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The Large Subunit of Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase is Fragmented into 37-kDa and 16-kDa Polypeptides by Active Oxygen in the Lysates of Chloroplasts from Primary Leaves of Wheat

Hiroyuki Ishida, Yoshito Nishimori, Miki Sugisawa, Amane Makino and Tadahiko Mae

Department of Applied Biological Chemistry, Faculty of Agriculture, Tohoku University, 1-1 Tsutsumidori-Amamiyamachi, Sendai, 981 Japan

Lysates of chloroplasts isolated from wheat (*Triticum aestivum* L. cv. Aoba) leaves were incubated on ice (pH 5.7) for 0 to 60 min in light (15 μmol quanta m^{-2} s^{-1}), and degradation of the large subunit (LSU) of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco: EC 4.1.1.39) was analyzed by applying immunoblotting with site-specific antibodies against the N-terminal, internal, and C-terminal amino acid sequences of the LSU of wheat Rubisco. The most dominant product of the breakdown of the LSU and that which was first to appear was an apparent molecular mass of 37-kDa fragment containing the N-terminal region of the LSU. A 16-kDa fragment containing the C-terminal region of the LSU was concomitantly seen. This fragmentation of the LSU was inhibited in the presence of EDTA or 1,10-phenanthroline. The addition of active oxygen scavengers, catalase (for H_2O_2) and *n*-propyl gallate (for hydroxyl radical) to the lysates also inhibited the fragmentation.

When the purified Rubisco from wheat leaves was exposed to a hydroxyl radical-generating system comprising H_2O_2, $FeSO_4$ and ascorbic acid, the LSU was degraded in the same manner as observed in the chloroplast lysates.

The results suggest that the large subunit of Rubisco was directly degraded to the 37-kDa fragment containing the N-terminal region and the 16-kDa fragment containing the C-terminal region of the LSU by active oxygen, probably the hydroxyl radical, generated in the lysates of chloroplasts.

Key words: Active oxygen — Chloroplast — Protein degradation — Rubisco (EC 4.1.1.39) — Wheat (*Triticum aestivum* L.).

Rubisco (EC 4.1.1.39) is a bifunctional enzyme which catalyzes two competing reactions, photosynthetic CO_2 assimilation and photorespiratory carbon oxidation in the stroma of the chloroplasts, and is the most abundant protein in leaves. In the mature leaves of C_3 plants, it accounts for 40 to 55% of soluble protein and 20 to 30% of the total leaf nitrogen (Makino et al. 1984, 1985a, Evans and Seemann 1989). The amount of Rubisco is well correlated with the rate of CO_2 assimilation throughout the life span of leaves (Makino et al. 1985b). Rubisco content increases during leaf expansion and reaches its maximum soon after full expansion. Thereafter, during leaf senescence, Rubisco is gradually degraded in leaves and its nitrogen is remobilized and translocated into growing organs. Thus, the degradation of Rubisco through leaf senescence is closely related to photosynthetic capacities and nitrogen economy in leaves and plants. However, the mechanisms of Rubisco degradation in leaves are as yet not clearly understood (Dalling 1987, Huffaker 1990, Miyadai et al. 1990).

The first event of Rubisco degradation in leaves must occur within chloroplasts (Huffaker 1990) because the amount of Rubisco decreases much faster than the decrease in the number of chloroplasts in barley and wheat leaves (Martinoia et al. 1983, Mae et al. 1984, Wardley et al. 1984) during senescence. Considerable attention has been paid to proteases in chloroplasts. Bushnell et al. (1993) purified a zinc protease from the stroma of pea chloroplasts and demonstrated that the partially-purified enzyme was able to degrade the LSU to a smaller polypeptide at about 36 kDa. However, the process of the LSU breakdown triggered by the enzyme has not yet been characterized. Several other peptide hydrolase activities have been found in chloroplasts and some of these peptidases were purified (Robinson and Ellis 1984a, b, Musgrove et al. 1989, Kuwabara and Hashimoto 1990, Kuwabara 1992, Oblong and Lamppa 1992). However, their relationship to Rubisco degradation has not been shown.

Rubisco degradation is often accelerated by illumination or oxidative-stress conditions in leaves and isolated chloroplasts (Mehta et al. 1992, Mitsuhashi et al. 1992, Landry and Pell 1993, Casano et al. 1990, 1994, Garcia-Ferris and Moreno 1994). These results indirectly suggest the involvement of active oxygen as a trigger in the degradation of Rubisco. Active oxygen species are now known to cause damage to various biomolecules in all aerobic organisms.

Abbreviations: AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride; anti-LSU-N, anti-LSU-I, or anti-LSU-C, site-specific antibody against N-terminal, internal, or C-terminal portion of the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase; BSA, bovine serum albumin; DABCO, 1,4-diazabicyclo-[2.2.2]octane; DTT, dithiothreitol; LSU, large subunit; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene difluoride; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; SSU, small subunit.

They cause non-enzymatic fragmentation, aggregation, and modification of proteins (Davies 1987, Davies et al. 1987a). Such altered proteins are more susceptible to degradation by intracellular proteases (Davies et al. 1987b, Davies and Goldberg 1987). Mehta et al. (1992) reported that Rubisco was highly susceptible to oxidative stress, resulting in intermolecular cross-linking of large subunits by disulfide bonds within the holoenzyme. They also suggested that translocation of oxidized Rubisco to chloroplast membranes might be a necessary step in its turnover. Eckardt and Pell (1995) reported that oxidative inactivation of Rubisco by O_3 was associated with the formation of carbonyl derivatives on amino acid side chains of the LSU.

In illuminated chloroplasts, the production of superoxide anion increases during the early stage of leaf senescence (McRae and Thompson 1983). Several degradation products of the LSU of Rubisco were found in the chloroplasts after incubation of pea chloroplasts in light (Mitsuhashi et al. 1992). Recently, Desimone et al. (1996) reported that oxidative treatment stimulated partial degradation of the LSU of Rubisco in isolated chloroplasts of barley and that the most prominent degradation product of the LSU had an apparent molecular mass of 36 kDa. However, the role of active oxygen in the degradation process of Rubisco remains uncertain.

In this paper we first report that the large subunit of Rubisco is directly degraded by active oxygen, probably the hydroxyl radical, generated in the lysates of wheat chloroplasts, and that this fragmentation gives rise to an apparent molecular mass of a 37-kDa fragment derived from the N-terminal side and a 16-kDa fragment from the C-terminal side.

Materials and Methods

Plant material—Wheat (*Triticum aestivum* L. cv. Aoba) seeds were planted on a Saran net floating on tap water in a pot and grown in a phytotron with a day/night temperature of 20°C/18°C and 70% relative humidity. The photoperiod was 12 h, with a quantum flux density of 300 μmol quanta $m^{-2} s^{-1}$ at plant height. Under these conditions, the primary leaves were fully expanded at the 7th day after sowing.

Chloroplast isolation—Chloroplasts were isolated from the primary leaves of 12-day-old seedlings by a mechanical method using continuous Percoll gradient centrifugation as described by Miyadai et al. (1990). Intactness of the chloroplasts thus obtained was more than 75% based on the latency of the Hill reaction in the presence of ferricyanide (Lilley et al. 1975). Contamination from mitochondria and peroxisomes was less than 4% and 0.1%, respectively (Mae et al. 1989).

Purification of Rubisco—Rubisco was purified from the primary and secondary leaves of 9-day-old seedlings by the following procedure based on that of Makino et al. (1983a). All steps were done at 0 to 4°C. The leaves (20 g) were homogenized with 60 ml of a buffer containing 100 mM Tris-HCl (pH 7.6 at 4°C), 12.5% (v/v) glycerol, 1% (v/v) 2-mercaptoethanol, 1 mM Na_2-EDTA, 10 mM Na_2HPO_4, 0.1% (w/v) BSA, and 10 μM E-64 in a mortar and pestle with 0.3 g of polyvinylpyrrolidone and 6.0 g of acid-washed quartz sand. The homogenate was passed through four layers of cheesecloth, and centrifuged at 39,800 × *g* for 30 min. The supernant fraction was subjected to $(NH_4)_2SO_4$ fractionation between 33 and 55% (w/v) saturation. The precipitate was dissolved in 5 ml of a buffer containing 20 mM Tris-HCl (pH 7.6 at 4°C), 12.5% (v/v) glycerol, 2 mM DTT, and 0.5 mM Na_2-EDTA and passed through a column (Econo-Pac 10DG, Bio-Rad) previously equilibrated with the same buffer. The eluted protein fractions were applied to a column of RESOURCE Q (gel volume 6 ml, Pharmacia) previously equilibrated with the same buffer and eluted with 120 ml of the same buffer containing a linear gradient of 0 to 0.5 M NaCl at a flow rate of 1 ml min^{-1} using the FPLC system of Pharmacia. The fractions containing Rubisco were pooled and the protein was precipitated with $(NH_4)_2SO_4$ at 60% (w/v) saturation. The precipitate, dissolved in 1.3 ml of a buffer containing 50 mM Na-phosphate (pH 7.5), 12.5% (v/v) glycerol, 1 mM DTT and 0.1 mM EDTA, was loaded onto a column of Hiload16/60 Superdex-200 p.g. (Pharmacia) previously equilibrated with the same buffer and eluted with the same buffer at a flow rate of 0.5 ml min^{-1} using the FPLC system. The purity of the enzyme was confirmed by SDS-PAGE (Laemmli 1970).

Preparation of site-specific anti-LSU antibodies—Three different kinds of site-specific anti-LSU antibodies against the amino acid sequences of N-terminal, internal, and C-terminal portions of wheat LSU were prepared. Synthetic 15-residue peptides were first produced by a peptide synthesizer (Model 431A, Applied Biosystems) according to the deduced amino acid sequence for wheat LSU (Terachi et al. 1987). The oligopeptides synthesized were as follows: N-terminal portion of LSU corresponding to residues 3 to 17 (for anti-LSU-N); internal portion of LSU corresponding to residues 223 to 237 (for anti-LSU-I); and C-terminal portion of LSU corresponding to residues 463 to 477 (for anti-LSU-C). The synthetic oligopeptides were conjugated with ovalbumin as described by Goodfriend et al. (1966), and mixed with an equal volume of Freund's complete adjuvant. Samples containing 1 mg of peptides were injected into rabbits. A booster injection of 0.5 mg of peptides emulsified in an equal volume of complete adjuvant was given 2 weeks later. Two additional booster injections were given every 2 weeks. After 1 week from the third booster injection, blood samples were collected, and antisera were separated.

Affinity purification of antibodies—Anti-LSU antibody was purified from anti-Rubisco antiserum (Makino et al. 1983b) by the method of Miyadai et al. (1990) based on that of Snyder et al. (1987). The purified Rubisco was subjected to SDS-PAGE (12.5%) and the Rubisco subunit bands separated on the gel were electrophoretically transferred to a PVDF membrane (Millipore) by the method of Towbin et al. (1979). The part of the PVDF membrane corresponding to the position of LSU was cut out, and blocked with PBS containing 5% (w/v) non-fat dry milk. The membrane was incubated with anti-Rubisco antiserum diluted in PBS containing 0.3% (w/v) BSA and 0.05% (w/v) NaN_3 at 37°C for 18 h, and rinsed three times in PBS containing 5% (w/v) non-fat dry milk and 0.05% (v/v) Tween-20. The antibodies were eluted from the membrane by adding 1 ml of 0.2 M glycine-HCl (pH 2.5) and incubating for 2 min. The solution was neutralized immediately with 0.17 ml of 1.0 M Tris-HCl (pH 8.8) containing 7% (w/v) BSA. After the addition of 0.05% (w/v) NaN_3 at the final concentration, this antibody solution was stored at 4°C until its use for immunoblotting. Three kinds of site-specific anti-LSU antibodies were also purified from antisera by this method for each. It was confirmed that the respective antibodies cross-reacted

only with the corresponding oligopeptides used as antigen.

SDS-PAGE and immunoblotting—Samples were boiled for 3 min after the addition of an equal volume of 200 mM Tris-HCl (pH 8.5) containing 2% (w/v) SDS, 20% (v/v) glycerol, and 5% (v/v) 2-mercaptoethanol, and subjected to SDS-PAGE (Laemmli 1970). The acrylamide concentration in the separation gel was 12.5% (w/v). Molecular masses of fragments of LSU were estimated by using the marker proteins (Bio-Rad). Separated polypeptides were transferred to a nitrocellulose membrane (Bio-Rad) with an electroblotting apparatus (Bio-Rad Mini Trans-Blot, 1 h at 100 V and 4°C) as described by Towbin et al. (1979), except that the methanol concentration in the transfer buffer was changed to 10% (v/v). The membrane was reacted with affinity-purified antibodies, as described above, and further reacted with alkaline phosphatase-conjugated goat anti-rabbit IgG antibodies (Promega). Immunoreacted bands were visualized by the reaction with nitroblue tetrazolium and bromochloroindolyl phosphate (Promega) according to the manufacturer's instructions.

Others—Protease inhibitors were all prepared as stock solutions at 100 times their final concentration, except for EDTA, which was prepared at ten times its final concentration. PMSF, 1,10-phenanthroline and Pepstatin A were dissolved in methanol. EDTA was first dissolved in MES buffer, and then its pH was adjusted to 5.5 with 1 M NaOH. The final concentration of MES buffer was 0.5 M in the stock solution and 50 mM in the reaction mixture. Other inhibitors were dissolved in Milli Q water (Millipore). Active oxygen scavengers were all prepared as stock solutions at ten times their final concentrations in Milli Q water. Leupeptin, E-64, Phosphoramidon, and Pepstatin A were purchased from Peptide Institute (Osaka, Japan). PMSF, EDTA, and 1,10-phenanthroline were purchased from Wako Pure Chemical Industries (Osaka, Japan). Other inhibitors, catalase from bovine liver, superoxide dismutase from bovine erythrocytes, and all active oxygen scavengers were purchased from Sigma.

Results

Degradation of Rubisco in the lysates of chloroplasts—Intact chloroplasts, isolated from the primary leaves of 12-day-old seedlings of wheat, were ruptured in a hypotonic buffer and the lysates were incubated on ice at pHs of 4.0 to 8.0 for 0 to 60 min in weak light (15 μmol quanta $m^{-2} s^{-1}$). The degradation of Rubisco was analyzed by immunoblotting with affinity-purified anti-LSU antibody following SDS-PAGE. The results of the incubation at pH 5.7 are shown in Fig. 1 because accumulation of the fragments was the highest at this pH. The LSU fragment having an apparent molecular mass of 37 kDa appeared first and was the most dominant fragment throughout the experimental period. It clearly increased in amount with the time of incubation. The appearance of the 37-kDa fragment was not observed when the lysates were incubated in the dark. On the other hand, the degradation was more pronounced when light intensity increased from 15 to 500 μmol quanta $m^{-2} s^{-1}$. A few minor fragments also increased with the time of incubation. The amount of the 37-kDa fragment at 60 min after incubation was estimated to be around 1% of the LSU, judged by densitometric measurement of the 37-kDa fragment. A similar pattern of the LSU degradation

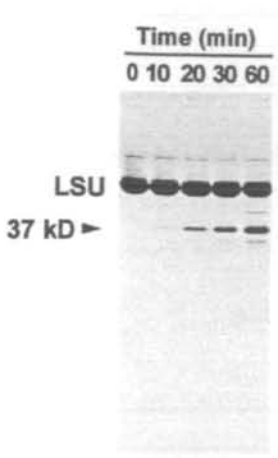

Fig. 1 Time-dependent changes in immunoblot profiles of the LSU fragments in the lysates of chloroplasts isolated from the primary leaves of wheat. Intact chloroplasts were ruptured in a buffer containing 10 mM HEPES-NaOH (pH 7.5) and 5% (v/v) glycerol. After adding one tenth volume of 0.5 M MES-NaOH (pH 5.5) to the lysates, the reaction mixtures were incubated on ice for indicated times in light (15 μmol quanta $m^{-2} s^{-1}$). After incubation, the reaction mixtures were analyzed by immunoblotting with affinity-purified anti-LSU antibody following SDS-PAGE.

was observed at all the pHs examined here (data not shown). When the lysates were incubated at higher temperatures, 20 to 30°C, the 37-kDa fragment was also found in the lysates after incubation, but its accumulation was less (data not shown).

The effects of protease inhibitors on the degradation of Rubisco were tested (Fig. 2). Degradation of the LSU was clearly inhibited by metal ion chelators, EDTA, and 1,10-phenanthroline. Phosphoramidon, which is a specific inhibitor for bacterial metallo-proteases, such as thermolysin, had no inhibitory effect. Inhibitors for other classes of protease, AEBSF, PMSF (serine protease), Leupeptin, E-64 (cysteine protease), and Pepstatin A (aspartic protease), were also ineffective. On the other hand, the breakdown of LSU into the 37-kDa polypeptide was clearly enhanced in the presence of DTT.

Two possible mechanisms were suggested from the effects of chelators on the fragmentation of LSU in the chloroplast lysates. One was the involvement of metallo-protease. It has been reported that chloroplasts contain a metallo-protease, designated as EP1 (Liu and Jagendorf 1986). Bushnell et al. (1993) reported that a partially-purified zinc protease, EP1, from pea chloroplasts, could degrade LSU into a 36-kDa polypeptide. The other possible mechanism was the involvement of active oxygen, which resulted from a metal ion-catalyzed oxidation (MCO) system (Stadtman and Oliver 1991) in the lysates. The active oxygen species, especially hydroxyl radical or a combination of hydroxyl radical and superoxide anion, can directly cleave the peptide bonds of protein (Kim et al. 1985, Davies

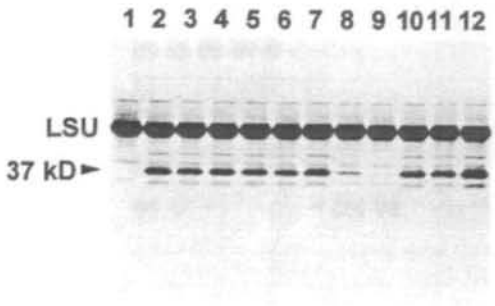

Fig. 2 Effect of protease inhibitors on the fragmentation of the LSU in the lysates of chloroplasts from the primary leaves of wheat. Chloroplast lysates were incubated with various protease inhibitors on ice for 1 h in light (15 μmol quanta $m^{-2} s^{-1}$) under the same buffer condition as described in Fig. 1. After incubation, the reaction mixtures were analyzed by immunoblotting with affinity-purified anti-LSU antibody following SDS-PAGE. Lane 1, 0 time; lane 2, incubated without inhibitors; lane 3, incubated with 1% (v/v) methanol; lane 4-12, incubated with 100 μM AEBSF (lane 4), 2 mM PMSF (lane 5), 100 μM Leupeptin (lane 6), 10 μM E-64 (lane 7), 5 mM EDTA (lane 8), 2 mM 1,10-phenanthroline (lane 9), 10 μM Phosphoramidon (lane 10), 1 μM Pepstatin A (lane 11), or 4 mM DTT (lane 12).

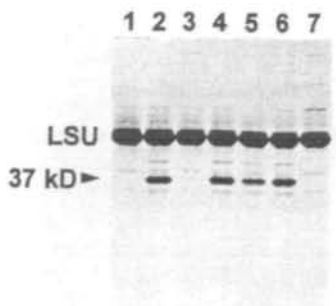

Fig. 3 Effect of catalase, superoxide dismutase, and active oxygen scavengers on the fragmentation of the LSU in the lysates of chloroplasts from the primary leaves of wheat. Chloroplast lysates were incubated in the presence of various active oxygen scavengers on ice for 1 h in light (15 μmol quanta $m^{-2} s^{-1}$) under the same buffer condition as described in Fig. 1. After incubation, the reaction mixtures were analyzed by immunoblotting with affinity-purified anti-LSU antibody following SDS-PAGE. Lane 1, 0 time; lane 2, incubated without scavengers; lane 3-7, incubated with 0.1 mg ml^{-1} catalase (lane 3), 0.1 mg ml^{-1} superoxide dismutase (lane 4), 10 mM histidine (lane 5), 1 mM DABCO (lane 6), or 1 mM *n*-propyl gallate (lane 7).

1987). Moreover, susceptibility of proteins to proteolytic attack is altered by their conformational changes, resulting from the amino acid modification of proteins by active oxygen species (Stadtman 1990, 1993).

The fragmentation of the LSU into the 37-kDa polypeptide in the lysates of chloroplasts after incubation did not seem to be caused by the action of metallo-protease, EP1, because the effect of DTT was not consistent with that observed for EP1 by Liu and Jagendorf (1986) and Bushnell et al. (1993). In this experiment, the addition of DTT showed a stimulative effect on the appearance of the 37-kDa fragment in the chloroplast lysates, while it had an inhibitory effect on EP1 activity. Moreover, the optimal pH for the appearance of the 37-kDa fragment in our experiment was different from that for EP1 activity. Mg^{2+}-dependency was not found for the degradation of the LSU in the chloroplast lysates in this experiment, although the activity of EP1 against synthetic peptides showed a clear dependency on a divalent cation, Mg^{2+} or Ca^{2+} (Liu and Jagendorf 1986).

The effect of active oxygen scavengers on the appearance of the 37-kDa fragment was investigated to clarify whether the degradation of the LSU into the 37-kDa fragment in chloroplast lysates might be derived from direct fragmentation by active oxygen. Catalase (for hydrogen peroxide: H_2O_2), superoxide dismutase (for superoxide anion: O_2^-), histidine and DABCO (for singlet oxygen: $^1O_2$, Foote 1976), and *n*-propyl gallate (for hydroxyl radical: $^{\cdot}OH$ and alkoxyl radicals, Bors et al. 1989) were used as scavengers for respective active oxygen species. As shown in Fig. 3, catalase and *n*-propyl gallate had clear suppressive effects on the appearance of the 37-kDa fragment. Other scavengers, superoxide dismutase, histidine, and DABCO did not have any remarkable suppressive effects.

These results strongly suggest that the hydroxyl radical ($^{\cdot}OH$), generated from hydrogen peroxide by an MCO system, possibly via Fenton reaction (Stadtman 1993), caused the fragmentation of the LSU in the chloroplast lysates. The positive effect of DTT on the degradation of the LSU in the chloroplast lysates shown in Fig. 2 would be caused by the supply of electrons from DTT to such an MCO system.

Degradation of purified Rubisco by hydroxyl radical—Figure 4 shows the effects of active oxygen species on the degradation of purified wheat Rubisco. When the purified Rubisco was incubated alone or only with hydrogen peroxide at the same conditions as in Fig. 1, fragmentation of the LSU into the 37-kDa was not detected. It could, however, be observed when the purified Rubisco was incubated with the hydroxyl-radical generating system, composed of hydrogen peroxide, ferrous sulfate, and ascorbic acid. These results confirm that the LSU is degraded into the 37-kDa fragment by one of the active oxygen species, hydroxyl radical, but not by protease.

Estimation of putative cleavage site of the LSU by active oxygen—To estimate the putative cleavage site of the

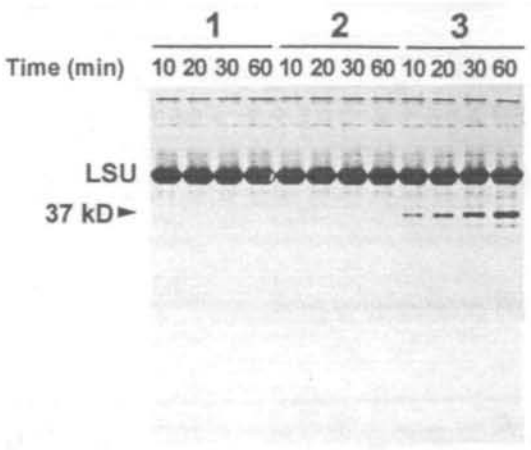

Fig. 4 Fragmentation of purified Rubisco by active oxygen. Purified Rubisco from wheat leaves was incubated on ice for the indicated times under the same buffer condition as described in Fig. 1 in the absence or presence of active oxygen. (1) No addition; (2) 1 mM hydrogen peroxide; (3) 1 mM hydrogen peroxide, 10 μM ferrous sulfate, and 1 mM ascorbic acid. Concentration of Rubisco in the reaction mixture was 0.8 mg ml^{-1}. After incubation, the reaction mixtures were analyzed by immunoblotting with affinity-purified anti-LSU antibody following SDS-PAGE.

primary sequence of the LSU by active oxygen, three different kinds of site-specific anti-LSU antibodies, anti-LSU-N, anti-LSU-I, and anti-LSU-C were prepared. The cross-reactivity of the 37-kDa fragment in the chloroplast lysate with each site-specific antibody was examined (Fig. 5). The 37-kDa fragment cross-reacted with anti-LSU-N and anti-LSU-I, but not with anti-LSU-C, suggesting that the 37-kDa fragment includes the N-terminal side. We also found another LSU fragment having an apparent molecular mass of 16 kDa, which cross-reacted with anti-LSU-C, but not with anti-LSU-N or anti-LSU-I (Fig. 5). This 16-kDa fragment also cross-reacted with the purified anti-LSU antibody. However, since the intensity of the band was weaker than that of the 37-kDa fragment, detection was very slight (see Fig. 1). This may be because the titer of the anti-LSU antibodies against the 16-kDa fragment was weaker than that against the 37-kDa fragment. Alternatively there may be a smaller pool of the 16-kDa fragment in the lysates because this product is more susceptible to additional fragmentation by the active oxygen or to proteolysis.

The cross-reactivities of the 37-kDa and 16-kDa fragments of the LSU derived from purified Rubisco were examined with the site-specific anti-LSU antibodies (Fig. 6). They exhibited the same cross-reactivities with anti-LSU-N or with anti-LSU-C as those in the chloroplast lysates, respectively. A few bands were found above the LSU band after incubation of purified Rubisco. They seemed to be aggregates of LSU and/or those of LSU and SSU. Considering the molecular mass of whole LSU (53 kDa) and that of the 37-kDa and 16-kDa fragments, and their cross-reactivities with the site-specific antibodies, it is strongly suggested that LSU is degraded into an N-terminal side fragment of 37 kDa and a C-terminal side fragment of 16 kDa by active oxygen, probably the hydroxyl radical.

Characteristics of degradation products of Rubisco in whole leaf extract—It has been reported that more than 95% of proteolytic activity against Rubisco in crude leaf

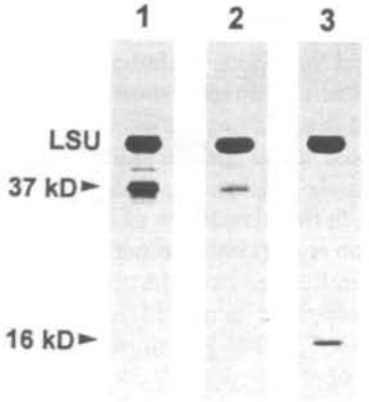

Fig. 5 Cross-reactivity of the site-specific anti-LSU antibodies to the LSU fragments in the lysates of chloroplasts from the primary leaves of wheat. Chloroplast lysates were incubated on ice for 3 h in a buffer containing 50 mM MES-NaOH (pH 5.7), 4.5% (v/v) glycerol, 4 mM DTT and 30 μg ml^{-1} chloramphenicol in light (15 μmol quanta m^{-2} s^{-1}). Immunoblot analysis was performed with anti-LSU-N (lane 1), anti-LSU-I (lane 2), or anti-LSU-C (lane 3) following SDS-PAGE.

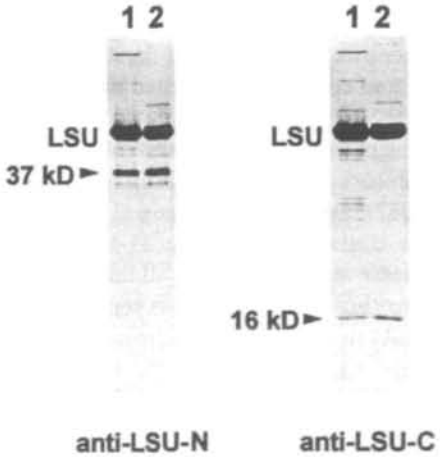

Fig. 6 Comparison of the LSU fragments produced by the exposure of purified Rubisco to the hydroxyl radical-generating system with those in the chloroplast lysates after incubation in light (15 μmol quanta m^{-2} s^{-1}) for 1 h. The reaction mixtures were analyzed by immunoblotting with site-specific anti-LSU antibodies, anti-LSU-N, or anti-LSU-C. Lane 1, the purified Rubisco exposed to the hydroxyl radical-generating system; lane 2, chloroplast lysates after incubation.

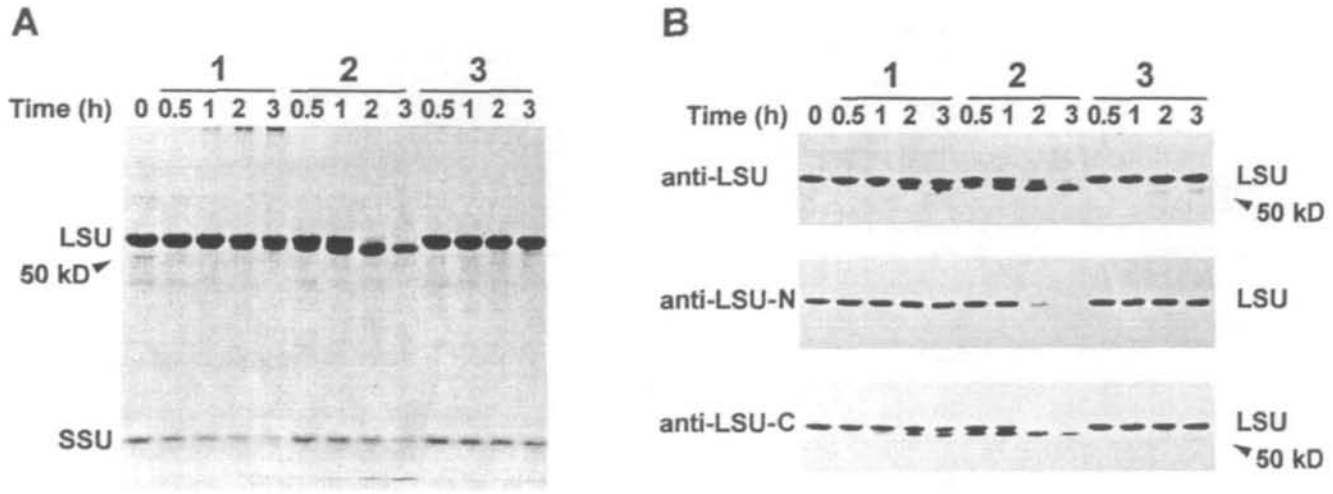

Fig. 7 Characteristics of Rubisco degradation in crude leaf extracts of wheat. (A) Polypeptide profiles after staining with Coomassie Brilliant Blue. (B) Immunoblot profiles of the LSU and its fragment analyzed with affinity-purified anti-LSU antibody, or with site-specific anti-LSU antibodies, anti-LSU-N or anti-LSU-C. Crude leaf extracts were incubated at 30°C for indicated times, and then analyzed by SDS-PAGE and immunoblotting. The reaction buffers used were 50 mM MES-NaOH (pH 5.7) and 4.5% (v/v) glycerol in the absence (1) or in the presence of 4 mM DTT (2), or in the presence of 4 mM DTT and 10 μM E-64 (3).

extracts is derived from vacuolar proteases (Miller and Huffaker 1981, Thayer and Huffaker 1984). Miyadai et al. (1990) pointed out that studies on Rubisco degradation with purified chloroplasts should be treated cautiously because of easy contamination of vacuolar proteases adhering to the outer envelope of the chloroplasts during their isolation. We therefore characterized the degradation products of Rubisco in crude leaf extracts after incubation and compared them with those found in the chloroplast lysates after incubation (Fig. 7). The degradation of the LSU to a 50-kDa fragment was observed. The degradation was stimulated by DTT and completely inhibited by E-64. The activities of vacuolar proteases are stimulated at acidic pHs, 4.8 to 5.5, or in the presence of DTT, and inhibited by cysteine protease inhibitors (Thayer and Huffaker 1984, Wittenbach et al. 1982). Bhalla and Dalling (1986) reported that the LSU was degraded to 50- to 51-kDa fragments by vacuolar protease at pH 5.2. Since all the results in Fig. 7A were similar to those in the previous reports, the degradation of the LSU to the 50-kDa fragment in the crude leaf extracts seemed to be caused by the action of vacuolar protease. This 50-kDa fragment did not cross-react with anti-LSU-N, but cross-reacted with anti-LSU-C (Fig. 7B), suggesting that the 50-kDa fragment of the LSU does not contain the N-terminal region of LSU. In addition, the manner in which LSU was degraded is quite similar to that by vacuolar cysteine protease from French bean leaves (Yoshida and Minamikawa 1996). Thus, it is obvious that the degradation of the LSU in the chloroplast lysate shown in this study is quite different from that caused by vacuolar protease.

Discussion

Some indirect evidence exists which supports the involvement of active oxygen species, generated under photo-oxidative conditions, in the increased rate of degradation of Rubisco (Weckenmann and Martin 1984, Casano et al. 1990, Casano and Trippi 1992). Mitsuhashi et al. (1992) reported that the degradation of Rubisco was stimulated in intact pea chloroplasts in light. Mehta et al. (1992) showed that Cu^{2+}-induced oxidative stress caused inactivation, cross-linking, membrane translocation, and finally degradation of Rubisco in wheat and *Spirodela* plants and in intact wheat chloroplasts. Although the involvement of active oxygen was not directly shown in their reports, it may be inferred that the production of active oxygen species was enhanced under these conditions of illumination or oxidation (Asada and Takahashi 1987, Jakob and Heber 1996), resulting in the stimulation of Rubisco degradation. Recently, Casano et al. (1994) compared the degradation of whole proteins in isolated oat chloroplasts exposed to light with that in chloroplasts incubated in darkness in the presence of a hydroxyl radical-generating system, and found that the pattern of protein degradation in the hydroxyl radical-treated chloroplasts was almost identical to that in light. They proposed that a high dose of light irradiation promotes proteolysis by increasing the formation of hydroxyl radical, which may modify proteins so that they become more susceptible to protease-mediated degradation. Although these authors suggested the involvement of the hydroxyl radical in the degradation of protein in chloroplasts in light, it was not shown how Rubisco was specifical-

ly modified by the hydroxyl radical in chloroplasts. In this study, we demonstrated that the large subunit of Rubisco was non-enzymatically cleaved at a specific site by active oxygen, probably hydroxyl radical, generated in the lysates of wheat chloroplasts in light, and that this fragmentation gave rise to a 37-kDa fragment of the N-terminal side and a 16-kDa fragment of the C-terminal side.

Recently, Desimone et al. (1996) reported that active oxygen generated by varying the light intensity, the oxygen concentration, or the addition of herbicides or ADP-$FeCl_3$-ascorbate to the incubation medium could cause the degradation of the LSU of Rubisco into the 36-kDa fragment in barley chloroplasts or its lysates. However, they did not conclude that it was caused by the direct fragmentation of the LSU with hydroxyl radical, because the degradation of the LSU was not inhibited in the presence of the scavengers for hydroxyl radical such as mannitol, ethanol, and dimethylsulfoxide in their experiment. On the other hand, we used *n*-propyl gallate as a hydroxyl radical scavenger (Bors et al. 1989) and found that it completely inhibited the degradation of the LSU (Fig. 3). This is because each protein shows a different sensitivity to hydroxyl radical scavengers depending on its specific metal binding site (Stadtman 1993). In addition, from the analysis of the N-terminal sequence of the 36-kDa fragment, Desimone et al. (1996) suggested that one of the cleavage sites of the LSU might be between the residue 100 and 101. This means that the 36-kDa fragment does not have the N-terminal portion of the LSU. In contrast, by using site-specific anti-LSU antibodies, we demonstrated that the 37-kDa fragment in the lysates of wheat chloroplasts certainly contains the N-terminal portion of the LSU (Fig. 5). Thus, the cleavage site of the LSU indicated in barley chloroplasts is clearly different from ours. Identification of the exact cleavage site of the LSU to the 37-kDa and 16-kDa fragments by hydroxyl radical, and its mechanism are now under investigation. However, it probably cannot be easily identified by simply applying a routine method for amino acid sequencing because the oxygen-mediated cleavage of the polypeptide chain is assumed to occur by the α-amidation pathway, yielding one side of amide derivative and the other side of an α-ketoacyl derivative (Stadtman 1993).

One example of direct fragmentation of chloroplastic protein by the action of active oxygen is D1 protein (Miyao 1994, Miyao et al. 1995). Miyao et al. (1995) demonstrated that the D1 protein could be cleaved to specific fragments directly by the action of active oxygen species inside PSII under illumination. D1 protein has a rapid turnover and is specifically degraded under illumination (Mattoo et al. 1984). The exact cleavage sites of the polypeptide chain have not yet been reported. Another well known example of direct fragmentation of proteins is by hydroxyl radical generated by ^{60}Co radiation in the presence of oxygen (Davies 1987).

The production of superoxide anion by illuminated chloroplasts increases by about fourfold during the early stages of leaf senescence (McRae and Thompson 1983). The activities of CuZn-superoxide dismutase and ascorbate peroxidase decrease during senescence in tobacco leaves (Polle 1996). Thus, it can be considered that under some physiological conditions such as oxidative stress and/or during leaf senescence, scavenging capacities (activities) of active oxygen become insufficient to decrease the concentration of produced active oxygen to a non-harmful level in chloroplasts. As a consequence, the fragmentation of the LSU might occur.

Fragments of the LSU were not detected in intact chloroplasts after isolation (see the lane for 0 time in figures) or in the extracts from young to senescing leaves of wheat (data not shown). These indicate that degradation of Rubisco in intact chloroplasts or in intact leaves proceeds quite rapidly and smoothly, without any accumulation of detectable amounts of their breakdown products. This may be physiologically essential, because their presence could interfere with a multitude of protein/protein interactions (Vierstra 1993). In this study, the accumulation of LSU fragments was most pronounced at relatively non-physiological conditions (pH 5.7 and 0 to 4°C). When the lysates were incubated at the physiological pH of chloroplast stroma and 20 to 30°C, the same fragments were also detected, but their amount was much less than that observed at pH 5.7 and 0 to 4°C (data not shown). The reason for this is not known, but one possible explanation for this would be that further proteolysis of the fragments into smaller peptides or amino acids was accelerated more under conditions near to physiological conditions.

In the present study, we were able to demonstrate that the LSU was degraded into the 37-kDa and the 16-kDa fragments in the lysates of chloroplasts; however, the role of active oxygen in Rubisco degradation in vivo, during senescence and under various stress conditions, still remains uncertain and requires further study. It will be particularly interesting to examine how the once-modified 37-kDa and 16-kDa fragments are further degraded in chloroplasts or their lysates by intrinsic protease(s).

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