Plant Physiol. (1996) 111: 441–445

# Isolation of a Protein Containing Covalently Linked Large and Small Subunits of Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase from *Botryococcus braunii*[1]

**Xing Wang and Pappachan E. Kolattukudy***

Neurobiotechnology Center and Medical Biochemistry Department, The Ohio State University, 206 Rightmire Hall, 1060 Carmack Road, Columbus, Ohio 43210

**Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and a 66-kD protein were co-purified from solubilized microsomal preparations of the green alga *Botryococcus braunii* by Green A agarose, sucrose density gradient, MonoQ, and gel filtration. The 66-kD protein remained intact after 6 M urea treatment and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. It could be detected in the soluble fraction of the cell-free extract but appeared to be more abundant in the microsomal preparations. It cross-reacted with antibodies raised against Rubisco holoenzyme, large and small subunits, indicating that the 66-kD protein contains both the large and the small subunits of Rubisco. The N-terminal amino acid sequence of this protein and that of a proteolytic fragment showed high homology with the mature Rubisco small subunits, and the sequence of another proteolytic fragment showed high homology with that of the Rubisco large subunit. It is concluded that the 66-kD protein is produced by cross-linking of large and small subunits of Rubisco in the cell.**

Rubisco plays a pivotal role in photosynthesis and photorespiration. The majority of Rubiscos found in prokaryotic and eukaryotic organisms are composed of eight large and eight small subunits with a molecular mass of about 50 to 55 kD and 12 to 18 kD, respectively (Andrews and Lorimer, 1987). In bacteria, it has been known that the genes encoding Rubisco large and small subunits are adjacent and co-transcribed (Tabita, 1988), but the proteins are synthesized separately and assembled into the holoenzyme later. In eukaryotic organisms, however, the gene for the large subunit is encoded by chloroplast DNA, whereas the small subunit gene is encoded by the nuclear genome (Ellis, 1981). The small subunit is synthesized in the cytoplasm and transported to the chloroplast for assembly into the functional holoenzyme. There have been reports that both Rubisco large and small subunits are encoded by chloroplast DNA in some algae, although the two are not co-translated (Reith and Cattolico, 1986; Hwang and Tabita, 1989, 1991; Valentin and Zetsche, 1989; Fujiwara et al., 1993).

We report that while purifying enzymes involved in lipid biosynthesis from the microsomes of the green alga *Botryococcus braunii*, we found a 66-kD protein that cross-reacted with antibodies against Rubisco holoenzymes, large and small subunits. Its N-terminal and one internal amino acid sequence showed high homology with the mature Rubisco small subunit, and another internal amino acid sequence showed high homology with the Rubisco large subunit, therefore suggesting that this protein is composed of both Rubisco large and small subunits. Reverse transcription-PCR failed to detect transcripts that can encode both subunits, leading to the conclusion that the two subunits must be covalently attached but not co-translated. This protein might be similar to the 66-kD variant of Rubisco recently reported to be produced by UV-B photomodification in higher plants (Wilson et al., 1995).

## MATERIALS AND METHODS

### Organism and Growth

Green alga (*Botryococcus braunii*, race A) was grown in a modified CHU13 medium of 2-fold strength (Dennis and Kolattukudy, 1992) in a 20-L Nalgene carboy with stirring at 25°C and continuously illuminated at 10 $\mu$E $m^2$ $s^{-1}$ by cool-white fluorescence tubes. After 10 to 14 d of growth, cultures were harvested by continuous centrifugation.

### Rubisco and the 66-kD Polypeptide Purification

Cells (about 120 g wet weight) collected by centrifugation were washed with 0.5 M KCl and resuspended in 50 mM Tris buffer, pH 8, containing 10 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, and 0.3 M Suc. After brief homogenization in a Ten-Broeck glass homogenizer, the cell suspension was passed through a French press (American Instrument Company, Silver Spring, MD) three times under 18,000 p.s.i. The lysate was centrifuged at 3,000*g* for 1 min at 4°C, and the supernatant was centrifuged at 105,000*g* for 90 min to pellet the microsomes. The final supernatant was used to purify the typical $L_8S_8$ Rubisco, whereas the microsomes served as the source of the 66-kD polypeptide. The microsomal pellet was resuspended in the solubilization buffer (Tris buffer indicated above plus 0.1% octyl $\beta$-glucoside) with a glass homogenizer. After 4 h of gentle shaking at 4°C, the mixture was centrifuged at 105,000*g* for 90 min, and the super-

[1] This was work was supported in part by grant no. DE-FG02-93 ER 20109 from the Department of Energy.

* Corresponding author; e-mail kolattukudy.2@osu.edu; fax 1-614-292-5379.

natant was passed through a 0.8-$\mu$m filter and placed on ice.

The microsome-solubilized preparation and the original high-speed supernatant from the cell lysate were applied to a Green A agarose column (40 mL, 2.5 cm diameter) with a flow rate of 20 mL/min. The column was washed with 400 mL of Tris buffer, and the bound proteins were eluted by 1 M NaCl (50 mL). The eluant was concentrated and loaded directly onto a 0.2, 0.4, 0.6, and 0.8 M Suc step gradient. The samples were centrifuged in an SW28 rotor (Beckman L8-M ultracentrifuge) at 83,000*g* for 18 h. Fractions containing Rubisco activity were collected and loaded directly on a Mono-Q anion-exchange column (Pharmacia), and the bound Rubisco was eluted with a 0 to 1 M NaCl gradient. For the enzyme preparation from solubilized microsomes, after the Mono-Q column, the fractions containing Rubisco activity were concentrated and subjected to gel filtration with a Superose-6 column by fast protein liquid chromatography. If not specified, all of the purification steps were carried out at 4°C.

### Rubisco Activity Assay

Rubisco activity was measured as described by Whitman and Tabita (1976); 1 unit of Rubisco activity is defined as the amount of enzyme needed to fix 1 $\mu$mol of $CO_2$/min at 30°C.

### Antibody Production and Western Blot

The Rubisco purified from the soluble fraction was subjected to SDS-PAGE, and the gel bands corresponding to Rubisco large and small subunits were cut out separately, mixed thoroughly with Freund's adjuvant (complete for the first injection, incomplete for the subsequent injections), and injected subcutaneously into rabbits; two additional booster injections were made at 4-week intervals, after which blood was collected by heart puncture. Antibodies against the spinach holoenzyme was kindly provided by Dr. F.R. Tabita (Department of Microbiology, The Ohio State University, Columbus). Immunoblots were done by the standard protocol (Towbin et al., 1979), with 5% nonfat dry milk as the blocking agent and $^{125}$I-protein A (New England Nuclear, 7-10 $\mu$Ci/$\mu$g) for detection.

### N-Terminal and Internal Peptide Amino Acid Sequencing

The section of gel corresponding to the 66-kD polypeptide, separated from Rubisco large and small subunits by SDS-PAGE, was cut out, 1 $\mu$g of Endoproteinase Glu-C (1 $\mu$g/$\mu$L, Sigma) was added to the gel (which contained about 15 $\mu$g of protein) after homogenization, and the mixture was incubated at 37°C overnight. The digested protein products were subjected to SDS-PAGE (11%). The electrophoretically separated peptides were transblotted onto ProBlot (Applied Biosystems), and two protein fragments (18 and 32 kD) and the parent 66-kD protein were subjected to amino acid sequencing on an Applied Biosystems 475A protein sequencer.

## RESULTS

### Solubilization and Purification of a 66-kD Rubisco

Rubisco was purified from high-speed supernatants and from solubilized microsomal fractions obtained with 0.1% octyl glucoside. The following procedure was used for purification of both Rubisco and a 66-kD polypeptide. The soluble protein solution was passed through a Green A agarose column, and the bound Rubisco was eluted with 1 M NaCl in Tris buffer. The eluted Rubisco preparation was subjected to Suc density gradient centrifugation. Rubisco preparation obtained from this step was subjected to ion-exchange chromatography on a Mono-Q column, and the bound Rubisco was eluted at 0.15 M NaCl. The purification of the 66-kD polypeptide was done by the same procedure except that octyl $\beta$-glucoside was present during the purification procedures. The 66-kD polypeptide preparation was then subjected to gel filtration. SDS-PAGE of the final preparation under reducing conditions (with dithioerythritol) indicated that Rubisco from the soluble fraction was more than 90% pure (Fig. 1A), and the 66-kD polypeptide was co-purified with Rubisco (Fig. 1B).

### Characterization of the 66-kD Polypeptide

The 66-kD polypeptide showed very similar properties to those of the typical Rubisco. It eluted in the same fraction as Rubisco from the Mono-Q ion-exchange column, indicating that this protein has similar electrostatic properties as Rubisco. The binding on the Green A agarose

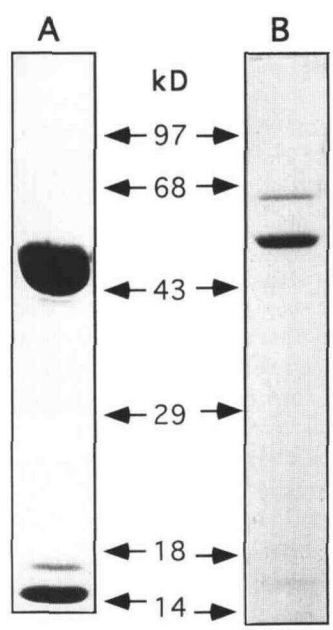


**Figure 1.** SDS-PAGE of Rubisco and the 66-kD polypeptide co-purified with Rubisco. A, Rubisco purified from the soluble protein fraction. B, Rubisco and the co-purified 66-kD polypeptide from the microsome preparation. In both cases fractions eluted from the Mono-Q column containing Rubisco activity were concentrated and subjected to gel filtration on a Superose-6 column; fractions containing the highest Rubisco activity were subjected to SDS-PAGE.

column suggested that it shares a common binding site with Rubisco. It occupied the same position in the Suc density gradient and was eluted at the same volume as Rubisco from the gel filtration column, indicating that it has a molecular size similar to that of the native Rubisco. It was obvious that under the denaturing SDS-PAGE conditions the 66-kD polypeptide remained intact. When the 66-kD polypeptide-containing preparation from the Mono-Q step was treated overnight with 6 M urea and subjected to gel filtration in the presence of 6 M urea, the typical Rubisco dissociated into large and small subunits. Therefore, under this dissociating condition, the fraction containing the most protein upon SDS-PAGE showed only the large subunit (Fig. 2A), whereas under the nondissociating condition, the corresponding fraction (without urea) from gel filtration showed both large and small subunits (Fig. 2B). In both cases the 66-kD polypeptide remained intact. In Figure 2A, the 66-kD protein band is faint because the total protein concentration in this fraction was low. When green alga was disrupted by a French press and the lyase was subjected directly to SDS-PAGE, immunoblots indicated that the 66-kD polypeptide was present in the cell-free supernatant (Fig. 3A), indicating that the 66-kD protein was not generated as an artifact during the solubilization and purification procedure.

### Immunoblot Analysis

Immunoblot analysis of the 66-kD protein-containing fraction indicated that this protein cross-reacted with antibodies against Rubisco from spinach (Fig. 3B). Since this antibody preparation was prepared against the holoenzyme, these results suggested that the 66-kD polypeptide might contain the Rubisco large or small subunit or both. When antibodies prepared against the separated Rubisco large and small subunits isolated from *B. braunii* were used for immunoblot analysis, the 66-kD polypeptide cross-reacted with both antibodies (Fig. 3, C and D). Since the antibodies were specific for the large and small subunits of Rubisco as seen in Figure 3, C and D, this result indicated that the 66-kD polypeptide probably contains both the large and small subunits of Rubisco.

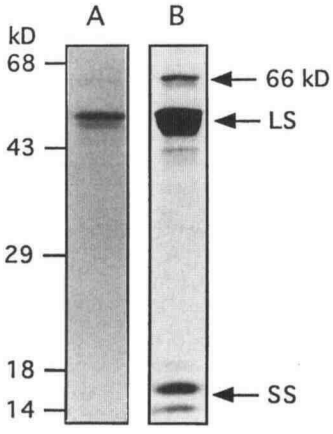


**Figure 2.** SDS-PAGE analyses of the major protein fraction from Superose-6 gel filtration, in the presence or absence of 6 M urea, of partially purified microsomal Rubisco. A, The microsomal Rubisco preparation obtained from the Mono-Q step was treated overnight with 6 M urea at 4°C and subjected to gel filtration on a Superose-6 analytical column in the presence of 6 M urea. B, Same as in A except that no urea treatment was involved. In both cases, the fraction containing the highest amount of protein was subjected to SDS-PAGE. The presence of 6 M urea caused the proteins to emerge from the column in a larger number of fractions, and this dilution was reflected in the lower amount of protein found in the SDS-PAGE shown in A. LS, Large subunit; SS, small subunit.

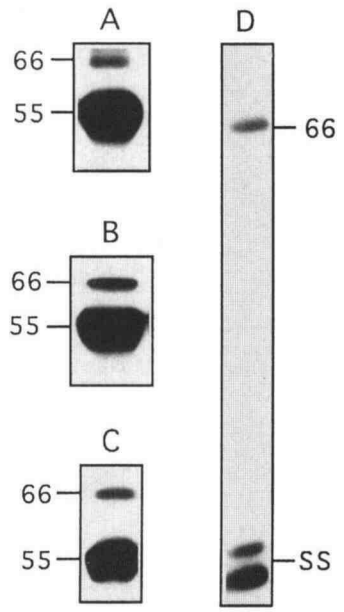


**Figure 3.** Immunoblot analyses of Rubisco in crude extracts and partially purified preparations showing an immunologically cross-reacting 66-kD protein. A, Immunoblot of crude cell-free extract with antibodies against the Rubisco large subunit of *B. braunii*. B, Immunoblot of partially purified (through the Mono-Q step) microsomal Rubisco with antibodies against the spinach Rubisco holoenzyme. C, Immunoblot of the same protein as in B with antibodies prepared against the large subunit of *B. braunii* Rubisco. D, Immunoblot of the same protein as in B with antibodies against the small subunit of *B. braunii* Rubisco (SS). In all cases, SDS-PAGE was done in 11% acrylamide gel and $^{125}$I-protein A was used for detection.

### N-Terminal and Internal Amino Acid Sequences

To further test whether the 66-kD polypeptide co-purified with Rubisco was composed of both large and small subunits of Rubisco, the 66-kD protein was subjected to N-terminal sequencing. The N-terminal amino acid sequence of the 66-kD polypeptide showed high homology with the N-terminal sequence of the mature Rubisco small subunit (Fig. 4A). To test whether amino acid sequences of the large subunit (Fig. 4A) could be detected in the 66-kD protein, this protein was treated with Endoproteinase Glu-C, and the N-terminal sequences of two peptides with molecular sizes of 18 and 32 kD derived by the proteolytic cleavage were determined. The amino acid sequence from the 32-kD peptide demonstrated high homology with the internal sequence of the Rubisco large subunit (Fig. 4B), and the 18-kD peptide gave a se-

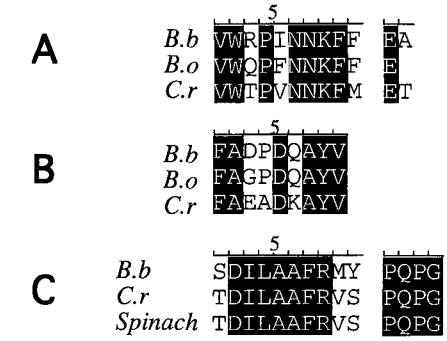


**Figure 4.** Sequence comparisons of three segments of the 66-kD Rubisco from *B. braunii* with those of Rubisco from other sources. A, Alignment of the N-terminal amino acid sequence. B, Alignment of the N-terminal amino acid sequence of an 18-kD fragment proteolytically derived from the 66-kD protein. C, Alignment of the N-terminal amino acid sequence of a 32-kD fragment derived from the 66-kD polypeptide. *B.b, B. braunii; B.o, Batophora oerstedii; C.r, Chlamydomonas reinhardtii.*

quence that showed high homology with an internal segment of the Rubisco small subunit (Fig. 4C). Because the large subunit is conserved through evolution (Ellis, 1981), this amino acid sequence has high homology with a large number of Rubisco proteins from various sources (data not shown). All of the above results strongly suggest that the 66-kD polypeptide is composed of both the large and small subunits of Rubisco.

## DISCUSSION

The 66-kD protein that we detected in the microsomal and soluble fractions of *B. braunii* extracts contains both the large and the small subunits of Rubisco. This conclusion is strongly supported by the immunological cross-reactivity of the 66-kD species with the antibodies prepared against the large and the small subunits separately and against the antibodies prepared against the holoenzyme. Further strong evidence in support of this conclusion is that the 66-kD protein contained amino acid sequences of both the large and small subunits. Since the 66-kD species was not altered by denaturing conditions, such as urea treatment and exhaustive SDS treatment under reducing conditions, the two subunits are most probably covalently linked. Since SDS-PAGE was done under reducing conditions, the two subunits could not be held together by disulfide bonds. Identification of amino acid sequences homologous to the large and small subunits within the 66-kD protein confirms the conclusion that this protein is composed of both subunits. Since the small subunit is smaller than the 18-kD proteolytic fragment derived from the 66-kD protein, the finding that the N-terminal sequence of 18-kD fragment was homologous to an internal sequence of the small subunit shows that this 18-kD fragment contained the small subunit and a portion of the large subunit. How, exactly, the two subunits are linked together in the 66-kD protein remains unknown. Since the N-terminal sequencing of the 66-kD protein yielded sequences characteristic of the small subunit, the large subunit must be attached in such a way that the N terminus of the small subunit remains free. That N-terminal amino acid residues from the large subunit were not released by the sequencing could either mean that the N terminus of the large subunit of the *B. braunii* Rubisco is blocked as found in other organisms (Hwang and Tabita, 1989, 1991) or the N terminus of the large subunit could be attached to the carboxy terminus of the small subunit, possibly representing co-translation of the two subunits. Even though the genes for the two subunits are co-transcribed in some photosynthetic bacteria, the two subunits are not co-translated in such cases (Reith and Cattolico, 1986; Hwang and Tabita, 1989, 1991).

To test whether the genes for the two subunits are co-transcribed in *B. braunii* we made many attempts to detect such transcripts by reverse transcription and PCR using several sets of primers. No evidence for co-transcription was obtained. While we were wondering about the meaning of these results, a report appeared in which it was concluded that UV-B exposure led to the formation of a 66-kD species of Rubisco in higher plants (Wilson et al., 1995), in which the two subunits are known to be transcribed and translated separately. Our results are consistent with a covalent attachment between the two subunits through Trp residues that are known to be at the interface of the two subunits in Rubisco from the other species as suggested by these authors. Although such attachment in higher plants was suggested to be produced by UV cross-linking, our cultures were not deliberately exposed to UV. However, since the algal cultures were grown under light, photo cross-linking could have happened under such growth conditions. Another possibility is an isopeptide-type cross-linking between the large and small subunits catalyzed by transglutaminase, which has been demonstrated to co-fractionate with Rubisco in crude extracts and is thought to interact with Rubisco in intact chloroplasts (Margosiak et al., 1989). Since the fractionation procedures, such as Suc density gradient centrifugation that would not disrupt intermonomer interactions, did not separate the 66-kD species from native Rubisco, it is likely that the cross-linked species is produced while the two types of subunits are in the native complex and remain in the complex during the purification procedures. Whether the type of cross-links observed would have any biological consequences remains uncertain.

## ACKNOWLEDGMENT

We thank Debra Gamble for her assistance in preparing the manuscript.

Received December 6, 1995; accepted March 5, 1996.
Copyright Clearance Center: 0032–0889/96/111/0441/05.

## LITERATURE CITED

**Andrews TJ, Lorimer GH** (1987) Rubisco: structure, mechanisms, and prospects for improvement. *In* MD Hatch, NK Boardman, eds, The Biochemistry of Plants, Vol 10. Academic Press, New York, pp 131–219

**Dennis MW, Kolattukudy PE** (1992) A cobalt-porphyrin enzyme converts a fatty aldehyde to a hydrocarbon and CO. Proc Natl Acad Sci USA **89:** 5306–5310

**Ellis RJ** (1981) Chloroplast proteins: synthesis, transcript, and assembly. Annu Rev Plant Physiol **32:** 111–137

**Fujiwara S, Iwahashi H, Somega J, Nishikawa S, Minaka N** (1993) Structure and cotranscription of the plastid-encoded rbcL and rbcS genes of *Pleurochrysis corferae* (PRYMNESIOPHYTA). J Phycol **29:** 347–355

**Hwang S-R, Tabita FR** (1989) Cloning and expression of the chloroplast-encoded rbcL and rbcS genes from the marine diatom *Cylindrotheca* sp. strain N1. Plant Mol Biol **13:** 69–79

**Hwang S-R, Tabita FR** (1991) Cotranscription, deduced primary structure, and expression of the chloroplast-encoded rbcL and rbcS genes of the marine diatom *Cylindrotheca* sp. strain N1. J Biol Chem **266:** 6271–6279

**Margosiak SA, Dharma A, Bruce-Carver MR, Gonzales AP, Louie D, Kuehn GD** (1989) Identification of the large subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase as a substrate for transglutaminase in *Medicago sativa* L. (Alfalfa). Plant Physiol **92:** 88–96

**Reith ME, Cattolico RA** (1986) Inverted repeat of *Olistho discus luteas* chloroplast DNA contains genes for both subunits of ribulose-1,5-bisphosphate carboxylase and the 32,000-dalton $Q_B$ protein: phylogenetic implication. Proc Natl Acad Sci USA **83:** 8599–8603

**Tabita FR** (1988) Molecular and cellular regulation of autotrophic carbon dioxide fixation in microorganisms. Microbiol Rev **52:** 155–189

**Towbin H, Staehelin T, Gordon J** (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. Proc Natl Acad Sci USA **76:** 4350–4354

**Valentin K, Zetsche K** (1989) The genes of both subunits of ribulose-1,5-bisphosphate carboxylase constitute an operon on the plastome of a red alga. Curr Genet **16:** 203–209

**Whitman W, Tabita FR** (1976) Inhibition of D-ribulose 1,5-bisphosphate carboxylase by pyridoxal 5′-phosphate. Biochem Biophys Res Commun **71:** 1034–1039

**Wilson MI, Ghosh S, Gerhardt KE, Holland N, Babu S, Edelman M, Dumbroff EB, Greenberg BM** (1995) *In vivo* photomodification of ribulose-1,5-bisphosphate carboxylase/oxygenase holoenzyme by ultraviolet-B radiation. Plant Physiol **109:** 221–229