Orderly Disposition of Heterogeneous Small Subunits in \(\Delta\)-Ribulose-1,5-bisphosphate Carboxylase/Oxygenase from Spinach*

(Received for publication, July 26, 1996, and in revised form, August 30, 1996)

Naoki Shibata, Tsuyoshi Inoue, Kazuhiro Fukuhara, Yoshitaka Nagara, Ryouichi Kitagawa, Shigeharu Harada, Nobutami Kasai, Koichi Uemura, Ko Kato, Akihiko Yokota, and Yasushi Kai

From the Department of Applied Chemistry, Faculty of Engineering, Osaka University, Suita, Osaka 565, Japan and the National Institute of Innovations for Technology Promotion, the Foundation for Earth Environmental Disabilities in Kyoto 619-02, Japan

We determined the crystal structure of spinach ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) by x-ray diffraction at 1.8-Å resolution and found that the enzyme contained two kinds of S, S1, and S2, present in equal number and disposed in an orderly way within the Rubisco holoenzyme. The electron density maps suggested that leucine was at residue 56 in S2, although histidine was at that position in S1. There were other residue differences. Thus, spinach Rubisco has a \(L_5S_2S_1S_4\) subunit structure. The orderly disposition of the heterogeneous small subunits in the Rubisco holoenzyme provides accounts of a multigene family of S in plants.

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39) is the key enzyme catalyzing the primary reactions in photosynthesis as well as photorespiration (1). This enzyme is an important enzyme involved in regulation and synthesis in complex ways (1). Cells of green plants contain 50–100 chloroplasts, each of which has 20–900 copies of the genome (2). Because of these large numbers, cells can synthesize much enzyme rapidly. Rubisco from some bacteria and eukaryotes is composed of eight large (L) and eight small (S) subunits (3). The gene for L is encoded in the plastid genome, and 4–13 S genes compose the multigene family in higher plants (4). A multigene family of S subunits in plants probably facilitates fine tuning of the rate of synthesis of these subunits relative to L subunits (5). All S genes are expressed in green leaves of plants. The multiplicity in the genome construction may make possible transitory, organ-specific, or signal-specific expression of different genes that have individual promoters (6). Where do the translations of this multigene family in green leaves reside in the structure of Rubisco holoenzyme? Crystal structures of Rubiscos from tobacco (7–10), spinach (11–14), and a cyanobacterium (15, 16) are consistent with a hexadecameric \(L_5S_8\) structure in which all S subunits are identical. We analyzed the crystal structure of spinach Rubisco at 1.8-Å resolution to answer the question.

EXPERIMENTAL PROCEDURES

Purification and Crystallization—Rubisco was purified from spinach leaves with polyethylene glycol 4000 (PEG 4000)/MgCl2 (17) instead of ammonium sulfate as reported previously. The enzyme was stored as a precipitate in a mixture of 20% PEG 4000 and 20 mM MgCl2, collected by centrifugation at 15,000 × g for 20 min, and dissolved in 10 mM potassium phosphate buffer (pH 7.0). The enzyme was dialyzed against the same buffer overnight and put on a column (1.5 × 7 cm) of hydroxyapatite that had been washed with the phosphate buffer. The effluent from the column contained Rubisco, and the enzyme, free from contaminants, was collected by precipitation in a mixture of PEG 4000 and MgCl2 at 8°C.

Crystals were grown by vapor diffusion with 6 ml drops containing a protein solution (15 mg/ml) in 7% PEG 4000, 20 mM MgCl2, 20 mM NaHCO3, 1 mM dithiothreitol, and 50 mM Bicine (pH 7.9), and reservoir solutions containing 9% PEG 4000, 20 mM MgCl2, 20 mM NaHCO3, 1 mM dithiothreitol, and 50 mM Bicine (pH 7.9) at 20°C. Crystals were of space group C222, with unit cell dimensions of \(a = 157.8\), \(b = 157.8\), and \(c = 200.9\) Å, isomorphous to crystals prepared with ammonium sulfate (18). If we assume that there are four large subunits and four small subunits in an asymmetric unit, the solvent content of the crystal is \(46\% V_s = 2.27\AA^3 D\)

Data Collection and Refinement of Structure—Diffraction data from the Rubisco crystals were collected at room temperature with a Weissenberg camera for macromolecules (20) at the Photon Factory. A total of 586,168 observations was recorded from two crystals and was reduced to 216,085 unique reflections. The data were 67% complete to 1.6-Å resolution with an \(R_p = 7.5\%\)

The crystal structure to 2.4-Å resolution of spinach Rubisco was used as an initial model (Brookhaven Protein Data Bank code, 8RUB) (12). After rigid body and positional refinement, a simulated annealing method (21) was used on SGI indigo2 and NEC EWS4800 workstations. Data between 6.0 and 2.5 Å were used for these calculations. After positional refinement and individual temperature factor refinement, an atomic model was fitted to a 2\(F_o - F_c\) electron density map with the program FRODO (22). Noncrystallographic restraints were used throughout the refinement process. Gradual expansion of the resolution range gave the final model.

Two-dimensional Gel Electrophoresis—Thirty micrograms of purified spinach Rubisco was analyzed by two-dimensional electrophoresis and Coomassie Blue staining with a horizontal electrophoresis system (Multiphore II, Pharmacia Biotech Inc.). A broad pH gradient gel ranging from pH 3 to 10.5 (Immobile DryStrip, Pharmacia), was used for isoelectrofocusing in the first dimension, and a 15% SDS-polycrylamide gel was used in the second dimension. Sample preparation,
electrophoresis, and staining were done as recommended by the manufacturer of the system.

RESULTS AND DISCUSSION

The results of the structure analysis of the four pairs of an L subunit with an S subunit in an asymmetric unit of the crystal lattice are shown in Table I. While refining the structures, we found that the side chain skeletons of some residues in the S subunits, reported by Martin (23), deviate significantly from our electron density maps, especially at residue 56, and that the shapes of the maps of the four crystallographically independent small subunits, named as S1, S2, S3, and S4, were different. Examination of the electron density maps suggested that residue 56 in S1 and S3 was leucine instead of the aspartate reported elsewhere (23) and that residue 56 in S2 and S4 was histidine (Fig. 1). The electron density maps at residue 93 suggested further structural differences; the reported alanine side chains of S2 and S4 fit the maps well, but S1 and S3 had a much longer side chain at this position (the residue could not be identified). Still other differences were observed in residue 8. These findings are evidence that spinach Rubisco had two S

![Figure 1](image1)

**Fig. 1.** Ball-and-stick stereo models around residue 56 of four small subunits with 2Foobs – Foobs electron density maps. A, S1; B, S2; C, S3; and D, S4. The green broken lines in B and D show the His-56Ne–Glu-259-O hydrogen bonds. These density maps were calculated without the contribution of residue 56. The contour level of all maps is set at 1.5σ. These figures were drawn with a program Proteus (System Co., Ltd., Japan).

![Figure 2](image2)

**Fig. 2.** Two-dimensional gel electrophoresis of purified spinach Rubisco. LSU and SSU indicate the L and S peptides, respectively. The stained gel between pI 5.88 and 6.60 is shown in the figure.
chains. Two-dimensional electrophoresis showed two peptides of S with one L peptide (Fig. 2) as reported before (24). With the pl of L taken to be 6.13, as calculated from the reported amino acid sequence (25) with GENETYX-MAX, Version 8 (Software Development Co., Ltd.), the pl points of the two S peptides were 6.10 and 6.42. The observed difference in the pl’s was partly due to the residue at position 56 being leucine in one peptide and histidine in the other. This difference should give rise to a difference in the pl of 0.13. The larger difference (0.32) actually found might be explained by other differences in the amino acid residues at positions 8 and 93 or elsewhere; these could not be identified in this study. Thus, the results of x-ray diffraction analysis showed that spinach Rubisco had two kinds of small subunits: Si, with Leu-56, and Sii, with His-56. Spinach Rubisco, therefore, has a L8S4S4 structure, not L8S8 as reported before (11–14).

Fig. 3 shows the L8S4S4 structure schematically. Earlier spinach Rubisco was described as having D4 point symmetry (11–14), which would be possible only if Rubisco were composed of eight identical large subunits and eight identical small subunits. Our x-ray results showed that what might be the 4-fold symmetry of spinach Rubisco was broken by the heterogeneity of the S subunits. The same kind of small subunits occupies the positions furthest from each other, maintaining 2-fold symmetry along the central core.

The Ne atom of His-56 in the Shi subunits forms a hydrogen bond with the carbonyl oxygen atom of Glu-259 in the neighboring large subunit (Fig. 1). On the other hand, the side chain of Leu-56 of the Sii subunits does not interact electrostatically. The additional ShiHis-56 – Glu-259-O hydrogen bond must cause the difference in the dissociation constants; the L-Sii interaction must be more stable because of the hydrogen bond between His-56 and Glu-259, and the Sii subunit will construct the L-S pair more readily than Sii. In this context, it is interesting to recall the finding that a highly conserved sequence of 16 amino acids, including that at position 56, is essential for the assembly of L and S in the plant enzyme (26).

In terms of its different interactions of the two S subunits, L also may be of two kinds; the structure of spinach Rubisco may be \([L^I S^I]_8 S^I ([L^II S^II]_8 S^II]_4\), where \(L^I\) is the L that has the \(S^I\)His-56 – Glu-259 hydrogen bond and \(L^I\) does not have such a bond, and \(L^I S^I\) and \(L^II S^II\) are mean \(L_2\) dimers (12) formed by the same kind of large subunits. Glu-259 participates in a dimer-dimer interaction with \(Arg-258\) in the neighboring \(L_2\) dimer and may be involved in the transfer of signals of an \(L_2\) dimer to the next one. Plant Rubisco gradually decreases in activity to a constant level during reaction (17). The decrease is smaller if there is binding of the substrate ribulose 1,5-bisphosphate to the noncatalytic substrate-binding sites (27). Binding of ribulose 1,5-bisphosphate to these sites proceeds cooperatively; binding to the first four sites suppresses binding to the remaining four sites (28, 29). The grouping of eight large and eight small subunits into two different structures as described above may give an account for the cooperativity in plant Rubisco. Thus, a multigene family for S may be related to a genetic mechanism that has given the enzyme the ability to fine tune its own catalysis.

Acknowledgments—We thank N. Sakabe and A. Nakagawa for support in data collection at KEK, Japan.

REFERENCES
1. Andrews, T. J., and Lorimer, G. H. (1987) The Biochemistry of Plants, Vol. 10, pp. 131–218, Academic Press, New York
2. Muñoz, J. E. (1988) Annu. Rev. Plant Physiol. Plant Mol. Biol. 39, 475–502
3. Akazawa, T. (1979) Encyclopedia of Plant Physiology, Vol. 6, pp. 208–229, Springer-Verlag, Berlin
4. Manzara, T., and Gruissem, W. (1988) Photosynth. Res. 16, 117–139
5. Dean, C., Dunsmuir, P., and Bedbrook, J. (1987) Tailoring Genes for Crop Improvement: An Agricultural Perspective, pp. 59–68, Plenum Press, New York
6. Meier, T., Callan, K. L., Fleming, A. J., and Gruissem, W. (1995) Plant Physiol. 107, 1105–1118
7. Chapman, M. S., Suh, S. W., Casacio, D., Smith, W. W., and Eisenberg, D. (1987) Nature 329, 354–356
8. Chapman, M. S., Suh, S. W., Curmi, P. M. G., Casacio, D., Smith, W. W., and Eisenberg, D. S. (1988) Science 241, 71–74
9. Curmi, P. M. G., Casacio, D., Sweet, R. M., Eisenberg, D., and Schreuder, H. G. (1992) J. Biol. Chem. 267, 16980–16989
10. Schreuder, H. A., Knight, S., Curmi, P. M. G., Andersson, I., Casacio, D., Sweet, R. M., Branden C.-I., and Eisenberg, D. (1993) Protein Sci. 2, 1136–1146
11. Knight, S., Andersson, I., and Brandén, C.-I. (1999) Science 244, 702–705
Heterogeneous Small Subunits in Rubisco

12. Knight, S., Anderson, I., and Brandén C.-I. (1990) J. Mol. Biol. 215, 113–160
13. Taylor, T. C., and Andersson, I. (1996) Nat. Struct. Biol. 3, 95–101
14. Andersson, I. (1996) J. Mol. Biol. 259, 160–174
15. Newman, J., and Gutteridge, S. (1993) J. Biol. Chem. 268, 25876–25886
16. Newman, J., Branden, C.-I., and Jones, T. A. (1993) Acta Crystallogr. Sec. D 49, 548–560
17. Yokota, A. (1991) J. Biochem. (Tokyo) 110, 246–252
18. Andersson, I., and Branden C.-I. (1984) J. Mol. Biol. 172, 363–366
19. Matthews, B. W. (1968) J. Mol. Biol. 33, 491–497
20. Sakabe, N. (1983) J. Appl. Crystallogr. 16, 542–547
21. Brünger, A. T., Kuriyan, J., and Karplus, M. (1987) Science 235, 458–460
22. Johns, T. A. (1985) Methods Enzymol. 115, 157–171
23. Martin, P. G. (1979) Aust. J. Plant Physiol. 6, 401–408
24. Ren, L., Salnikow, J., and Vater, J. (1991) Plant Sci. 74, 1–6
25. Zarawski, G., Perrot, B., Bottomley, W., and Whitfeld, P. R. (1981) Nucleic Acids Res. 9, 3251–3270
26. Wasmann, C. C., Ramage, R. T., Bohnert, H. J., and Ostrem, J. A. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 1198–1202
27. Yokota, A., Wadano, A., and Murayama, H. (1996) J. Biochem. (Tokyo) 119, 487–499
28. Yokota, A., Higashioka, M., Taïra, T., Usuda, H., Wadano, A., and Murayama, H. (1994) Plant Cell Physiol. 35, 317–321
29. Yokota, A., Higashioka, M., and Wadano, A. (1991) J. Biochem. (Tokyo) 110, 253–256
30. Ferrin, T. E., Huang, C. C., Jarvis, L. E., and Langridge, R. (1988) J. Mol. Graphics 6, 13–27
Orderly Disposition of Heterogeneous Small Subunits in D-Ribulose-1,5-bisphosphate Carboxylase/Oxygenase from Spinach
Naoki Shibata, Tsuyoshi Inoue, Kazuhiro Fukuhara, Yoshitaka Nagara, Ryouichi Kitagawa, Shigeharu Harada, Nobutami Kasai, Koichi Uemura, Ko Kato, Akiho Yokota and Yasushi Kai

J. Biol. Chem. 1996, 271:26449-26452.
doi: 10.1074/jbc.271.43.26449

Access the most updated version of this article at http://www.jbc.org/content/271/43/26449

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 29 references, 7 of which can be accessed free at http://www.jbc.org/content/271/43/26449.full.html#ref-list-1