limits were determined by fitting these data (N=3/group) to a logistic sigmoidal function and compared by fit coefficients. Data beyond stationary phase were excluded from the fit.

Compared to group 2, group 1 had significantly higher MAX (p=0.003) and ET50 (p=0.01), but they had the same TAU. Group 3 failed to demonstrate significant growth.

**Microbial Population (% normalized to max OD_{660}**

**Fit Coefficients ± SD**

- MAX₁ (%): 100 ± 3
- ET50₁ (hrs): 133 ± 3
- TAU₁ (hrs): 26 ± 3
- MAX₂ = 73 ± 6
- ET50₂ = 118 ± 5
- TAU₂ = 20 ± 3

maximum OD of WT (squares) = 0.753
CGA2087 (∆RLP2/∆RLP1)

This mutant had both the RLP genes deleted via double recombination based on the lethal \textit{sacB} gene. As expected, with a full complement of RubisCO genes, this strain showed no effect on anaerobic MTA dependent growth (Figure 4.11)

Drawing analogies from the RLP-RubisCO system in \textit{R. rubrum}, it was expected that the RLP genes would be involved in the MTA metabolism under aerobic conditions. However, it was seen that the strain could grow with a long lag but that growth was noticeable (50\% of that of the positive control i.e growth on sulfate), yet highly compromised (Figure 4.12).

However, it could not be confirmed if the RLP genes were responsible for MTA metabolism under aerobic conditions in \textit{Rps. palustris}. There is the possibility that either \textit{cbbM} or \textit{cbbLS} is expressed at lower levels under aerobic conditions in this organism, which in the absence of the RLP genes, could support the function of sulfur salvage in this mutant. Indeed, there is precedent for aerobic expression of RubisCO genes in another strain of \textit{Rps. palustris} (Yoshida et al., 2006).
Microbial population data [mean ± SE] are plotted (ordinate) in normalized (top panel) & absolute (right) values vs. time (abscissa) for anaerobic photoheterotrophic growth of \textit{Rps. palustris} strain CGA2087 (\(\Delta RLp1/\Delta RLp2\)) in media containing either: 1. Sulfate (squares); 2. MTA (circles); 3. No sulfur (diamonds). Growth curves (lines) with 95% confidence limits were determined by fitting these data (N=3/group) to a logistic sigmoidal function and compared by fit coefficients. Data beyond stationary phase were excluded from the fit. Group 1 had significantly higher MAX and ET50 (p< 0.05) than group 2 but similar TAU. Group 3 failed to demonstrate significant growth.

Maximum OD of WT (squares) = 1.763
Microbial population data [mean ± SE] are plotted (ordinate) in normalized (top panel) & absolute (right) values vs. time (abscissa) for aerobic chemoheterotrophic growth of *Rps. palustris* strain CGA2087 (ΔRLP1/ΔRLP2) in media containing either: 1. Sulfate (squares); 2. MTA (circles); 3. No sulfur (diamonds). Growth curves (lines) with 95% confidence limits were determined by fitting these data (N=3/group) to a logistic sigmoidal function and compared by fit coefficients. Data beyond stationary phase were excluded from the fit. Group 1 had a significantly higher MAX (p=0.0002) than group 2 but similar TAU. The ET50 values were significantly higher in group 2 (p=0.0004) than in group 1. Group 3 failed to demonstrate significant growth.
**CGA2096 (ΔRLP1)**

Similar to strain CGA2046, one of the RLP genes (RLP1 in this instance) was knocked out from the WT *Rps. palustris* strain (CGA010) by a double recombination based on the lethal *SacB* gene. This strain was grown as explained in Materials and Methods, with OM media and MTA as sole sulfur source under aerobic and anaerobic conditions.

As expected, the anaerobic (PH) growth was not affected due to the lack of the RLP2 gene in the strain (*Figure 4.13*). However under aerobic conditions, the growth was somewhat affected as there was a lag before the growth ensued compared to the CGA2096 grown with ammonium sulfate as a sulfur source (*Figure 4.14*). Because the other genes of the RubisCO family, RLP2, *cbbM* and *cbbLS* were still intact on the chromosome of the organism, any of these genes could have complemented the growth of the organism with MTA, particularly under anaerobic growth conditions.
Microbial population data [mean ± SE] are plotted (ordinate) in normalized (top panel) & absolute (right) values vs. time (abscissa) for anaerobic photoheterotrophic growth of *Rps. palustris* strain CGA2087 (ΔRLP2) in media containing either:

1. **Sulfate** (squares); 2. **MTA** (circles); 3. **No sulfur** (diamonds). Growth curves (lines) with 95% confidence limits were determined by fitting these data (N=3/group) to a logistic sigmoidal function and compared by fit coefficients, i.e., population at stationary phase (MAX), time to reach 50% MAX (ET50), time constant (TAU). Data beyond stationary phase were excluded from the fit. Group 1 had a significantly higher MAX and ET50 (p< 0.05) than group 2 but similar TAU. Group 3 failed to demonstrate significant growth.
FIGURE 4.14: Aerobic growth of *Rps. palustris* strain CGA2096

Microbial population data [mean ± SE] are plotted (ordinate) in normalized *(top panel)* & absolute *(right)* values vs. time *(abscissa)* for aerobic chemoheterotrophic growth of *Rps. palustris* strain CGA2096 (∆RLP1) in media containing either

1. **Sulfate** (squares); 2. **MTA** (circles); 3. **No sulfur** (diamonds). Growth curves (lines) with 95% confidence limits were determined by fitting these data (N=3/group) to a logistic sigmoidal function and compared by fit coefficients. Data beyond stationary phase were excluded from the fit. Group 1 had a significantly higher MAX (p=0.0001) than group 2 but similar TAU values. Group 2 had significantly higher ET50 (p=0.009) than group 1. Group 3 failed to demonstrate significant growth.

*maximum OD of WT(squares) = 0.78*
CGA 2099 (ΔRLP1/ΔRLP2/ΔcbbLS/ΔcbbM)

This mutant was derived from CGA2053; and a knockout was made in the RLP1 gene described in the Materials and Methods. In the earlier sections of this chapter, the results indicated that under anaerobic conditions, either form I or form II of RubisCO was required for PH growth in the presence of MTA and also that either form could concurrently catalyze sulfur and carbon metabolism with similar efficacies. These results suggested that both forms either share a similar mechanism or have distinct mechanisms that encounter similar energetic barriers (i.e., similar activation energies).

By contrast to anaerobic conditions, the mechanism and regulation is completely unknown under aerobic conditions. Little is known about the contribution of RLP-1 and RLP-2 in *Rps. palustris* under aerobic conditions. When the quadruple knockout (ΔRLP1/ΔRLP2/ΔcbbLS/ΔcbbM) was introduced to aerobic conditions, in the presence of MTA, there was a growth lag and somewhat reduced growth (Figure 4.15).
FIGURE 4.15: Aerobic growth of *Rps. palustris* strain CGA2099

Microbial population data [mean ± SE] are plotted (ordinate) in normalized (top panel) & absolute (right) values vs. time (abscissa) for aerobic chemoheterotrophic growth of *Rps. palustris* strain CGA2099 (∆RLP1/∆RLP2/∆cbbLS/∆cbbM) in media containing either: 1. **Sulfate** (squares); 2. **MTA** (circles); 3. **No sulfur** (diamonds). Growth curves (lines) with 95% confidence limits were determined by fitting these data (N=3/group) to a logistic sigmoidal function and compared by fit coefficients. Data beyond stationary phase were excluded from the fit. Group 1 had significantly higher MAX and TAU (p<0.005) than group 2. The ET502 was significantly higher for group 2 (p=0.006) than group 1. Group 3 failed to demonstrate significant growth.

Maximum OD of WT (squares) = 0.55
These results raised the possibility that under aerobic conditions, additional genes are involved in sulfur metabolism. Consistent with this hypothesis, the observed lag may represent the time it takes for some genes to turn on to enable growth on MTA.

In summary, these results highlight the concept of redundancy, which is a well described feature inherent to any complex system and would be an interesting model for further study in *Rps. palustris*. Some of the potential challenges in exploring the lack of expected phenotype with this quadruple mutant include:

1. The fact that this mutant strain was derived from strain 2053, which had the RLP2 deleted by a *SacB* deletion scheme with about 90 base pairs were deleted in the gene (S. Romagnoli). It is quite possible that this deletion was not sufficient to completely inactivate the gene and a functional protein was produced.

2. An unknown alternate enolase-phosphatase gene (similar to *K. oxytoca*) might play a role under aerobic conditions.
3. Because of the genetic complexity of *Rps. palustris*, a "failsafe"
mechanism in response to stress or certain environmental triggers
could turn on expression of additional genes in order to increase the
organism's chances for survival under the new condition. Such
phenomena are not uncommon in nature and are a fundamental
attribute inherent to most biological systems.

**Discussion**

By virtue of several combinations of knockout strains made in *Rps. palustris*, the
role of RLP’s and RubisCO might be deciphered. Our experimental data
indicates that this organism was not absolutely depended on either RLPs as a
DKMTP enolase under aerobic or anaerobic growth conditions. In certain strains,
where the RLPs were knocked out from the chromosome, the growth of the
organism under aerobic CH conditions seemed to be somewhat compromised, as
there was a lag in growth and growth was much reduced compared to the
positive controls. Unlike *R. rubrum*, *Rps. palustris* is incapable of PH growth
without RubisCO, and thus, it was difficult to ascertain which protein might be acting as a DKMTP enolase under anaerobic conditions.

Under anaerobic conditions, RubisCO not only helps the organism use the CBB cycle as an electron sink, but also supports the organism to grow in MTA conditions. This would mean that the enzyme has a dual function in *Rps. palustris* as well (as in *R. rubrum*). Hence by these results we assume that RubisCO might serve DKMTP enolase or catalyze another alternate reaction in the anaerobic sulfur salvage pathway in this organism.

Under aerobic conditions, it was observed that the triple knockout CGA2053 (ΔcbbLS/ΔcbbM/ΔRLP2) was capable of growth, but a double RLP knockout CGA2087 (ΔRLP1/ΔRLP2) could only slightly grow and CGA2096 (ΔRLP1) had compromised growth. These results provide an insight into the regulation of genes for sulfur salvage in this organism. It seems that RLP1 is the primary protein for the aerobic MSP. Nonetheless, the organism seems to have alternate functional homologous proteins, which in the absence of either RLP acts as an
enolase for sulfur metabolism. Though under aerobic conditions, RubisCO is not expressed, in R. rubrum, there is precedent for aerobic expression of the RubisCO genes in some strains of Rps palustris (Yoshida et al., 2006). Thus perhaps low levels of RubisCO are expressed in Rps. palustris under aerobic growth conditions and at least partially compensates for the absence of the two RLPs.

*Rps. palustris* has a diverse genetic profile. To elucidate the role of the different RubisCO family genes makes it an interesting model for research. To demonstrate the relative growth of the *Rps. palustris* mutants under either aerobic or anaerobic conditions, Figures 4.16 (a and b) provide a three dimensional representation of the MTA-dependent growth curves.

In Figure 4.16, the abscissa plots magnitude of photoheterotrophic anaerobic growth, the ordinate plots magnitude of chemoheterotrophic aerobic growth and the applicate/Z axis plots time in hours.
Igor Pro was used to create the 3D plot showing the relative PA and PH growth of *Rps. palustris* strains with MTA as a sole sulfure source. The abscissa plots magnitude of photoheterotrophic anaerobic growth, the ordinate plots magnitude of chemoheterotrophic aerobic growth and the applicate/Z axis plots time in hours. The first axis-orientation of the 3-D plot demonstrates how gene knockouts affect MTA-dependent growth under aerobic and anaerobic conditions.
The first axis-orientation of the 3-D plot (Figure 4.16-A) demonstrates how gene knockouts affect MTA-dependent growth under aerobic and anaerobic conditions. The dynamics of organism growth is demonstrated in the plots.

There is two-stage cell growth which accounts for the cell behavior. The long lag in certain mutants implicates an adjustment to the absence of metabolic genes products and up-regulation of alternate homologous stress genes.

The 3D model shows that CGA2028 ($\Delta cbbLS$) and CGA2071 ($\Delta cbbM$) are more inclined towards anaerobic growth conditions, though they are capable of growth under both conditions. In these mutants, one of the RubisCOs is present. However, under aerobic conditions, the growth of mutant CGA 2046 ($\Delta RLP1/\Delta RLP2$) is highly reduced with a long lag phase. This could mean that the organism requires RLP as a metabolic gene under aerobic conditions.
FIGURE 4.16-B: 3D plot of overall growth dynamics of *Rps. palustris* mutant strains with MTA as a sole sulfur source (view 2)

Igor Pro was used to create the 3D plot showing the relative PA and PH growth of *Rps. palustris* strains with MTA as a sole sulfur source. The abscissa plots magnitude of photoheterotrophic anaerobic growth, the ordinate plots magnitude of chemoheterotrophic aerobic growth and the applicate/Z axis plots time in hours. The first axis-orientation of the 3-D plot demonstrates how gene knockouts affect MTA-dependent growth under aerobic and anaerobic conditions.
Interestingly, strain CGA2053, which fails to grow under anaerobic conditions, can grow under aerobic conditions. CGAs 2046, 2096, and 2087 illustrate a long lag in the 3D plot. Presumably owing to the lack of the RLP genes, these strains show a longer lag phase before alternate genes for MTA metabolism are expressed).

Overall, interesting aspects to MTA metabolism have been uncovered in Rps. palustris. As mentioned earlier, MTA is a toxic compound and the cell is required to either get rid of it or metabolize it. If genes involved in MTA metabolism are lacking, there will be accumulation of MTA, which by a feedback mechanism inhibits the synthesis of essential cell molecules. The redundancies of the genes in the organism, which perform the same function, are possibly a help for such stress conditions.

In the final analysis, it is clear that additional research on the regulation of genes involved in aerobic and anaerobic MTA metabolism is warranted. Such studies will shed light on the requirements for RubisCO/RLP family members under the
different growth conditions.

The *in vivo* studies performed this far on this organism offers limited answers at this time since the organism is depended in the form I and form II genes for growth under phototrophic conditions. However, much like *R. rubrum*, strains have recently been selected that allow for PH growth in the absence of RubisCO (via redox balancing performed by nitrogenase derepression) (Rick Laguna, unpublished observations). In addition, transcriptome and shot-gun proteomic studies might be useful to follow the expression of these (and other) genes under specific growth conditions. Furthermore, the data thus far obtained suggest that the mutant RubisCO proteins may be very useful; e.g. proteins in which the carboxylase function of RubisCO are unaffected while the enolase function is disrupted. Clearly, the initial findings reported here indicate that this interesting and versatile organism would be a good model for the follow-up studies as discussed above.
TABLE 4.4: Quantitative comparison of growth parameters between strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>MAX (%)</th>
<th>ET50 (hours)</th>
<th>TAU (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PH growth of R. palustris strain 2028 with ammonium sulfate</td>
<td>100 ± 7</td>
<td>228 ± 6</td>
<td>41 ± 4</td>
</tr>
<tr>
<td>2028 with MTA</td>
<td>85 ± 7</td>
<td>210 ± 8</td>
<td>44 ± 3</td>
</tr>
<tr>
<td>2040 with ammonium sulfate</td>
<td>NG</td>
<td>NG</td>
<td>NG</td>
</tr>
<tr>
<td>2040 with MTA</td>
<td>NG</td>
<td>NG</td>
<td>NG</td>
</tr>
<tr>
<td>2046 with ammonium sulfate</td>
<td>95 ± 8</td>
<td>179 ± 6</td>
<td>38 ± 4</td>
</tr>
<tr>
<td>2046 with MTA</td>
<td>68 ± 4</td>
<td>155 ± 3</td>
<td>33 ± 4</td>
</tr>
<tr>
<td>2053 with ammonium sulfate</td>
<td>NG</td>
<td>NG</td>
<td>NG</td>
</tr>
<tr>
<td>2053 with MTA</td>
<td>NG</td>
<td>NG</td>
<td>NG</td>
</tr>
<tr>
<td>2071 with ammonium sulfate</td>
<td>99 ± 4</td>
<td>107 ± 3</td>
<td>25 ± 2</td>
</tr>
<tr>
<td>2071 with MTA</td>
<td>61 ± 4</td>
<td>109 ± 6</td>
<td>25 ± 5</td>
</tr>
<tr>
<td>2087 with ammonium sulfate</td>
<td>100 ± 4</td>
<td>218 ± 4</td>
<td>42 ± 4</td>
</tr>
<tr>
<td>2087 with MTA</td>
<td>74 ± 2</td>
<td>180 ± 6</td>
<td>42 ± 5</td>
</tr>
<tr>
<td>PH growth of strain 2096 with (NH₄)₂SO₄</td>
<td>100 ± 3</td>
<td>214 ± 3</td>
<td>43 ± 1</td>
</tr>
<tr>
<td>2096 with MTA</td>
<td>61 ± 8</td>
<td>153 ± 9</td>
<td>45 ± 9</td>
</tr>
<tr>
<td>CH growth of strain 2028 with (NH₄)₂SO₄</td>
<td>100 ± 3</td>
<td>116 ± 3</td>
<td>26 ± 3</td>
</tr>
<tr>
<td>2028 with MTA</td>
<td>79 ± 2</td>
<td>93 ± 3</td>
<td>22 ± 3</td>
</tr>
</tbody>
</table>
### TABLE 4.4: Quantitative comparison of growth parameters between strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>MAX (%)</th>
<th>ET50 (hours)</th>
<th>TAU (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2040 with (NH₄)₂SO₄</td>
<td>98 ± 3</td>
<td>50 ± 3</td>
<td>12 ± 4</td>
</tr>
<tr>
<td>2040 with MTA</td>
<td>80 ± 11</td>
<td>136 ± 11</td>
<td>29 ± 5</td>
</tr>
<tr>
<td>2046 with (NH₄)₂SO₄</td>
<td>100 ± 7</td>
<td>117 ± 4</td>
<td>27 ± 4</td>
</tr>
<tr>
<td>2046 with MTA</td>
<td>36 ± 3</td>
<td>123 ± 6</td>
<td>22 ± 3</td>
</tr>
<tr>
<td>2053 with (NH₄)₂SO₄</td>
<td>100 ± 3</td>
<td>126 ± 3</td>
<td>25 ± 2</td>
</tr>
<tr>
<td>2053 with MTA</td>
<td>75 ± 9</td>
<td>136 ± 8</td>
<td>21 ± 3</td>
</tr>
<tr>
<td>2071 with (NH₄)₂SO₄</td>
<td>100 ± 3</td>
<td>133 ± 3</td>
<td>26 ± 3</td>
</tr>
<tr>
<td>2071 with MTA</td>
<td>73 ± 6</td>
<td>118 ± 5</td>
<td>20 ± 3</td>
</tr>
<tr>
<td>2087 with (NH₄)₂SO₄</td>
<td>100 ± 6</td>
<td>130 ± 5</td>
<td>33 ± 5</td>
</tr>
<tr>
<td>2087 with MTA</td>
<td>44 ± 4</td>
<td>194 ± 9</td>
<td>46 ± 7</td>
</tr>
<tr>
<td>2096 with (NH₄)₂SO₄</td>
<td>100 ± 8</td>
<td>122 ± 7</td>
<td>29 ± 5</td>
</tr>
<tr>
<td>2096 with MTA</td>
<td>47 ± 3</td>
<td>144 ± 4</td>
<td>22 ± 3</td>
</tr>
<tr>
<td>2099 with (NH₄)₂SO₄</td>
<td>100 ± 8</td>
<td>60 ± 5</td>
<td>32 ± 4</td>
</tr>
<tr>
<td>2099 with MTA</td>
<td>43 ± 3</td>
<td>84 ± 6</td>
<td>13 ± 3</td>
</tr>
</tbody>
</table>

Microbial population growth data were fit to a logistic sigmoidal function using an iterative, nonlinear, least-squares method, and compared by fit coefficients (mean ± SD), i.e., population size at stationary phase (MAX), estimated time to reach 50% MAX (ET50), time constant (TAU). PH=photoheterotrophic, CH=chemoheterotrophic, NG=no growth.
Table 4.4 summarizes some of the key results from the sigmoidal fits of the normalized growth data in this chapter. A remarkable difference in growth of the mutant strains of *Rps. palustris* for MTA dependent growth as compared to sulfate dependent growth is observed. Under PH conditions, *Rps. palustris* strains 2046 (ΔRLP2), 2071 (ΔcbbM), 2087 (ΔRLP1/ΔRLP2) and 2096 (ΔRLP1) demonstrate a significant difference in MAX (between growth on sulfate and MTA). The rate constant or TAU remains similar for these strains. Strains 2040 and 2053 did not demonstrate any significant growth.

For CH growth, *Rps. palustris* mutant strains 2046 (ΔRLP2), 2053 (ΔcbbLS/ΔcbbM/ΔRLP2), 2087 (ΔRLP1/ΔRLP2), 2096 (ΔRLP1) and 2099 (ΔcbbLS/ΔcbbM/ΔRLP2A/RLP1) demonstrate a significantly higher MAX for growth with sulfate as compared to growth with MTA. The normalized curves distinctly reveal the comparative growth characteristics of the mutant strains. It is distinctly apparent from the normalized curves, that strains lacking either of the RLP genes showed statistically significant growth differentiation.
Chapter Five

Summary and Implications
Chapter 5

Summary and Implications

The objective of this study was to investigate the dynamic molecular nature of a key metabolic enzyme under physiological conditions. Using a combination of molecular, genetic, in vitro, in vivo, and computer simulation methods, this dissertation establishes a novel role of RubisCO in a sulfur salvage pathways. In addition this work establishes the manner by which all forms of RubisCO may catalyze two simultaneous and/or concurrent reactions for two different necessary and physiologically significant metabolic pathways of the cell. Moreover, it is apparent that the active site of RubisCO has evolved to the point where the two activities may be differentiated in that mutant proteins were identified where residues may be important for one activity to the exclusion of the other. In addition, this dissertation identifies residues that are important for both activities and allow the organism to perform the enolase reaction.
In Chapter 2 and 3, experiments were aimed at understanding the roles of different forms of RubisCO via complementation in *R. rubrum* strain IR. It appears that all bonafide RubisCOs are able to catalyze the DKMTP reaction. Results from *in vivo* experiments were complemented by *in vitro* measurements with purified enzymes, i.e., using an analog of DKMTP for a convenient assay (Jaya Sriram, unpublished observations). These elemental findings provided an unprecedented opportunity for further research into how the active site of a single protein may be modified to affect the course of both carbon and sulfur metabolism, and this question was addressed in the next chapter of this dissertation.

In Chapter 3, it was noted that RubisCO has been broadly studied and much is known about its active site and the mechanism for the carboxylation/oxygenation reaction. However, an important previously unanswered question addressed in this chapter is how the same enzymes accommodate two different substrates. Results from Chapter 3 demonstrate that in order to orient the two different substrates, the enzyme offers different active sites for each reaction. Moreover,
residues that are involved in the enolase reaction or involved in the ligand binding process for DKMTP were identified. Interestingly, certain residues were found to be differentially involved in the carboxylation and enolase reactions.

Investigation of the protein-substrate interaction was performed by first, testing active site mutants of RubisCO (with respect to the carboxylase reaction) for their role in the enolase reaction. At the same time, residues based on the models were tested for enolase and hence carboxylase reaction. The results indicate that certain amino acid residues are involved in both enolase and carboxylase reactions, suggesting that there are conserved residues for binding of both substrates in the active site. More specifically, residues near the N terminal of the protein probably interact with DKMTP (the substrate for the enolase reaction).

Whereas some of the RubisCO active site residues (e.g., Lys at 191) seem to partially affect the enolase activity, a mutation in residues such as Asp at 111 and Ser-368 affected the enolase reaction and prevented growth on MTA. On the other hand, residues like Lys at 166 or His at 321 had no effect on the enolase
activity. Of the site-directed mutations that were tested, H at 288, A at 305, H at 291, and T at 391 affected the enolase reactions, either partially or fully.

Interestingly, some of these residues were also involved in the carboxylase reaction. For example, mutations at His 291 to Glu supported the carboxylase dependent growth, albeit partially. Whereas mutated residues like A 305 to V and Thr 391 to Val affected the enolase but not the carboxylase reactions.

To demonstrate the relative growth of the *R. rubrum cbbM* mutants with either carbon or sulfur as the energy sources, *figures 5.1 and 5.2* provide a three dimensional representation of the MTA-dependent growth curves. In these figures, the abscissa plots magnitude of autotrophic growth, the ordinate plots magnitude of heterotrophic growth and the applicate/Z axis plots time in hours.
FIGURE 5.1: 3D plot of *R. rubrum cbbM* mutant growth curves under heterotrophic (in IR) and autotrophic (in SBI/II) conditions (view 1)

Igor Pro was used to create the 3D graph. In this graph, the abscissa plots magnitude of autotrophic growth, the ordinate plots magnitude of heterotrophic growth and the applicate/Z axis plots time in hours. The first axis-orientation of the 3-D plot demonstrates how certain mutations differentially affect autotrophic growth (i.e., carboxylase reaction) and heterotrophic-MTA based growth (i.e., enolase reaction). All curves proceed along the applicate (i.e., towards the top of this page) and indicate growth over time.
The first axis-orientation of the 3-D plot (Figure 5.1) demonstrates how certain mutations differentially affect autotrophic growth (i.e., carboxylase reaction) and heterotrophic-MTA based growth (i.e., enolase reaction). All curves proceed along the applicate (i.e., towards the top of this page) and indicate growth over time. Mutant growth curves that deviate to the left indicate heterotrophic growth and those that deviate to the right indicate autotrophic growth, but those with minimal deviation indicate a lag in the earlier or later phase of growth.

Transposition of the axes provides an alternate view (Figure 5.2) and more clearly demonstrates the time course and magnitude of MTA-dependent growth for the other mutants (i.e., not N111S or A305V). These mutants do not grow under autotrophic conditions. This 3-dimensional representation of the growth model highlights the differential behavior of certain residues in the hydrophobic pocket of the enzyme, suggesting an active center for the dual role. One may appreciate the relative lag of the mutants while growing under specific conditions. The degree of deviation towards one side or another represents the importance of the mutant in the corresponding function.
FIGURE 5.2: 3D plot of R.rubrum cbbM mutant growth curves under heterotrophic (in IR) and autotrophic (in SBI/II') conditions (view 2)

Igor Pro was used to create the 3D graph. In this graph, the abscissa plots magnitude of autotrophic growth, the ordinate plots magnitude of heterotrophic growth and the applicate/Z axis plots time in hours. The first axis-orientation of the 3-D plot demonstrates how certain mutations differentially affect autotrophic growth (i.e., carboxylase reaction) and heterotrophic-MTA based growth (i.e., enolase reaction). All curves proceed along the applicate (i.e., towards the top of this page) and indicate growth over time.
Results from chapter 4 further indicate that under anaerobic conditions, RubisCO either form I or form II, is required for PH growth, and either form can simultaneously catalyze a sulfur and carbon metabolism with similar efficacies. These results suggest that both forms either share a similar mechanism or, have distinct mechanisms that encounter similar energetic barriers (i.e., similar activation energies). This hypothesis is further supported by the similar doubling time of the growth curves.

In summary, this dissertation advances our knowledge of the dynamic molecular nature of RubisCO under physiological conditions and provides a comprehensive description of a novel role of RubisCO in a sulfur salvage pathway. This dissertation has also raised several interesting and key questions, yet at the same time several new hypotheses have been generated that may be tested in future projects.

1. It remains to be established what the fate of the RubisCO-catalyzed enolase reaction product might be in vivo because there are no
recognizable genes in the genome that encode proteins for its subsequent metabolism.

2. A distinct and novel sulfur salvage pathway that involves *R. rubrum* RLP participating in an isomerase reaction (Imker et al., 2008) occurs under aerobic growth conditions and appears to be linked to isoprenoid biosynthesis (Erb at al., 2012; *in press*). Further investigation is warranted to determine how these diverse sulfur salvage pathways, involving both RubisCO and RLP, are differentially regulated in the cell.

3. Evidence in this dissertation asserts that RubisCO accommodates the substrates in two different but overlapping, active sites and certain residues (based on docking models) are involved in ligand binding (i.e., DKMTP). It would be interesting to further examine the dynamic molecular nature of these interactions using molecular techniques, such as more specific site-directed mutagenesis experiments that carefully dissect side chain properties of each residue (e.g., based on charge, polarity, bulk).
Collaborative studies are currently underway with an established X-ray crystallography laboratory as it will be essential to obtain structures of RuBP and DKMTP RubisCO complexes. By virtue of these experiments, further insight would be gained into the ligand-binding process.

Furthermore, a few amino acid residues (from the protein-ligand model) that were evaluated in this dissertation did not definitively affect the enolase reaction. Again, more precise targeting of specific residues, in combination with structural studies, may provide insight into the chemical bonds and interactions of the side chain with the ligand.

4. The novel reaction catalyzed by the *R. rubrum* RLP can be assayed by NMR methods. Our *in vivo* experiments show that RubisCO is involved in both enolase and carboxylase reactions. Thus using NMR or other techniques, it will be important to provide detailed in vitro evidence for the capabilities of different RubisCOs and RLPs to carry out the reaction. These studies can be extended to the RubisCO mutants generated in this dissertation.
5. This dissertation demonstrates the absence of the RLP1 gene reduces the growth of *Rps. palustris* mutant as compared to the wild type. However, some growth was also seen under aerobic conditions with MTA, thus raising the possibility that RubisCO in *Rps. palustris* is expressed under aerobic conditions, albeit at low levels. RT-PCR based experiments may provide additional insight by checking the transcript levels or performing proteomic studies under aerobic and anaerobic conditions using MTA as a sulfur source. The different mutants along with the WT *Rps. palustris*, could be grown with MTA as a sole sulfur source and the transcript/proteome levels might show the differential expression of the RubisCO family (and other) genes. These experiments could provide important insight and lead to further studies that will establish the definitive role of each protein in sulfur metabolism.

6. A detailed analysis based on complementation studies with *Rps. palustris* quadruple mutants could enrich our knowledge of the apparent redundancy present in this complex system. The quadruple mutant may
be complemented by form I, II RubisCO, RLP1 and RLP2 from R. palustris. Apart from this, since RLP1 is closely related to R. rubrum RLP, the R. rubrum gene can also be complemented into the quadruple mutant and could be the focus of extensive studies.

7. RubisCO, the most abundant protein, has demonstrated to be the rate limiting enzyme for photosynthesis. Studies are going on to increase the catalytic proterty of the enzyme by making genetic manipulations. Alternatively, it may be possible to bioengineer the RLP such that it can catalyze the bonafide RubisCO reaction. Both RLP and RubisCO share the same fundamental protein scaffold as they are structurally related molecules. By starting with a molecule (engineered RLP) with rudimentary RubisCO activity, it may be more feasible and easier to design the active site to achieve an enzyme with improved catalytic properties.
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