Acta Cryst. (1998). D**54**, 668–670

Preliminary X-ray crystallographic study of wild-type and mutant ribulose-1,5-bisphosphate carboxylase/oxygenase from *Chlamydomonas reinhardtii*

Ann Yen,[a] Eric J. Haas,[a] Kathy M. Selbo,[a] Charles R. Ross II,[a] Robert J. Spreitzer[b] and John J. Stezowski[a]* at [a]*Chemistry Department, University of Nebraska-Lincoln, Lincoln, NE 68588-0304, USA, and* [b]*Biochemistry Department, University of Nebraska-Lincoln, Lincoln, NE 68588-0304, USA. E-mail: jjs@unlinfo.unl.edu*

(*Received 28 May 1997; accepted 10 November 1997*)

Abstract

Ribulose-1,5-bisphosphate carboxylase/oxygenase is the key enzyme for photosynthesis. The wild-type and mutant (amino-acid substitutions in the catalytically important loop 6 region) enzymes from *Chlamydomonas reinhardtii*, a unicellular green alga, were crystallized. Wild-type, single-mutant (V331A) and two double-mutant (V331A/T342I and V331A/G344S) proteins were activated with cofactors CO_2 and Mg^{2+}, complexed with the substrate analog 2′-carboxyarabinitol-1,5-bisphosphate, and crystallized in apparently isomorphous forms. Unit-cell determinations have been completed for three of the enzymes. They display orthorhombic symmetry with similar cell parameters: wild type a = 130.4, b = 203.3, c = 208.5 Å; single mutant (V331A) a = 128.0, b = 203.0, c = 207.0Å; and double mutant (V331A/T342I) a = 130.0, b = 202.1, c = 209.7 Å. Crystals of the wild-type and single-mutant (V331A) enzymes diffracted to ∼2.8 Å. A small crystal of the double-mutant (V331A/T342I) enzyme diffracted to ∼6 Å. A partial data set (68% complete) of the wild-type protein has been collected at room temperature to about 3.5 Å.

1. Introduction

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco; E.C. 4.1.1.39) is an essential bifunctional enzyme that catalyzes both carboxylation and oxygenation of D-ribulose 1,5-bisphosphate (see Fig. 1). The enzyme initiates photosynthetic CO_2 fixation or the wasteful process of photorespiration (reviewed by Spreitzer, 1993). The mutual competition between CO_2 and O_2 at the active site is the rate-limiting step for catalyis (reviewed by Hartman & Harpel, 1994). The photosynthetic efficiency of the enzyme is characterized by the Rubisco CO_2/O_2 specificity factor, which is equal to the ratio of carboxylation to oxygenation at any specific concentrations of CO_2 and O_2 (Laing *et al.*, 1974). Alteration of selected residues within the enzyme may lead to an improved CO_2/O_2 specificity and improved crop productivity.

Rubisco is found in all photosynthetic organisms. A number of crystal structures for two types of Rubisco holoenzyme are known: L_2 (large subunit dimers) for some photosynthetic bacteria (Lundqvist & Schneider, 1991), and L_8S_8 (eight large and eight small subunits) for cyanobacteria and plants (Newman & Gutteridge, 1993; Schreuder *et al.*, 1993; Andersson, 1996). The function of the small subunit is not yet known (reviewed by Spreitzer, 1993). The active site is highly conserved between L_2 and L_8S_8 Rubisco. It is located at the interface between the N-terminal domain of one large subunit and the C-terminal α/β-barrel domain of an adjacent large subunit (Knight *et al.*, 1990; Curmi *et al.*, 1992; Newman & Gutteridge, 1993; Andersson, 1996). The comparison of crystal structures for activated and unactivated Rubisco enzymes has indicated that loop 6 (see Fig. 2) may serve as a flexible flap that covers the active site upon substrate binding (Schreuder *et al.*, 1993).

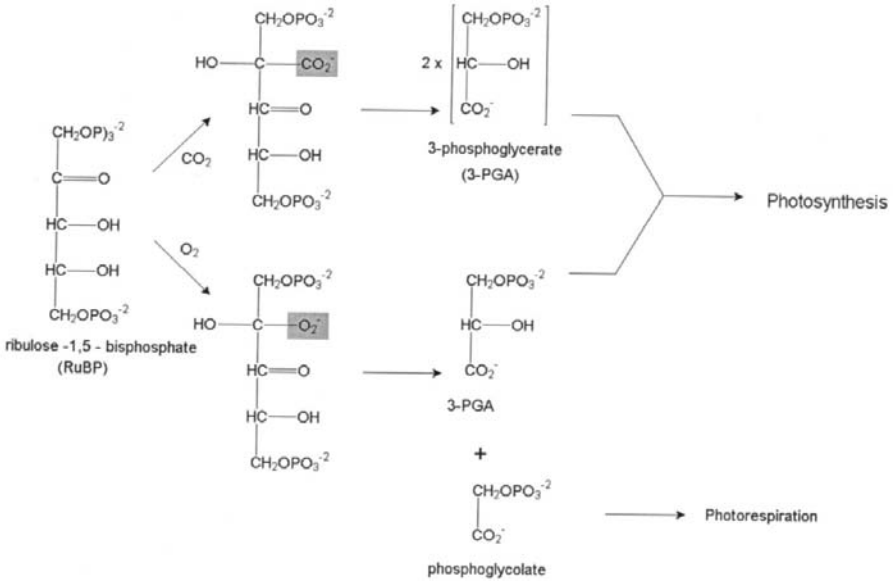

Fig. 1. Reactions catalyzed by ribulose-1,5-bisphosphate carboxylase/oxygenase. Whereas photosynthesis leads to carbon fixation, photorespiration leads to the net loss of cellular carbon.

In contrast to higher plants and photosynthetic prokaryotes, the green alga *C. reinhardtii* has allowed the application of chloroplast genetic methods for the recovery of Rubisco large-subunit mutants (Spreitzer, 1993). The *C. reinhardtti* large subunit has high sequence identity (~90%) with higher plant large subunits, whereas there is low sequence identity (~30%) with the L_2 holoenzyme. Analysis of *C. reinhardtii* large-subunit mutants initially identified the importance of loop 6 in the control of the carboxylase to oxygenase ratio (Chen & Spreitzer, 1989; Chen *et al.*, 1991). The V331A mutation caused a decrease in the CO_2/O_2 specificity factor, whereas either of two second-site mutations (T342I or G344S) improved specificity compared to V331A, but did not reach the level of the wild type. These amino-acid substitutions occur on opposite sides of loop 6 (see Fig. 2) and may complement by size within the hydrophobic core of the loop (Chen & Spreitzer, 1989; Chen *et al.*, 1991). By determining the crystal structures of the wild-type and mutant enzymes, it may be possible to understand better the structure–function relationships of Rubisco catalytic efficiency.

2. Experimental

C. reinhardtii cells were concentrated from 7 l cultures and lysed by sonication. The Rubisco enzymes were then purified from supernatants by differential precipitation with 35 and 55% ammonium sulfate followed by linear 10–30% sucrose gradient fractionation (Spreitzer & Chastain, 1987). The enzyme was maintained in 10 m*M* $MgCl_2$, 10 m*M* $NaHCO_3$, 1 m*M* dithiothreitol, 50 m*M* Bicine (pH 8.0) throughout the purification process to provide Rubisco in an activated form. Prior to crystallization, the enzyme was incubated with the substrate analog, 2′-carboxyarabinitol 1,5-bisphosphate (CABP).

Single crystals of wild-type and mutant enzymes were obtained by hanging-drop and sitting-drop vapor-diffusion methods with and without the use of microseeds (regular or cross seeding). Microseeds were prepared by mechanical agitation in a Jencons® glass homogenizer. Seeds produced by this method were found to be useful for up to three months. Crystals were grown at ~282 K in two weeks without seeding or 1–2 d with seeding (Fig. 3). Each drop contained a mixture of 5 µl of Rubisco at a concentration of ~7–8 mg ml^{-1} and 5 µl of well solution containing 50–150 m*M* KH_2PO_4 (pH 4.7), 6–16% polyethylene glycol 6000, and 1 m*M* $MnCl_2$. The well contained 1 ml of well solution. When seeding was used, 1 µl of seed stock was added to a mixture of 5 µl of protein and 5 µl of well solution.

Best results have been obtained when growing crystals with 6–10% PEG 6000, 75–100 m*M* KH_2PO_4, and 1 m*M* $MnCl_2$. Crystals typically reach sizes of 0.1 × 0.2 × 0.2 mm. Crystals at least double this size have been obtained with microseeding.

3. Results

Crystals of wild-type and mutant (V331A, V331A/T342I and V331A/G344S) Rubisco enzymes were characterized at room temperature with a MAR Research image-plate area detector on a Siemens rotating-anode X-ray generator. A partially complete data set (68% completeness) of wild-type *C. reinhardtii* Rubisco was collected at room temperature with a Rigaku X-ray generator equipped with a Siemens CCD detector. The wild-type and mutant enzymes were found to display orthorhombic symmetry with similar cell parameters: wild-type a = 130.4, b = 203.3, c = 208.5 Å; single-mutant

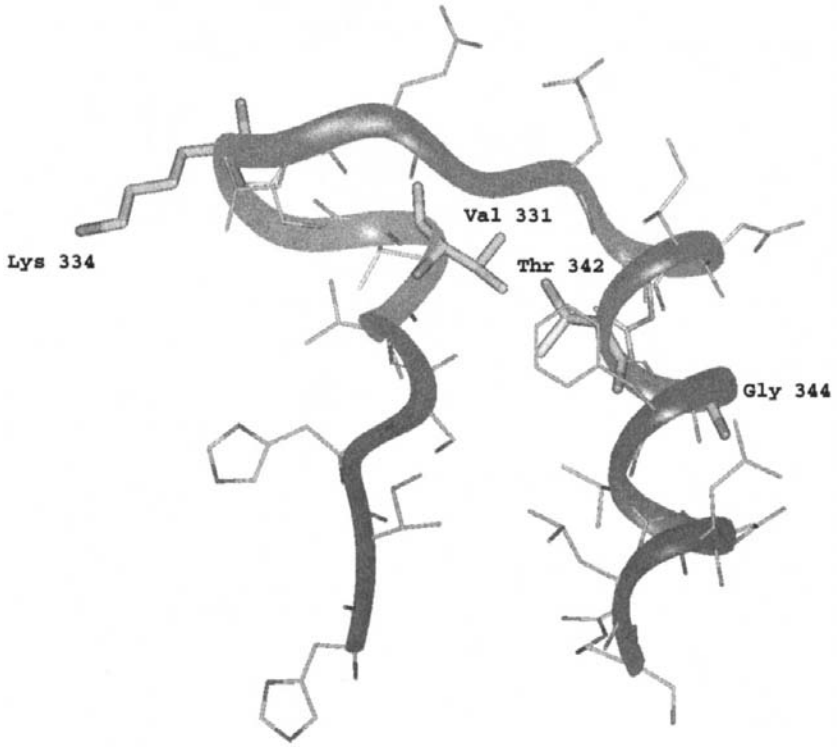

Fig. 2. X-ray structure of loop 6 from spinach Rubisco (8ruc.pdb) (Bernstein *et al.*, 1977; Knight *et al.*, 1990). Lys334 interacts with the CO_2 of the carboxylation transition state. (Figure produced with *InsightII*® software, Molecular Simulations, Inc., San Diego, CA.)

(V331A) a = 128.0, b = 203.0, c = 207.0 Å; and double-mutant (V331A/T342I) a = 130.0, b = 202.1, c = 209.7 Å. Due to incompleteness of the current data set, the identity of the space group could not be uniquely determined, but it is either $P2_12_12_1$ or $P2_12_12$. Examples of Rubisco crystallizing in both of these space groups exist in the literature (Andersson *et al.*, 1983; Newman & Gutteridge, 1990; Newman *et al.*, 1993; Taylor *et al.*, 1996). Assuming a holoenzyme molecular weight of 560 kDa and four molecules per unit cell (one per asymmetric unit), the volumes per unit molecular weight for each Rubisco form are: wild type, 2.45 $Å^3\ Da^{-1}$; single mutant (V331A), 2.40 $Å^3\ Da^{-1}$; and double mutant (V331A/T342I), 2.46 $Å^3\ Da^{-1}$.

Crystals of sufficient quality for data collection have not been obtained without the inclusion of $MnCl_2$. It is important to note that Rubisco is maintained in the active form by the irreversible binding of CABP (Zhu & Spreitzer, 1996). It is unlikely that the final pH upon mixing buffered Rubisco with well solution is sufficiently low to cause release of the CABP.

In conclusion, wild-type and three mutant Rubisco enzymes from *C. reinhardtii* have been crystallized. Initial phases for the crystal structures of wild type and the mutants from *C. reinhardtii* Rubisco will be pursued using molecular replacement and one of three candidate probe structures from the Protein Data Bank (Bernstein *et al.*, 1977). The structure of activated spinach Rubisco with bound CABP is 8ruc.pdb (Andersson, 1996). The corresponding tobacco Rubisco structure is 1rlc.pdb (Schreuder *et al.*, 1993).

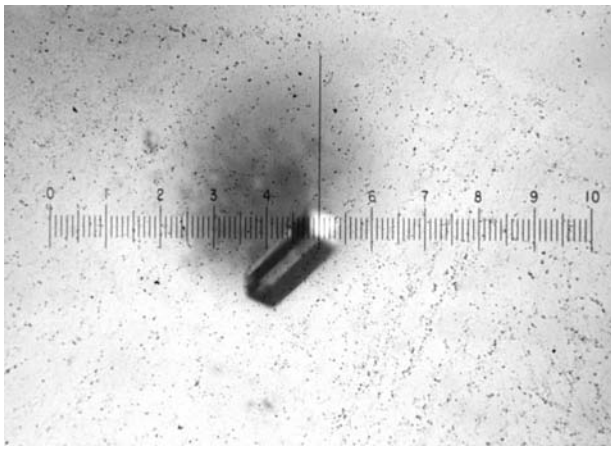

Fig. 3. Crystal of wild-type Rubisco (0.2 × 0.2 × 0.1 mm) from *Chlamydomonas reinhardtii*. The crystal shown is representative of the crystals we have obtained. Protein (~8 mg ml^{-1}) was crystallized in 50 m*M* KH_2PO_4, 1 m*M* $MnCl_2$, 16% polyethylene glycol 6000.

In the mutant enzymes, the altered amino acids are not catalytic residues. Nonetheless, these substitutions influence the CO_2/O_2 specificity factor. Eventual structure determination may reveal how such changes affect catalytic efficiency. A deeper understanding of the structure–function relationships of Rubisco may provide the necessary information for engineering a better enzyme.

This work was supported in part by USDA/NRI grant 94-37306-0349 to RJS, and NSF grants CHE9214428 and OSR-9255225 to JJS.

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