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PREVIEW

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**Genetic modification of ribulose-1,5-bisphosphate carboxylase/oxygenase  
from *Chlamydomonas reinhardtii***

Chen, Zhixiang, Ph.D.

The University of Nebraska - Lincoln, 1990

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PREVIEW

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**GENETIC MODIFICATION OF RIBULOSE-1,5-BISPHOSPHATE  
CARBOXYLASE/OXYGENASE FROM *CHLAMYDOMONAS*  
*REINHARDTII***

by

**Zhixiang Chen**

**A DISSERTATION**

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The Graduate College in the University of Nebraska  
In Partial Fulfillment of Requirements  
For the Degree of Doctor of Philosophy**

**Major: Biological Sciences**

**Under the Supervision of  
Professors Robert J. Spreitzer and Raymond Chollet**

**Lincoln, Nebraska**

**September, 1990**

DISSERTATION TITLE

Genetic Modification of Ribulose-1,5-bisphosphate Carboxylase/oxygenase

from Chlamydomonas reinhardtii

BY

Zhixiang Chen

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GENETIC MODIFICATION OF RIBULOSE-1,5-BISPHOSPHATE  
CARBOXYLASE/OXYGENASE FROM *CHLAMYDOMONAS*  
*REINHARDTII*

Zhixiang Chen, Ph.D.

University of Nebraska, 1990

Advisors: Robert J. Spreitzer and Raymond Chollet

The 45-3B mutant of *Chlamydomonas reinhardtii* has a mutation in the chloroplast large-subunit gene of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) that causes valine-331 to be replaced by alanine. This mutation causes a photosynthesis-deficient, acetate-requiring phenotype by reducing the specific activity of the Rubisco enzyme by 95% and the CO<sub>2</sub>/O<sub>2</sub> specificity by 37%. Revertant selection identified two intragenic suppression mutations, named S40-9D and S61-2J, that cause threonine-342 to be replaced by isoleucine and glycine-344 to be replaced by serine, respectively. Both of these amino acid substitutions appear to complement the original 45-3B mutation through steric interaction to improve the specific activity and CO<sub>2</sub>/O<sub>2</sub> specificity of the mutant enzyme.

Temperature-sensitive mutant 68-4PP resulted from a chloroplast mutation that causes a phenylalanine-for-leucine substitution at residue 290 in the Rubisco large subunit. The mutant Rubisco enzyme had about 50% of wild-type enzyme activity and its CO<sub>2</sub>/O<sub>2</sub> specificity was reduced by 13%. Reversion experiments at the restrictive temperature (35°C) identified a nuclear mutation which suppressed the temperature-sensitive phenotype of 68-4PP by enhancing both the specific activity and protein level of the

mutant Rubisco enzyme. More significantly, the reduced  $\text{CO}_2/\text{O}_2$  specificity factor of the 68-4PP mutant enzyme was restored to the wild-type level by this nuclear mutation. DNA sequencing showed that this mutation was not in either of the two Rubisco small-subunit genes, suggesting that some other nuclear-encoded protein is able to influence the structure and, in turn, the  $\text{CO}_2/\text{O}_2$  specificity of Rubisco.

Binding assays with the transition-state analogue 2-carboxyarabinitol-1,5-bisphosphate, in combination with other biochemical analyses, revealed that the mutant forms of Rubisco with reduced  $\text{CO}_2/\text{O}_2$  specificities had defects in stabilizing the transition state of the partial reaction of carboxylation. This observation was consistent with a prediction derived from transition state theory which indicates that the relative stabilities of the transition states for the competitive partial reactions of carboxylation and oxygenation determine the  $\text{CO}_2/\text{O}_2$  specificity of Rubisco.

PREVIEW

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## ABBREVIATIONS

<b>ac</b>	<b>acetate-requiring</b>
<b>Bicine</b>	<b>N,N-bis(2-hydroxyethyl)glycine</b>
<b>CABP</b>	<b>2-carboxyarabinitol 1,5-bisphosphate</b>
<b>CRBP</b>	<b>2-carboxyribitol 1,5-bisphosphate</b>
<b>DTT</b>	<b>dithiothreitol</b>
<b>EDTA</b>	<b>ethylenediaminetetraacetate</b>
<b>FdUrd</b>	<b>5-fluorodeoxyuridine</b>
<b>K<sub>c</sub></b>	<b>K<sub>m</sub> for substrate CO<sub>2</sub></b>
<b>K<sub>o</sub></b>	<b>K<sub>m</sub> for substrate O<sub>2</sub></b>
<b>kDa</b>	<b>kilodalton</b>
<b>MMS</b>	<b>methyl methanesulfonate</b>
<b><i>mt</i></b>	<b>mating type</b>
<b><i>pf</i></b>	<b>paralyzed flagella</b>
<b>3-PGA</b>	<b>3-phosphoglycerate</b>
<b>PVPP</b>	<b>polyvinylpolypyrrolidone</b>
<b>Rubisco</b>	<b>ribulose-1,5-bisphosphate carboxylase/oxygenase</b>
<b>RuBP</b>	<b>ribulose 1,5-bisphosphate</b>
<b>SDS/PAGE</b>	<b>sodium dodecyl sulfate/polyacrylamide gel electrophoresis</b>
<b>ts</b>	<b>temperature-sensitive</b>
<b>TPCK</b>	<b>N-<math>\rho</math>-toluenesulfonyl-L-phenylalanine chloromethyl ketone</b>
<b>V<sub>c</sub></b>	<b>V<sub>max</sub> for carboxylation</b>
<b>V<sub>o</sub></b>	<b>V<sub>max</sub> for oxygenation</b>
<b>wt</b>	<b>wild type</b>

## Chapter I

### INTRODUCTION

#### 1.1 RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE/OXYGENASE

##### 1.1.1 General Information

Ribulose-1,5-bisphosphate (RuBP) carboxylase/oxygenase (Rubisco; EC 4.1.1.39) is a bifunctional enzyme with two catalytic activities (Ogren, 1984). The carboxylase activity of Rubisco is responsible for the first reaction in the C<sub>3</sub> photosynthetic carbon reduction cycle, in which the carboxylation of RuBP leads to the formation of two molecules of 3-phosphoglycerate (3-PGA). The oxygenase activity of Rubisco is responsible for the initial reaction in the C<sub>2</sub> photorespiratory carbon oxidation cycle where the oxygenation of RuBP results in the formation of one molecule of 3-PGA and one molecule of 2-phosphoglycolate. Thus, the reactions catalyzed by Rubisco link photosynthetic CO<sub>2</sub> fixation with photorespiration in photosynthetic organisms (Laing *et al.*, 1974; Somerville and Ogren, 1982). Since the oxygenase activity of Rubisco competes with the carboxylase activity and initiates the nonessential photorespiratory pathway, there has been a strong expectation that increasing the specificity of Rubisco for CO<sub>2</sub> relative to O<sub>2</sub> will allow the enhancement of photosynthetic rate, and, in turn, increase plant productivity.

There are basically two types of Rubisco enzymes with respect to subunit composition (Andrews and Lorimer, 1987). Rubisco from several species of purple, nonsulfur bacteria is an oligomer of only large subunits. For example, Rubisco from *Rhodospirillum rubrum* is a dimer of large subunits (L2) (Tabita and McFadden, 1974). However, from all eukaryotes

and the majority of prokaryotes, Rubisco is a hexadecamer (L8S8), composed of eight large subunits each with a molecular mass of 50 to 55 kDa, and eight small subunits each with a molecular mass of 12 to 18 kDa. In most eukaryotes, the large subunit of Rubisco is encoded by a single-copy gene in the chloroplast genome (Coen *et al.*, 1970), whereas the small subunit is encoded by the nuclear genome as a small multigene family of 2 to 15 members (Berry-Low *et al.*, 1982; Broglie *et al.*, 1983; Coruzzi *et al.*, 1983; Dunsmuir *et al.*, 1983). The small subunit protein is synthesized on cytoplasmic ribosomes as a precursor polypeptide, which is then imported into the chloroplast and processed to the mature form to assemble into the holoenzyme with the large subunit (Chua and Schmidt, 1978; Highfield and Ellis, 1978; Roy, 1990).

The post-translational assembly of Rubisco holoenzyme in both prokaryotes and eukaryotes is a complex process which requires other helper proteins (Roy, 1990). In higher plants, this helper protein (originally called Rubisco subunit binding protein) is synthesized as a precursor in the cytoplasm and imported into the chloroplast (Hemmingsen and Ellis, 1986). After processing, it appears to form a complex with Rubisco large subunits during or very shortly after synthesis of the subunits. Rubisco large subunits are then released in an ATP-dependent fashion from the Rubisco binding protein as dimeric complexes, followed by uncharacterized association with each other and with small subunits to form the hexadecameric Rubisco holoenzyme molecules (Roy, 1990). Recently, it has been demonstrated that higher plant Rubisco subunit binding protein is highly homologous to a 60 kDa-protein encoded by the *groEL* gene in *Escherichia coli* (Hemmingsen *et al.*, 1988). Even more recently,

Goloubinoff *et al.* (1989) found that Rubisco subunits cloned either from cyanobacterium (L8S8 type) or from *R. rubrum* (L2 type) could assemble into active holoenzymes only in the presence of a functional *E. coli groEL* gene. Thus, the Rubisco subunit binding protein in higher plants is closely related to the *E. coli groEL*-encoded protein, and both of these proteins function as molecular chaperones of Rubisco. It is for these reasons that Hemmingsen *et al.* (1988) proposed that the *groEL* class of proteins be called "chaperonins".

Primary structures of Rubisco from a wide range of organisms are known, determined either by direct amino acid sequencing of the protein or by inference from the nucleotide sequences of the corresponding genes. For the L8S8 type of Rubisco, there is an extraordinary homology (>80%) between all sequences of the large subunit (Andrews and Lorimer, 1987). Sequence homology between the large subunit of L8S8 Rubisco and the subunit of L2 Rubisco from *R. rubrum* was only about 25% (Nargang *et al.*, 1984). However, there are several regions where high homology of sequences exists between these two types of Rubisco large subunits, and a number of residues that were implicated as being near the active site of Rubisco are located within these conservative regions (described in section 1.1.3). There is a much lower level of homology in the small-subunit sequences for Rubisco from different species than that observed with the large subunit (Andrews and Lorimer, 1987). However, there are three regions of extensive sequence homology (residues 10-21, 66-76 and 98-116), evident from the comparison of the small-subunit sequences between prokaryotes and eukaryotes. In addition, the small subunit of higher-plant Rubisco contains a highly conserved sequence of 16 amino acids (residues

45-60) that is absent in the small subunit of cyanobacteria. A recent study indicated that a pea small subunit lacking this 16 amino acid sequence failed to assemble with endogenous pea large subunit, but addition of this sequence to the Rubisco small subunit of the cyanobacterium *Anacystis nidulans* enabled the fusion protein to assemble with pea large subunit (Wasmann *et al.*, 1989). Thus, this conserved sequence appears to be required for the assembly of the eukaryotic Rubisco holoenzyme.

Rubisco subunit structure and interactions have been best revealed by X-ray crystallography of Rubisco from *R. rubrum*, spinach and tobacco (Schneider *et al.*, 1986; Chapman *et al.*, 1988; Andersson *et al.*, 1989). The large subunit structure in L8S8 Rubisco from both spinach and tobacco is fairly similar to that of L2 Rubisco from *R. rubrum*. The subunit has two domains: a small N-terminal domain and a large C-terminal domain. The C-terminal domain forms an eight stranded  $\alpha/\beta$  barrel, a structure that has been found to be very common among enzymes whose structure is known (Farber and Petsko, 1990). The active site of Rubisco is in a pocket at the opening of the  $\alpha/\beta$  barrel structure, partially covered by the N-terminal domain of another large subunit. Thus, each active site of Rubisco is built from residues of two large subunits. For L8S8 Rubisco, the eight large subunits are arranged around the four-fold axis to make up the L8 core of the Rubisco molecule, whereas four small subunits cover the top and bottom of the elongated L8 core.

Rubisco from all organisms needs to be activated to form the catalytically competent form (Lorimer *et al.*, 1976). The activation process involves the slow, reversible reaction of a molecule of activator CO<sub>2</sub> with the  $\epsilon$ -amino group of lysine 201(L8S8 form) or lysine 191 ( L2 form) of the

Rubisco large subunit to form a carbamate, followed by the rapid addition of  $Mg^{2+}$  to create the active ternary complex (O'Leary *et al.*, 1979; Lorimer and Miziorko, 1980; Lorimer, 1981b). The activator  $CO_2$  is different from substrate  $CO_2$ , which becomes incorporated during catalysis (Lorimer, 1979; Miziorko, 1979). The *in vivo* regulation of Rubisco activation in higher plants appears to be mediated by Rubisco activase, which is able to fully  $CO_2/Mg^{2+}$ -activate Rubisco even at atmospheric levels of  $CO_2$  in the presence of RuBP (Portis, 1990). The series of chemical events for both the carboxylation and oxygenation catalyzed by Rubisco is shown in Fig. 1. The steady-state reaction mechanism of Rubisco is sequential and substrate addition is ordered. RuBP binding and enolization between C-2 and C-3 occur prior to interaction of this enzyme-enediol complex with the gaseous substrates  $CO_2$  and  $O_2$  (Pierce *et al.*, 1986). The carboxylation of RuBP at C-2 leads to the formation of the 6-carbon intermediate, 3-keto-2-carboxyarabinitol 1,5-bisphosphate, which is subsequently converted to two molecules of 3-PGA. The oxygenation of RuBP at C-2, on the other hand, is believed to result in the formation of a five-carbon hydroperoxide, which is then hydrolyzed into one molecule of 3-PGA and one molecule of 2-phosphoglycolate (Lorimer, 1981a).

### 1.1.2 Substrate Specificity

The competition between substrates  $CO_2$  and  $O_2$  at the active site of Rubisco determines the relative rates of carboxylation and oxygenation of RuBP, which, in turn, define the relative fluxes of carbon through the pathway of photosynthesis and photorespiration (Ogren, 1984). The ratio of carboxylase to oxygenase activities of Rubisco can be related to the kinetic