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Light-dependent fragmentation of the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase in chloroplasts isolated from wheat leaves

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Abstract. The large subunit (LSU) of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco; EC 4.1.1.39) is degraded into an N-terminal side fragment of 37 kDa and a C-terminal side fragment of 16 kDa by the hydroxyl radical in the lysates of chloroplasts in light (H. Ishida et al. 1997, Plant Cell Physiol 38: 471–479). In the present study, we demonstrate that this fragmentation of the LSU also occurs in the same manner in intact chloroplasts, and discuss the mechanisms of the fragmentation. The fragmentation of the LSU was observed when intact chloroplasts from wheat leaves were incubated under illumination in the presence of KCN or NaN_3, which is a potent inhibitor of active oxygen-scavenging enzyme(s). The properties, such as molecular masses and cross-reactivities against the site-specific anti-LSU antibodies, of the fragments found in the chloroplasts were the same as those found in the lysates. These results indicate that, as in the lysates, the fragmentation of the LSU in the intact chloroplasts was also caused by the hydroxyl radical generated in light. The fragmentation of the LSU was completely inhibited by 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea (DCMU), and only partially inhibited by methyl viologen in the lysates. The addition of hydrogen peroxide to the lysates stimulated LSU fragmentation in light, but did not induce any fragmentation in darkness. Thus, we conclude that both production of hydrogen peroxide and generation of the reducing power at thylakoid membranes in light are essential requirements for fragmentation of the LSU.

Key words: Active oxygen – Chloroplast – Light stress – Protein degradation – Ribulose-1,5-bisphosphate carboxylase/oxygenase – *Triticum* (light stress)

Introduction

Ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39), the most abundant protein in leaves, is a bifunctional enzyme that catalyzes two competing reactions, namely, photosynthetic CO_2 assimilation and photorespiratory carbon oxidation, in the stroma of the chloroplasts. The degradation of Rubisco is closely related to both the rate of photosynthesis and nitrogen economy in leaves. Although the synthesis, assembly, structure and regulation of Rubisco have been studied extensively (Knight et al. 1990; Hartman and Harpel 1994), the mechanisms of Rubisco degradation in leaves are as yet not known (for reviews, see Huffaker 1990; Vierstra 1993).

There have been many reports describing accelerated degradation of Rubisco 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; Eckardt and Pell 1995; Desimone et al. 1996). 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. Mitsuhashi et al. (1992) found that the degradation of Rubisco was stimulated in intact pea chloroplasts in light. Recently, Desimone et al. (1996) reported that oxidative treatment stimulated partial proteolysis of the large subunit (LSU) of Rubisco in isolated chloroplasts of barley. Under certain conditions, such as light- or oxidative-stress, the production of active oxygen species is stimulated in chloroplasts (Asada and Takahashi 1987; Jakob and Heber 1996). Therefore, it is suggested that active oxygen species are

Abbreviations: anti-LSU-C = site-specific antibody against the C-terminal portion of the large subunit of Rubisco; anti-LSU-N = site-specific antibody against the N-terminal portion of the large subunit of Rubisco; DCMU = 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; LSU = large subunit

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involved in the stimulation of Rubisco degradation under some stress conditions. Active oxygen species are now known to cause damage to various biomolecules in all aerobic organisms. They cause non-enzymatic fragmentation, aggregation, and amino acid modification of proteins (Davies 1987; Davies et al. 1987). It has been reported that active oxygen species participate in the inactivation and degradation of some chloroplastic proteins, such as glyceraldehyde-3-phosphate dehydrogenase and fructose-1,6-bisphosphatase (Asada and Takahashi 1987), D1 protein (Aro et al. 1993; Miyao 1994; Miyao et al. 1995), *psaB* gene product (Sonoike 1996), and superoxide dismutase (Casano et al. 1997).

Recently, we have reported that the LSU is degraded into an N-terminal side fragment of 37 kDa and a C-terminal side fragment of 16 kDa by an active oxygen species, the hydroxyl radical, in the lysates of chloroplasts in the light (Ishida et al. 1997). Direct degradation of proteins by active oxygen has previously been shown for D1 protein (Miyao et al. 1995) and recently for superoxide dismutase (Casano et al. 1997). However, it is not known whether this fragmentation of the LSU also occurs in intact chloroplasts. In the present study, we demonstrate that the same fragmentation of the LSU is induced inside the chloroplasts in light in the presence of KCN or NaN_3. Furthermore, we propose that both hydrogen peroxide and reducing power, produced and generated at thylakoids in the light, are essentially required for this fragmentation.

Materials and methods

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 °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.

Chloroplasts were isolated from the primary leaves of 12-d-old seedlings by a mechanical method using continuous Percoll gradient centrifugation as described previously (Ishida et al. 1997). Soluble (stromal) and insoluble (membrane) fractions of chloroplasts were prepared as follows. Intact chloroplasts were lysed in 10 mM Hepes-NaOH (pH 7.5) and the soluble fraction was separated from the lysates by centrifugation at 39 800 *g* for 20 min. Insoluble fractions were separated by centrifugation at 3000 *g* for 5 min and washed twice with 10 mM Hepes-NaOH (pH 7.5).

For light treatment, intact chloroplasts were suspended in an isotonic medium containing 50 mM Hepes-NaOH (pH 7.5) and 0.33 M sorbitol. The lysates and soluble and insoluble fractions of chloroplasts were suspended in 50 mM 2-(N-morpholino)-ethanesulfonic acid (Mes)-NaOH (pH 5.7), respectively. Ribulose-1,5-bisphosphate carboxylase/oxygenase, purified from wheat leaves as described previously (Ishida et al. 1997), was mixed with the insoluble fractions in 50 mM Mes-NaOH (pH 5.7). Each suspension (0.2–0.3 mg chlorophyll · ml^{-1} and 0.8–1.0 mg Rubisco · ml^{-1}) was illuminated at 500–2000 μmol quanta · m^{-2} · s^{-1} in a cuvette with white light from a projector lamp. The temperature was maintained at 4 °C under thermostatic control. Additional effectors, 100 μM 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea (DCMU), 100 μM methyl viologen, 1 mM hydrogen peroxide, 1 mM KCN or 1 mM NaN_3, were added to the medium before treatments. After incubation, the suspensions were boiled for 3 min with an equal volume of SDS sample buffer, which was 200 mM Tris-HCl (pH 8.5), 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) and molecular masses of the LSU fragments were estimated by using marker proteins (Bio-Rad, Richmond, Calif., USA).

Immunoblot analysis was performed with affinity-purified anti-LSU antibody from anti-Rubisco antiserum or site-specific anti-LSU antibodies against the amino acid sequences of N-terminal (residues 3–17) or C-terminal (residues 463–477) portions of wheat LSU, as described previously (Ishida et al. 1997).

Results

Intact chloroplasts, isolated from wheat leaves, were incubated in the light and the degradation of intrinsic Rubisco-LSU was analyzed by immunoblotting following SDS-PAGE (Fig. 1A). Fragmentation was not observed when intact chloroplasts suspended in an isotonic medium were incubated in light at 1000 μmol quanta · m^{-2} · s^{-1} without any additional effectors (Fig. 1A). It could, however, be observed when intact chloroplasts were incubated in light in a medium containing KCN or NaN_3. It was not observed when intact chloroplasts were incubated in darkness, either in the absence or in the presence of KCN or NaN_3 (data not shown). The 37-kDa fragment was the most dominant throughout the incubation time. Other minor fragments, having apparent molecular masses of 42, 35 and 16 kDa, were also observed. To verify that the fragmentation occurred within chloroplasts, the light-treated chloroplasts were re-purified by continuous

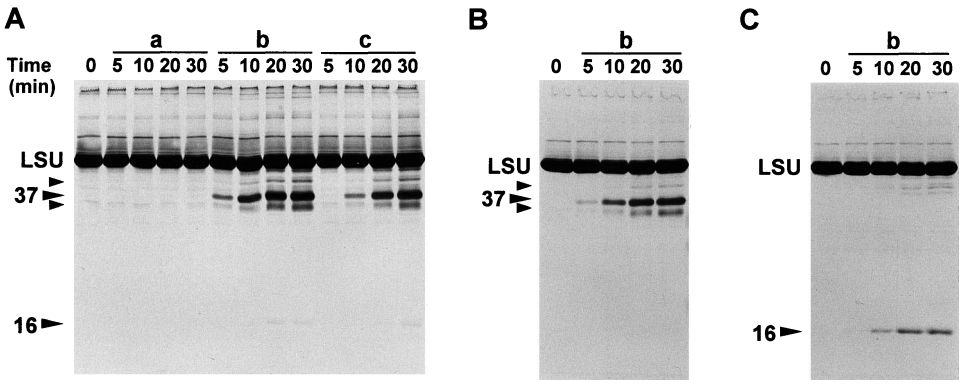

Fig. 1A–C. Fragmentation of the Rubisco-LSU in the intact chloroplasts from wheat leaves induced by the presence of KCN or NaN_3 in light. Intact chloroplasts were incubated at 4 °C for the indicated times in light at 1000 μmol quanta · m^{-2} · s^{-1} in the absence (*a*) or presence of 1 mM KCN (*b*), or in the presence of 1 mM NaN_3 (*c*). Immunoblot analysis was performed with affinity-purified anti-LSU antibody (**A**), or with site-specific anti-LSU antibodies, anti-LSU-N (**B**) or anti-LSU-C (**C**) following SDS-PAGE

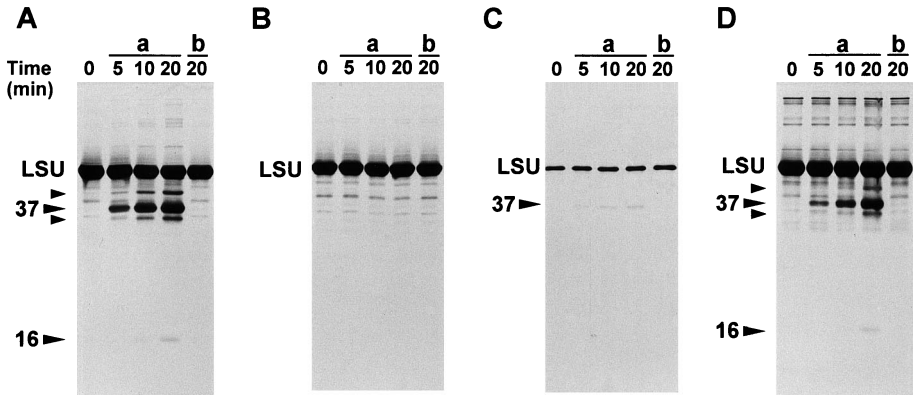

Fig. 2A–D. Fragmentation of the Rubisco-LSU in the chloroplast lysates, the stromal fraction, the membrane (thylakoid) fraction, and the membrane fraction incubated with purified Rubisco, from wheat leaves, in light. The chloroplast lysates (**A**), the stromal (**B**) or the membrane (**C**) fractions of chloroplasts, and the membrane fraction with added purified Rubisco (**D**) from intact chloroplasts were incubated at 4 °C for the indicated times in light at 2000 μmol quanta · m^{-2} · s^{-1} (*a*) or in darkness (*b*). Immunoblot analysis was performed with affinity-purified anti-LSU antibody following SDS-PAGE

Percoll gradient centrifugation and subjected to immunoblotting following SDS-PAGE, because it was suspected that the fragmentation of the LSU was due to chloroplasts which ruptured during the incubation. The same fragments of the LSU were also found in the repurified chloroplasts (data not shown). It was thus confirmed that the fragmentation certainly occurred within chloroplasts.

Cross-reactivities of the site-specific anti-LSU antibodies to the fragments were tested (Fig. 1B,C). The 37-kDa fragment cross-reacted with anti-LSU-N, but not with anti-LSU-C. The 42-kDa and 35-kDa fragments also cross-reacted with anti-LSU-N. The 16-kDa fragment cross-reacted with anti-LSU-C.

When the lysates of chloroplasts were incubated under strong illumination at 2000 μmol quanta · m^{-2} · s^{-1} without additional effectors, the time-dependent appearance of several fragments of the LSU was observed (Fig. 2A). In contrast to this, no fragments were detected in darkness. Fragmentation of the LSU did not occur in the stromal fraction of chloroplasts, irrespective of light or darkness (Fig. 2B).

It is known that a part of Rubisco is associated with thylakoid membranes (Akazawa 1978; Makino and Osmond 1991). In this study, when the membranes were directly prepared from the chloroplast lysates, a small amount of Rubisco was found in the membrane fractions as expected (Fig. 2C, lane of zero time). This membrane-bound Rubisco was fragmented into the 37-kDa fragment in light (Fig. 2C), although the amount of the fragment was much less than that detected in the lysates. Purified Rubisco, added to the washed membrane fractions, was also fragmented in the light in the same manner as observed in the intact chloroplasts and chloroplast lysates (Fig. 2D).

Figure 3 shows the effects of DCMU and methyl viologen on the light-dependent fragmentation of the Rubisco-LSU in the chloroplast lysates. Fragmentation of the LSU was completely inhibited by the addition of DCMU, which is known to inhibit the electron flow at PSII. The fragmentation of the LSU was clearly observed in the presence of methyl viologen, which is the electron acceptor of PSI, although the fragmentation was suppressed to a some extent compared with that occurring in the absence of the electron acceptors (Fig. 3).

The effect of hydrogen peroxide on the fragmentation of the LSU was investigated in light and in darkness (Fig. 4). In light, the fragmentation was stimulated by the addition of hydrogen peroxide. In contrast, the fragmentation did not occur with the addition of hydrogen peroxide in darkness.

Discussion

Fragmentation of the Rubisco-LSU in chloroplasts. Chloroplasts have strong defensive systems against light stress. Under favorable conditions, the concentration of active oxygen species produced is decreased to a non-harmful level (Asada 1992). The fragmentation of Rubisco was not observed in the illuminated intact

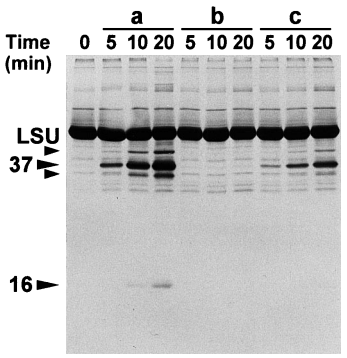

Fig. 3. Effects of DCMU and methyl viologen on the fragmentation of the Rubisco-LSU in the chloroplast lysates from wheat leaves in light. Chloroplast lysates were incubated at 4 °C for the indicated times in light at 500 μmol quanta · m^{-2} · s^{-1} in the absence (*a*) or presence of 100 μM DCMU (*b*) or in the presence of 100 μM methyl viologen (*c*). Immunoblot analysis was performed with affinity-purified anti-LSU antibody following SDS-PAGE

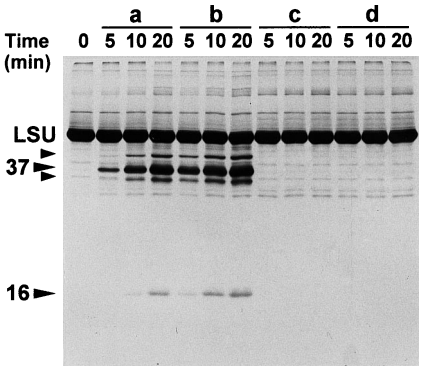

Fig. 4. Effect of hydrogen peroxide on the fragmentation of the Rubisco-LSU in the chloroplast lysates from wheat leaves in light and in darkness. Chloroplast lysates were incubated at 4 °C for the indicated times in light at 500 μmol quanta $\cdot m^{-2} \cdot s^{-1}$ (*a*, *b*) or in darkness (*c*, *d*) in the absence (*a*, *c*) or presence (*b*, *d*) of 1 mM hydrogen peroxide. Immunoblot analysis was performed with affinity-purified anti-LSU antibody following SDS-PAGE

chloroplasts without any addition of effectors (Fig. 1). This may be because the scavenging system of active oxygen species functioned normally in the chloroplasts. However, the fragmentation of Rubisco was observed in the chloroplast lysates in light (Fig. 2A). This may be due to the disintegration of the scavenging system caused by rupturing of chloroplasts and to the inactivation of the related enzymes in the lysates (Nakano and Asada 1987).

In chloroplasts, ascorbate peroxidase is the key enzyme in the scavenging of hydrogen peroxide (Nakano and Asada 1980; Asada and Takahashi 1987) and the activity of ascorbate peroxidase is inhibited by KCN and NaN_3 (Nakano and Asada 1987; Miyake and Asada 1992). Although KCN also inhibits CuZn-superoxide dismutase, the superoxide anion spontaneously disproportionates into hydrogen peroxide. Therefore, the addition of KCN or NaN_3 to the illuminated chloroplasts induces the accumulation of hydrogen peroxide (Nakano and Asada 1980), which is then converted to the hydroxyl radical by a Fenton-type reaction in the chloroplasts. As shown in Fig. 1, the Rubisco-LSU was fragmented into several polypeptides in light in the presence of KCN or NaN_3. The molecular masses of the fragments and their cross-reactivities to the site-specific anti-LSU antibodies matched those observed in the chloroplast lysates (Fig. 2A), as well as those observed in a previous study (Ishida et al. 1997). These results indicate that the fragmentation of the Rubisco-LSU in the intact chloroplasts is also caused by an active oxygen species, the hydroxyl radical, and that the mechanism of the fragmentation which occurred inside the chloroplasts is the same as that observed in the lysates.

Accumulated evidence indicates that scavenging enzymes of active oxygen species are inactivated in chloroplasts under some stressful conditions. For example, the activity of ascorbate peroxidase decreases in tobacco leaves during leaf senescence (Polle 1996). Oxidative-stress (Luna et al. 1994) or severe water-stress (Baisak et al. 1994) conditions also cause the decline of ascorbate peroxidase activity in oat and wheat leaves. Thus, under those conditions, the scavenging capacity for the hydrogen peroxide may become insufficient to detoxify it and to prevent the formation of the hydroxyl radical in chloroplasts. As a consequence, it is possible that the fragmentation of the LSU occurs in leaves, as well as in isolated chloroplasts. This possibility is now under investigation.

Roles of thylakoids and light in the fragmentation. The fragmentation of the Rubisco-LSU both in the intact chloroplasts and their lysates only occurred in light but not in darkness (Figs. 1, 2). These results clearly indicate that light is an essential requirement for the fragmentation. When the stromal fraction was incubated alone in light, fragmentation was not observed (Fig. 2B). However, Rubisco attached to the membranes or the purified Rubisco added to the membrane fraction was fragmented in light (Fig. 2C,D). Thus, the membrane fraction is basically required for the fragmentation, and stromal components other than Rubisco are not the necessary factors.

In the chloroplast lysates, DCMU completely inhibited the fragmentation (Fig. 3), indicating that the electron transport after PSII at the thylakoid membrane is involved in the process of fragmentation. Methyl viologen to some extent suppressed the fragmentation (Fig. 3). In illuminated thylakoids, the univalent reduction of oxygen occurs mainly at the reducing side of PSI. This reduction leads to the formation of an active oxygen species, the superoxide anion, which is converted into hydrogen peroxide by CuZn-superoxide dismutase or by spontaneous disproportionation (Asada 1994). In general, hydrogen peroxide can be converted to the hydroxyl radical in the Fenton reaction in the presence of metal ions, such as reduced iron or copper (Stadtman 1993). Recently, Jakob and Heber (1996) have investigated the mechanism of light-dependent production of the hydroxyl radical in the thylakoids of spinach. When ascorbate peroxidase was inactivated by the addition of KCN or by rupture of the chloroplast envelopes, the hydroxyl radical was produced as a consequence of O_2 reduction by the electron transport in illuminated thylakoids. They also found that the addition of methyl viologen decreased the production of the hydroxyl radical in the illuminated thylakoid membrane, but that production was partially recovered by the addition of Fe^{3+} to the reaction mixture. Methyl viologen acts as an electron acceptor of PSI and the electron donor for oxygen. Thus, the addition of methyl viologen stimulates the production of the superoxide anion, which converts into hydrogen peroxide, in the illuminated chloroplasts (Nakano and Asada 1980). In addition, Jakob and Heber (1996) indicated that light-dependent hydroxyl radical formation by the thylakoids might be brought about by a Fenton-type reaction between reduced iron species and hydrogen peroxide since methyl viologen reduction does not lead to the accumulation of reduced ferredoxin or reduced Fe-S-centers. In our study, iron was not added to the medium and no stromal components were required for the fragmentation of the LSU

(Fig. 2D). Thus, the partial suppression of the fragmentation by methyl viologen may be due to the decrease in the amount of reduced metal species in the reaction mixtures, because the fragmentation was not induced at all by the addition of hydrogen peroxide in darkness (Fig. 4). These results indicate that not only hydrogen peroxide but also the reducing power generated at the illuminated thylakoids for reduction of metals are essentially required for the fragmentation of the LSU. Further work aimed at the identification of the metal species is in progress.

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