The large subunit (LSU) of ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) in the illuminated lysates of wheat (*Triticum aestivum* L.) chloroplasts is broken down by reactive oxygen radicals into 37- and 16-kDa polypeptides. Analysis of the terminal amino acid residues of both fragments revealed that the C terminus of the 37-kDa fragment was Ser-328 and the N terminus of the 16-kDa fragment was Thr-330. Gly-329, which links the two fragments, was missing, suggesting that the fragmentation of the LSU in the lysates driven by oxygen-free radicals occurs at Gly-329. Purified rubisco, exposed to a hydroxyl radical-generating system, was also cleaved at the same site of the LSU. The cleavage site was positioned at the N-terminal end of the flexible loop (loop 6) within the β/α-barrel domain, constituting the catalytic site of rubisco. The binding of a reaction intermediate analogue, 2-carboxyarabinitol 1,5-bisphosphate, to the active form of rubisco completely protected the enzyme from the fragmentation. The fragmentation was differentially affected by CO$_2$, Mg$^{2+}$, ribulose 1,5-bisphosphate, or 2-carboxyarabinitol 1,5-bisphosphate. All these results indicate that the conformation of the catalytic site of the enzyme is involved as an important factor determining the breakdown of rubisco by reactive oxygen species. Reactive oxygen species generated at its catalytic site by a Fenton-type reaction may trigger the site-specific degradation of the LSU in the lysates of chloroplasts in the light.

Ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco, EC 4.1.1.39) catalyzes two competing reactions, photosynthetic CO$_2$ fixation and photorespiratory carbon oxidation, in the stroma of the chloroplasts. This enzyme is the most abundant protein, which accounts for 30–35% of total leaf protein in C$_3$ plants, and the amount of rubisco can be a limiting factor for light-saturated photosynthesis in air (1–4). During senescence rubisco is one of the early proteins that are broken down (5), therefore affecting photosynthesis and nitrogen economy in plants. Only limited information is available on the triggering mechanisms that cause rubisco degradation in plants (6–8).

It is widely accepted that the initial step of the rubisco degradation in leaves must occur within the chloroplast (6, 7). In illuminated chloroplasts, in which light energy is harvested and converted to chemical energy, production of reactive oxygen species occurs as an unavoidable event, particularly under light or oxidative stress conditions (9, 10) that cause degradation of rubisco (5, 11–15). Reactive oxygen species are known to inactivate and modify enzymes in diverse biological systems (16). Reactive oxygen species could be one means by which rubisco degradation is triggered. For example, Mehta et al. (5) showed that Cu$^{2+}$-induced oxidative stress caused intermolecular cross-linking of the large subunits of rubisco via disulfide bounds within the holoenzyme, rapid and specific translocation of the soluble enzyme complex to the chloroplast membranes, and finally rubisco degradation. In addition, Desimone et al. (15, 17) reported that light stress induces reactive oxygen-mediated denaturation of rubisco followed by proteolytic degradation of the large subunit (LSU) in chloroplasts (15) or its lysates (17). These reports indicated that oxidative stress induces a fragmentation of rubisco protein, which then leads to its subsequent degradation, perhaps by a protease. On the other hand, we recently found that the LSU of rubisco can be directly fragmented into 37- and 16-kDa polypeptides by reactive oxygen species in chloroplast lysates (18) and in intact chloroplasts (19) under illumination. This fragmentation of rubisco was completely inhibited in the presence of metal chelators (EDTA or 1,10-phenanthroline), catalase, or hydroxyl radical scavenger (n-propyl gallate), but not in the presence of protease inhibitors. A similar fragmentation was also observed for the purified rubisco exposed to a hydroxyl radical-generating system.

We have now identified the cleavage site where LSU of rubisco in the chloroplast lysates in the light and LSU of purified rubisco exposed to hydroxyl radicals are fragmented. In addition, we found that effectors potentially bound to its active site, such as CO$_2$, Mg$^{2+}$, ribulose 1,5-bisphosphate (RuBP), and 2-carboxyarabinitol 1,5-bisphosphate (CABP), affect to different extents the susceptibility of the enzyme to fragmentation mediated by hydroxyl radicals. Of particular significance is the finding that CABP, a reaction intermediate analogue of the carboxylation reaction of the enzyme, which is known to tightly bind to the catalytic site of the activated form of rubisco (20, 21), fully protected the enzyme from the fragmentation. We suggest that one mechanism of the site-specific fragmentation of rubisco in the illuminated lysates of chloroplasts may involve the generation of reactive oxygen species, probably hydroxyl radicals, at its catalytic site by a Fenton-type reaction.
EXPERIMENTAL PROCEDURES

Plant Material and Isolation of Chloroplasts—Wheat (Triticum aestivum L. cv. Aoba) seeds were planted on a plastic net floating on tap water in a pot and grown in a phytotron with a day/night temperature of 20/18°C and 70% relative humidity. The photoperiod was 12 h, with a quantum flux density of 300 μmol of quantum m−2 s−1 at plant height. Chloroplasts were isolated from the primary leaves of 12-day-old seedlings by a mechanical method using continuous Percoll gradient centrifugation as described previously (18).

Purification of Rubisco and Binding of CABP to Activated Enzyme—Rubisco was purified from wheat leaves as described previously (18). The enzyme-CO2-Mg2+-CABP complex was produced as follows. The purified enzyme (5 mg/ml) was pretreated at 37 °C for 1 h and then activated with 10 mM NaHCO3 and 20 mM MgCl2 at 25 °C for 20 min in 50 mM Hepes-NaOH, pH 8.0, containing 1 mM dithiothreitol (DTT). A 2-fold excess (with respect to active sites) of CABP (a gift from Dr. Tadaaki Yamashita) was added and allowed to stand for 20 min. After the reaction, the enzyme-CO2-Mg2+-CABP complex was separated from unbound CABP by gel filtration through a column of Sephadex G-25 (Amersham Pharmacia Biotech). Gel Electrophoresis and Immunoblotting—SDS-PAGE was performed by the method of Laemmli (22), except that SDS sample buffer was changed to 100 mM Tris-HCl, pH 8.5, containing 1% (v/v) SDS, 10% (v/v) glycerol, and 2.5% (v/v) 2-mercaptoethanol at final concentrations. Native PAGE was performed (22) with 5% (v/v) polycrylamide gel in which SDS was omitted. Two-dimensional electrophoresis was carried out by the method of O’Farrell (23), except that 2% (v/v) Ampholine (pH range, 3.5–10; Amersham Pharmacia Biotech) was used in isoelectric focusing. Immunoblot analysis was done as described previously (18).

Isolation of LSU Fragments from Chloroplast Lysates Exposed to Light—The chloroplast lysates were incubated at 4 °C for 1 h in the light at 2000 μmol of quantum m−2 s−1 in 50 mM 2-morpholinoethanesulfonic acid-NaOH, pH 5.7, containing 1 mM DTT and 10 μM E-64. After incubation, the lysates were centrifuged at 38,900 × g for 30 min, and the supernatant fraction was subjected to 35–55% (w/v) (NH4)2SO4 saturation. The precipitate was dissolved in 20 mM Tris-HCl, pH 7.6, containing 12.5% (v/v) glycerol, 1 mM DTT, and 1 mM EDTA, and passed through a column of Econo-Pac 10DG (Bio-Rad) previously equilibrated with the same buffer. The eluted protein fractions were applied to an ion exchange column of RESOURCE Q (Amersham Pharmacia Biotech) previously equilibrated with the same buffer using the FPLC system (Amersham Pharmacia Biotech). The proteins were eluted with 40 μl of the same buffer containing a linear gradient of 0–5% (w/v) NaCl at a flow rate of 1 ml/min. The fractions containing rubisco and its fragments were loaded onto a gel filtration column of Superdex 200 (Amersham Pharmacia Biotech) previously equilibrated with 50 mM Na-phosphate, pH 7.5, containing 12.5% (v/v) glycerol, 1 mM DTT, and 1 mM EDTA, and eluted with the same buffer at a flow rate of 0.5 ml/min using the FPLC system. The 37- and 16-kDa fragments co-eluted with intact rubisco throughout this purification step. For the isolation of 16-kDa fragments, the final fractions containing rubisco and its fragments were subjected to two-dimensional electrophoresis, and the separated fragment was electroblotted onto a polyvinylidene difluoride membrane. For the isolation of the relatively large quantity of 37-kDa fragment, the eluted proteins from the gel filtration column were reduced in 0.1M Tris-HCl, pH 8.5 containing 8 M urea at 4 °C for 2 h under N2. Then, the proteins were incubated with 0.6% (w/v) 4-vinylpyridine for 30 min. The pyrididethylated proteins were applied to a preparative SDS-PAGE system (Mini Prep Cell; Bio-Rad). The acrylamide concentration in the separation gel was 11% (w/v), and the procedure was followed according to the manufacturer’s instructions. The eluted fraction of the 37-kDa fragment was precipitated and washed with acetone to remove SDS. The precipitate was dissolved in 0.1M Tris-HCl, pH 8.5, containing 8 μl urea and loaded onto a reversed phase chromatography (ProRPC; Amersham Pharmacia Biotech) using HPLC (LC-10Aii series; Shimazu, Kyoto, Japan). The 37-kDa fragment was eluted with a 120-min linear gradient of 5–80% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid at a flow rate of 0.3 ml/min.

Isolation of LSU Fragments from the Purified Rubisco Exposed to Hydroxyl Radical-Generating System—Purified rubisco (1 mg/ml) was exposed to the hydroxyl radical-generating system comprising 1 mM H2O2, 10 μM FeSO4, and 20 mM ascorbic acid at 4 °C for 15 min in 50 mM Hepes-NaOH, pH 8.0, containing 3% (v/v) glycerol and 1 mM DTT. The mixture was passed through a column of Econo-Pac 10DG previously equilibrated with 0.1M Tris-HCl, pH 8.5, containing 1 mM EDTA and 1 mM DTT. The 16-kDa fragment was isolated from this fraction by two-dimensional electrophoresis as described above. The 37-kDa fragment was also isolated by the same procedure using the preparative SDS-PAGE system and reversed phase HPLC as described above.

Proteolytic Digestion of the 37-kDa Fragment and Peptide Characterization—The 37-kDa fragment (~20 μg) was digested with 0.4 μg of lysylendopeptidase (Wako Pure Chemical Industries, Osaka, Japan) in 10 mM Tris-HCl, pH 9.0, containing 2 μM urea at 37 °C for 15 h. The peptides were separated by HPLC using a reverse phase column of μRPC (Amersham Pharmacia Biotech). After the column was washed with 10% (v/v) acetonitrile in 0.06% (v/v) trifluoroacetic acid, the peptides were eluted with a 100-min linear gradient of 10–25% (v/v) acetonitrile in 0.06% (v/v) trifluoroacetic acid at a flow rate of 0.2 ml/min. N-terminal amino acid sequencing was performed using a protein sequencer (model 491; Applied Biosystems) equipped with an on-line analyzer of the phenylthiobodiesin derivative of the amino acids. Electrospray ionization-mass spectrometry analysis was performed using a triple-quadrupole mass spectrometer (API 365; Perkin-Elmer Sciex).

RESULTS

Identification of the Cleavage Site—The chloroplast lysates were incubated in the light, and proteins were electrophoretically separated and then immunoblotted. The N-terminal 37-kDa and C-terminal 16-kDa fragments of the rubisco-LSU were the most dominant degradation products. When a purified preparation of rubisco was exposed to a hydroxyl radical-generating system, the cleavage of the LSU into 37- and 16-kDa fragments was also observed (18). To identify the cleavage site, we isolated the 37- and 16-kDa fragments of the LSU from the chloroplast lysates incubated in the light and those generated from the purified rubisco after exposure to the hydroxyl radical-generating system. The 37- and 16-kDa fragments always co-purified with the intact rubisco throughout the purification steps. The LSU and 16-kDa fragments were separated by reverse phase chromatography (RESQ ( RESOURCE Q), and gel filtration (Superdex 200) (Fig. 1A). The purified fraction from gel filtration showed only a single band on native PAGE (Fig. 1B). These results indicate that both fragments co-exist as parts of the holoenzyme form comprising hexaomeric.
Rubisco Breakdown by Reactive Oxygen Species

The rubisco fraction containing the fragmented LSU obtained from the chloroplast lysates after a series of purification steps (Fig. 1) and the gels were subjected to two-dimensional gel electrophoresis, and the gels were stained with Coomassie Blue. The rubisco fraction containing the fragmented LSU obtained from the chloroplast lysates after a series of purification steps (Fig. 1) and the gels were stained with Coomassie Blue. Arrowheads indicate the positions of the LSU and the small subunit of rubisco and the fragments of the LSU with their molecular masses.

applied to a protein sequencer. The N-terminal sequences of the 16-kDa fragment generated in the chloroplast lysates and from the purified rubisco were TVVKLEGEREMLTG and XVVKLEGEXXMTLG, respectively. These sequences correspond to the deduced residues 330–344 in wheat LSU (24). When the 37-kDa fragment electroblotted onto the polyvinylidene difluoride membrane was applied to a protein sequencer, no phenylthiohydantoin derivatives of amino acids were released after three cycles of Edman degradative sequencing. This was expected because the N-terminal proline residue of intact LSU of rubisco; B, the 37-kDa fragment of the LSU purified from the lysates of chloroplasts and masks the metal and the substrate binding sites (20, 21, 26). Thus, it was of interest to examine the effect of the binding of CABP on the fragmentation. The enzyme CO2,Mg2+-CABP complex (active form-CABP complex) added to the thylakoid fraction was not fragmented even in the light, although the LSU of the purified rubisco (inactive form) added to the thylakoid fraction was fragmented in the light (Fig. 4). These results clearly indicated that the binding of CABP to the active form of rubisco protected the enzyme from the fragmentation.

Effects of CO2,Mg2+, RuBP, and CABP on the fragmentation of purified rubisco exposed to the hydroxyl radical-generating system—Purified rubisco was preincubated with various combinations of CO2,Mg2+, RuBP, and CABP. Then the mixtures bonds at the carboxylic side of lysine, and the C-terminal amino acid of peptides 2 and 3 is not lysine, both peptides are derived from the C-terminal region of the 37-kDa fragments. From electrospray ionization-mass spectrometry analysis, masses of 1278.9 and 1295.0 were obtained for peptide 2. Because the theoretical mass of ALRMSGGDHIHS is 1278.9, these results indicated that peptide 2 was terminated at Ser-328 and did not contain the Gly-329 residue. From these results, we concluded that the LSU is cleaved at Gly-329 or at both ends of this residue into the 37- and 16-kDa fragments.

CABP Binding Protects LSU from Fragmentation—The cleavage site of the LSU, Gly-329, is one of the residues that constitutes the catalytic site of the enzyme (26). It is known that CABP, a reaction intermediate analogue of the enzyme carboxylation reaction, tightly binds to the active form (ternary complex of enzyme,CO2,Mg2+). CO2,Mg2+-CABP complex (active form-CABP complex) added to the thylakoid fraction was not fragmented even in the light, although the LSU of the purified rubisco (inactive form) added to the thylakoid fraction was fragmented in the light (Fig. 4). These results clearly indicated that the binding of CABP to the active form of rubisco protected the enzyme from the fragmentation.

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we were exposed to the hydroxyl radical-generating system. The results are shown in Fig. 5. The fragmentation of the rubisco LSU was most pronounced when the enzyme was incubated alone (Fig. 5, lane 2). Preincubation of the enzyme with CO₂ (Fig. 5, lane 5) or Mg²⁺ (Fig. 5, lane 9) did not cause significant influence on the fragmentation. When the enzyme was, however, incubated with CO₂ and Mg²⁺ together, and once the ternary complex (enzyme-CO₂-Mg²⁺, active form) was formed, the fragmentation was significantly suppressed (Fig. 5, lane 6). The enzyme complex with RuBP (enzyme-RuBP) or CABP (enzyme-CABP) was degraded less than the enzyme alone but more than the enzyme-CO₂-Mg²⁺ complex (Fig. 5, lane 4 or 3). The complex of the active form with CABP (enzyme-CO₂-Mg²⁺-CABP) was not fragmented at all (Fig. 5, lane 7), as in the case of the complex of activated rubisco with the illuminated thylakoid (Fig. 4). The active form complexed with RuBP (enzyme-CO₂-Mg²⁺-RuBP) was resistant to fragmentation, as was the complex of the active form incubated with CABP (Fig. 5, lane 8).

**DISCUSSION**

The Cleavage Site of the LSU and Involvement of Hydroxyl Radicals—In the present study we determined the specific cleavage site of the LSU fragmented in the lysates of chloroplasts incubated in the light. The C-terminal amino acid residue of the 37-kDa fragment was Ser-328, and the N-terminal amino acid residue of the 16-kDa fragment was Thr-330, as found in the present study. However, it could not be explained why the Gly-329 was missing. It is not likely that the residue Gly-329 was removed by proteases in the lysates from the terminal end of the 37- or 16-kDa fragment after the cleavage of the LSU by reactive oxygen species, because Gly-329 was also missing in the fragments derived from the purified rubisco exposed to the hydroxyl radicals. Another, as yet unknown, mechanism of protein fragmentation by reactive oxygen species needs to be considered.

Mechanisms of Site-specific Fragmentation of the LSU—Rubisco from higher plants is a hexadecameric of eight LSU and eight small subunits. The fragmentation of rubisco was specific to the LSU; no fragmented product was found for the small subunits (data not shown). In addition, the cleavage of the LSU was observed at a specific site. As reviewed by Stadtman and Oliver (29), the metal-catalyzed oxidation of proteins is a site-specific process involving the interaction of oxygen or hydrogen peroxide and a redox-active metal at metal-binding sites on the protein. Rubisco requires Mg²⁺ or Mn²⁺ for the formation of a ternary complex of the enzyme-CO₂-Mg²⁺ (Mn²⁺), which is an active form of the enzyme. All the active sites of rubisco are on the LSU. Fe²⁺ is potentially able to bind to the metal binding site of the LSU, because Fe²⁺ can activate rubisco instead of Mg²⁺ (30). Chloroplasts are especially rich in iron and contain ~80% of the total iron of mesophyll cells (31). Rubisco possesses oxygenase activity, and the binding site of O₂ is the same as that of CO₂. Moreover, H₂O₂ competitively interacts with the binding site for O₂ and CO₂ (32). Therefore, it can be hypothesized that, under our experimental conditions, namely certain light- or oxygen-stressed conditions, the production of
hydroxyl radicals via a Fenton-type reaction occurs at the catalytic site of rubisco, which brings about the fragmentation of the LSU. The fragmentation of the LSU was completely protected by CABP binding to the catalytic sites of the enzyme (Figs. 4 and 5). The enzyme-CO₂-Mg²⁺-CABP complex is so stable that neither activator CO₂ nor Mg²⁺ can be readily replaced by unbound ligand (20, 21). Thus, Fe²⁺ or H₂O₂ (O₂) is unable to bind to their site at the active site, and the production of hydroxyl radical is prevented. As a consequence of this, under these conditions, LSU fragmentation does not occur even in the light. These results further support the above hypothesis.

No cleavage of the LSU was observed in the presence of 0.1% SDS even when the enzyme was exposed to the hydroxyl radical-generating system (data not shown). In the presence of SDS, rubisco disrupts into subunits and loses the enzyme activity (33). These results strongly indicate that a correctly folded structure of the enzyme is required for the fragmentation of rubisco by reactive oxygen species. This is largely different from rubisco degradation mediated by a protease (34, 35), because proteolytic degradation is often stimulated by SDS.

The LSU consists of two separate domains, a smaller N-terminal domain and a larger C-terminal domain. The N-terminal domain is built from a five-stranded β-sheet and two α-helices on one side of the sheet, and the C-terminal domain is from an eight-stranded parallel β/α-barrel, which has a flexible loop (loop 6), as commonly observed among β/α-barrel proteins. The catalytic site of rubisco is located at the interface between the C-terminal domain of one LSU and the N-terminal domain of the other LSU. The cleavage site of the LSU was between Ser-328 and Thr-330, possibly at Gly-329. This is located at the N-terminal end of loop 6 within the β/α-barrel domain and composed of one of the phosphate binding sites of RuBP (26). The residue Gly-329 is highly conserved in plant species (36). It is known that hydroxyl radicals are produced close to the site where the metal is bound, and that they can only attack atoms or chemical bonds located within a limited distance from their site of production. However, Gly-329 is likely not to be the closest amino acid residue to the O₂ binding site (37). For example, Asp-203 and Glu-204, constituting the metal binding site, or Gly-403 and Gly-404 are much closer to the O₂ binding site than Gly-329, respectively. Therefore, preferential attack of Gly-329 or both of its sides by reactive oxygen species could not be simply attributed to one specific reason. Other factors, such as a primary structure (kinds and properties) of amino acid residues juxtaposed to the Gly-329 or the flexibility of loop 6, may come into play.

The site-specific cleavage of the LSU strongly depends on the structural and conformational status of the catalytic site. Dissociation of the enzyme into its subunits did not cause any fragmentation. Binding of the activators (CO₂ and Mg²⁺), substrate (RuBP), and intermediate analogue (CABP) to their binding sites within catalytic site changed the susceptibility of the enzyme to fragmentation. This is perhaps attributable to the structural and conformational status of the catalytic site induced by the effectors. The conformational nature of rubisco seems to be very important in vivo, because under high light conditions rubisco is in the active form, and the level of RuBP in chloroplasts is maintained at a higher level (38). Such conditions would make rubisco more resistant to photo-oxidative stress.

Recently, Desimone et al. (17) reported that reactive oxygen species first modify rubisco, which then makes it susceptible to proteolysis. Although they did not show how rubisco is modified by reactive oxygen species, they demonstrated that once rubisco is exposed to reactive oxygen species, it is fragmented by a protease(s) in a stromal fraction in an ATP-dependent manner (17). Therefore, it seems that there are at least two triggering reactions driven by reactive oxygen species for the fragmentation of the LSU of rubisco in the lyses of chloroplasts: direct fragmentation shown here and modification followed by protease-dependent degradation reported by other authors.

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