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Quality Control of Photosystem II

*THYLAKOID UNSTACKING IS NECESSARY TO AVOID FURTHER DAMAGE TO THE D1 PROTEIN AND TO FACILITATE D1 DEGRADATION UNDER LIGHT STRESS IN SPINACH THYLAKOIDS**

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Photosystem II is vulnerable to light damage. The reaction center-binding D1 protein is impaired during excessive illumination and is degraded and removed from photosystem II. Using isolated spinach thylakoids, we investigated the relationship between light-induced unstacking of thylakoids and damage to the D1 protein. Under light stress, thylakoids were expected to become unstacked so that the photodamaged photosystem II complexes in the grana and the proteases could move on the thylakoids for repair. Excessive light induced irreversible unstacking of thylakoids. By comparing the effects of light stress on stacked and unstacked thylakoids, photoinhibition of photosystem II was found to be more prominent in stacked thylakoids than in unstacked thylakoids. In accordance with this finding, EPR spin trapping measurements demonstrated higher production of hydroxyl radicals in stacked thylakoids than in unstacked thylakoids. We propose that unstacking of thylakoids has a crucial role in avoiding further damage to the D1 protein and facilitating degradation of the photodamaged D1 protein under light stress.

In the chloroplasts of higher plants and green algae, thylakoid membranes are closely associated and stack to form grana. Under electron microscopy, cylindrical grana consisting of 10–20 layers of thylakoids have been observed. They have a diameter of 300–600 nm and are interconnected by lamellae of several hundred nm in length (1, 2). The structure of grana in the chloroplasts of higher plants is well known, but the precise role of grana is incompletely understood. Their possible functions in primary photochemical reactions and subsequent events have been discussed extensively (3–9). Photosystem I (PSI)[3] and II (PSII) complexes are segregated from each other in thylakoids, showing lateral heterogeneity in their distribution. The PSII complex is a multisubunit pigment-protein complex responsible for the photochemical oxidation of water and reduction of plastoquinone (8, 10–13). It comprises >25 protein subunits and other low molecular weight cofactors, including chlorophylls, carotenoids, plastoquinones, and manganeses. In the chloroplasts of higher plants, PSII complexes and the associated light-harvesting antenna complex LHCII are not present throughout the thylakoid membranes but are abundant in the grana (2, 14). A densely packed array of PSII complexes in the grana was visualized by electron microscopy (8, 15). Grana formation is more prominent in shade leaves (or shade plants) than in sun leaves (or sun plants), so it has been suggested that enrichment of the PSII·LHCII complex in grana is a strategy of plants to collect excitation energy by PSII under weak light (16). The grana structure probably provides an organized environment for PSII. PSI and ATP synthase are located exclusively in the stroma-exposed thylakoids, including the stroma thylakoids, grana end membranes, and grana margins, because these complexes protrude into the stroma. Cytochrome b_6/f complexes without this protrusion are present uniformly throughout the thylakoids (3). It has been suggested that separation of PSI and PSII complexes on the thylakoids through grana formation is important to prevent "spillover" of excitation energy from PSII to PSI, which lowers photosynthesis efficiency (17).

An active PSII complex comprises a homodimer of PSII monomers (13). When thylakoids are exposed to excessive visible light, the PSII dimer dissociates into two monomers (18), but the most significant change takes place inside the monomeric PSII, where the reaction center-binding D1 protein is photodamaged and degraded by specific proteases (19, 20). The photodamage to the D1 protein is a photooxidative process. This is caused by reactive oxygen species (ROS), most probably

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[3] The abbreviations used are: PSI and PSII, photosystem I and II, respectively; LHCII, light-harvesting chlorophyll-protein complex; ROS, reactive oxygen species; $^1O_2$, singlet oxygen; HO$^\bullet$, hydroxyl radical; DCBQ, 2,6-dichlorobenzoquinone; FeCN, potassium ferricyanide; Fd, ferredoxin; FNR, ferredoxin-NADP$^+$ oxidoreductase; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; POBN, 4-pyridyl-1-oxide-*N*-*tert*-butylnitrone; POBN-CH(CH_3)OH, α-hydroxyethyl radical adduct of POBN; O_2^-, superoxide anion radical; Mes, 4-morpholineethanesulfonic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

singlet oxygen ($^1O_2$) or the hydroxyl radical ($HO^\bullet$) produced by overreduction of the acceptor side of PSII under excessive illumination or by endogenous cationic radicals, such as the oxidized forms of the primary electron donor P680 and the secondary electron donor Tyr_Z (Tyr^{161} of D1) to PSII (21). Strong illumination of the grana may readily cause damage to the PSII complexes by ROS and endogenous cationic radicals, because the grana is rich in PSII complexes. Segregation of PSI and PSII in the stacked thylakoids should make the electron transport between PSI and PSII a rate-limiting step in the electron flow, and overexcitation of PSII under these conditions may stimulate ROS production at the acceptor side of PSII. Close association of LHCII with the PSII core complexes should also stimulate ROS generation in the grana. Unstacking of the thylakoids, which is also expected to lead to random distribution of PSI and PSII on the thylakoids and dissociation of the LHCII from the PSII core, may be important to avoid photodamage to PSII.

In the proteolysis of the damaged D1 protein in the chloroplasts of higher plants, the N-terminal Thr of the D1 protein is dephosphorylated, and the subsequent degradation produces 23- and 9-kDa fragments as the primary cleavage products (19, 20). The protease(s) and phosphatase(s) involved in these steps are presumably localized in the stroma thylakoids, grana end membranes, and grana margin. Lateral migration of the damaged PSII complexes from the grana to the membrane regions where the damaged PSII complexes are repaired is therefore important for degradation of the D1 protein. Thylakoid unstacking, if it occurs under light stress, should stimulate diffusion of the protein complexes on the thylakoids, thereby stimulating D1 turnover.

First, we examined if excessive visible light can induce unstacking of the thylakoids. Second, we studied the effects of strong illumination on stacked and unstacked thylakoids to see if they showed different responses to excessive light. We strongly suggest that unstacking of the thylakoids caused by light stress is necessary to avoid further photodamage to the D1 protein and to facilitate degradation and removal of the photodamaged D1 protein from PSII complexes.

EXPERIMENTAL PROCEDURES

Preparation of Thylakoids from Spinach—Fresh spinach leaves were purchased from a local market. Intact chloroplasts and intact thylakoid membranes were prepared according to a previously described method (22). Thylakoids were suspended in a buffer solution containing 0.1 M sorbitol, 15 mM NaCl, 5 mM $MgCl_2$, and 50 mM Tricine-KOH (pH 7.5) (solution A) and stored at −20 °C until use. Where indicated, 1 M glycine betaine was included during the preparation of intact thylakoids and in the final suspension medium. To obtain $MgCl_2$-free thylakoids, thylakoids were washed with solution A in which $MgCl_2$ was omitted (solution B) and resuspended in the same medium. NaCl (15 mM) was always included in the suspension medium to supply sufficient Cl^- to the oxygen-evolving site to sustain PSII activity. Procedures were carried out in darkness under green light. Chlorophyll was determined with 80% acetone extract using a Hitachi U-2000 spectrophotometer.

Light Stress on Thylakoid Membranes—For treatment of thylakoids with excessive visible light, thylakoids that had been suspended in solution B with or without 20 mM $MgCl_2$ at a chlorophyll concentration of 0.1 mg/ml chlorophyll for stacking or unstacking of the thylakoids were illuminated with white light (intensity 500–2,000 μmol of photons m^{-2} s^{-1}) for 10–60 min. Where indicated, 10 mM NH_4Cl, 1 mM 2,6-dichlorobenzoquinone (DCBQ), and 1 mM potassium ferricyanide (FeCN) were added to the suspension of thylakoids.

Measurement of PSII Activity—PSII activity of the thylakoids was measured by oxygen evolution using a Hansatech oxygen electrode connected to a thermoregulated water bath. The reaction mixture (1 ml) contained 0.4 M sucrose, 50 mM Mes (pH 6.5), 1 mM DCBQ, 1 mM FeCN, 10 mM NaCl, 5 mM $MgCl_2$, 10 mM $CaCl_2$, 10 mM NH_4Cl, and thylakoids equivalent to 30 μg of chlorophyll. For the measurement of oxygen evolution coupled with $NADP^+$ photoreduction, ferredoxin (Fd) (11 μg/ml), ferredoxin-$NADP^+$ oxidoreductase (FNR) (25 μg/ml), and 0.2 mM $NADP^+$ were added as electron acceptors to the reaction mixture. Fd and FNR were purchased from Sigma. Saturating actinic light was obtained from a W-lamp.

Digitonin Fractionation of Thylakoids and Estimation of the Degree of Thylakoid Stacking—Digitonin was used to separate stacked and unstacked thylakoids (23). To purify digitonin, 1 g of crude digitonin (Sigma) was dissolved in distilled water (20 ml). After centrifugation at 35,000 × *g* for 10 min, the supernatant containing pure digitonin was collected, freeze-dried, and stored at 4 °C in the dark. Freshly prepared thylakoids were suspended in a solution containing 0.2 M sorbitol and 1.5 mM K_2HPO_4 (pH 7.0) (solution C) at a chlorophyll concentration of 0.25 mg/ml and incubated in the dark at 4 °C for 15 min. Thylakoids were treated with 0.5% digitonin for 30 min with gentle stirring on ice. After dilution 6 times with solution C, the thylakoid suspension was centrifuged at 10,000 × *g* for 30 min, and pellets containing stacked thylakoids were suspended in solution C. The amount of chlorophyll in the pellet relative to that in the total thylakoids represented the degree of thylakoid stacking. When stacking of the thylakoids was induced by salts, NaCl or $MgCl_2$ was added before digitonin treatment. For examining the effects of light stress on the stacked thylakoids, thylakoids incubated with solution C with 15 mM NaCl and 25 mM $MgCl_2$ were illuminated before digitonin treatment. Where indicated, 10 mM NH_4Cl, 100 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), 1 mM DCBQ, 1 mM FeCN, Fd (11 μg/ml), FNR (25 μg/ml), and 0.2 mM $NADP^+$ were added to the reaction mixture before illumination.

SDS/Urea-PAGE and Western Blot Analysis—SDS/urea-PAGE and Western blot analysis were carried out as described previously (22). Acrylamide concentrations in the stacking and resolving gels were 5% (w/v) and 12.5% (w/v), respectively. In each lane of the gel, thylakoid sample equivalent to 10 μg of chlorophyll was included. The antibody against the DE-loop of the D1 protein was used for detection of the cleavage and aggregation products of the D1 protein after sample exposure to light stress. Anti-LHCb1–6 (antenna chlorophyll-binding proteins comprising LHCII) and plastocyanin antibodies were purchased from Agrisera (Vännäs, Sweden). A horseradish peroxidase-conjugated anti-rabbit antibody or an anti-hen antibody

(Bio-Rad) was the secondary antibody. Immunodecorated bands were detected by fluorography with ECL (Amersham Biosciences).

EPR Spin-trapping Spectroscopy—Spin trapping was accomplished by a 4-pyridyl-1-oxide-*N*-*tert*-butylnitrone (POBN)/ethanol spin-trapping system (23). Thylakoid membranes (0.3 mg/ml chlorophyll) were illuminated in a glass capillary tube (Blaubrand® intraMARK, Brand, Germany) in the presence of 50 mM POBN, 170 mM ethanol, 15 mM NaCl, and 25 mM K_2HPO_4 (pH 7.2). Illumination was with continuous white light (1,000 μmol of photons m^{-2} s^{-1}) using a halogen lamp with a light guide (KL 1500 Electronic, Schott, Germany), and spectra were recorded using an EPR spectrometer MiniScope MS100 (Magnet Tech GmbH, Berlin, Germany). Signal intensity was evaluated as the relative height of the central doublet of the first derivate of the absorption spectrum. EPR conditions were as follows: microwave power, 10 milliwatts; modulation amplitude, 1 G; modulation frequency, 100 kHz; sweep width, 100 G; scan speed, 1.62 G/s.

RESULTS

Unstacking of Thylakoids under Strong Illumination—The extent of membrane stacking was determined by fractionation of thylakoids into grana and stroma thylakoids with 0.5% digitonin. The amount of chlorophylls in the 10,000 × *g* pellets relative to the total chlorophyll in thylakoids after digitonin treatment gave the relative stacking of the thylakoids (24). Stacking of the thylakoids *in vitro* depends on the concentration of monovalent or divalent cations as well as the pH of the suspension medium (24). The degree of the thylakoid stacking was about 10% when thylakoids were incubated in solution C containing 0.2 M sorbitol and 1.5 mM K_2HPO_4 (pH 7.0) at a chlorophyll concentration of 0.25 mg/ml. Thylakoids showed a progressive increase of stacking by the addition of NaCl or $MgCl_2$, and the extent of stacking became ~50% at 20 mM $MgCl_2$ (Fig. 1*A*).

Illumination of thylakoids stacked in the presence of 15 mM NaCl and 20 mM $MgCl_2$ with strong light (500–2,000 μmol of photons m^{-2} s^{-1}) at 25 °C induced significant unstacking of the thylakoids (Fig. 1, *B* and *C*). Hereafter, NaCl (15 mM) was included in all of the thylakoid samples to supply sufficient Cl^- to the oxygen-evolving site to sustain PSII activity. Identical illumination caused only partial unstacking of the thylakoids when they had been preincubated in $MgCl_2$-free solution B because the membranes were already unstacked (data not shown). Light-induced unstacking of thylakoids was irreversible, and partial recovery was observed in the dark after strong illumination (Fig. 1*B*). This recovery may be due to the effects of 20 mM $MgCl_2$ remaining in the thylakoid suspension. The light-induced unstacking of thylakoids was inhibited in part by the addition of DCMU, whereas the electron acceptors FeCN + DCBQ or Fd + FNR + $NADP^+$ significantly suppressed the light-induced unstacking (Fig. 1*C*). These results suggest that light-induced electron transport, not only in PSII but also in linear electron transport through PSI and PSII, is responsible for the light-induced unstacking of thylakoids.

Inhibition of PSII Activity by Strong Illumination—The rate of steady state evolution of oxygen of thylakoids was measured with thylakoids suspended in solution A (Fig. 2*A*). The thylakoids showed PSII-dependent (electron acceptors: DCBQ and FeCN) and PSI + PSII-dependent (electron acceptors: Fd, FNR, and $NADP^+$) oxygen-evolving activities. The thylakoids used here seemed to have lost their own Fd and FNR during the preparation, because the sole addition of $NADP^+$ did not support oxygen evolution (data not shown). When Fd, FNR, and $NADP^+$ were all omitted from the reaction mixture, oxygen consumption was observed, suggesting that ROS are produced in the electron transport pathway. It is likely that the thylakoids used here were not disrupted significantly during the isolation procedure, because plastocyanin (marker protein localized in the lumen) was retained in thylakoids at much the same level as in intact chloroplasts (Fig. 2*B*).

Knowing the characteristics of the thylakoid samples, we next examined the effects of strong illumination on oxygen evolution of stacked and unstacked thylakoids (Fig. 2*C*). The oxygen-evolving activity of stacked thylakoids was about 15% higher than unstacked thylakoids before strong illumination. Importantly, the oxygen-evolving activity supported by DCBQ and FeCN of the thylakoids stacked in the presence of 20 mM $MgCl_2$ was inhibited by strong light 50% more than that in the unstacked thylakoids. The effects of strong light on the stacked and unstacked thylakoids were also examined with oxygen evolution coupled to $NADP^+$ photoreduction, but it was difficult to compare the light effects on the two different thylakoid samples because of the small oxygen-evolving activity. We used 1 M glycine betaine in the isolation of intact thylakoids and in the final suspension medium, but the results were identical (data not shown).

Photodamage to the D1 Protein—The D1 protein is photodamaged by excessive illumination. The damaged protein is degraded by a specific protease(s) or forms aggregates with neighboring polypeptides (25, 26). To estimate how much damage to the D1 protein occurs depending on a given change in the stacking of the thylakoids, we looked at the degradation and aggregation products of the D1 protein (as well as the loss of the original D1 band) with Western blot analysis using the antibody against the DE-loop of the D1 protein. Significant change was not detected by Coomassie staining of the SDS/urea-polyacrylamide gel between the dark-incubated and light-treated thylakoids (Fig. 3*A*), but Western blot analysis demonstrated that the amounts of aggregation products of the D1 protein were notably increased by strong illumination in the presence of increasing concentrations of $MgCl_2$ in the reaction mixture (Fig. 3*B*). The amounts of the degradation products of the D1 protein were also increased in the stacked thylakoids after illumination but not so significantly compared with D1 aggregates. This is probably because the aggregation and degradation of the D1 protein are competitive processes depending on the stacked and unstacked conditions of the thylakoids. A net loss of the original D1 band was clearly detected by strong illumination of thylakoids in the presence of $MgCl_2$ (Fig. 3*C*). We therefore concluded that photodamage to the D1 protein was larger in stacked thylakoids than in unstacked thylakoids.

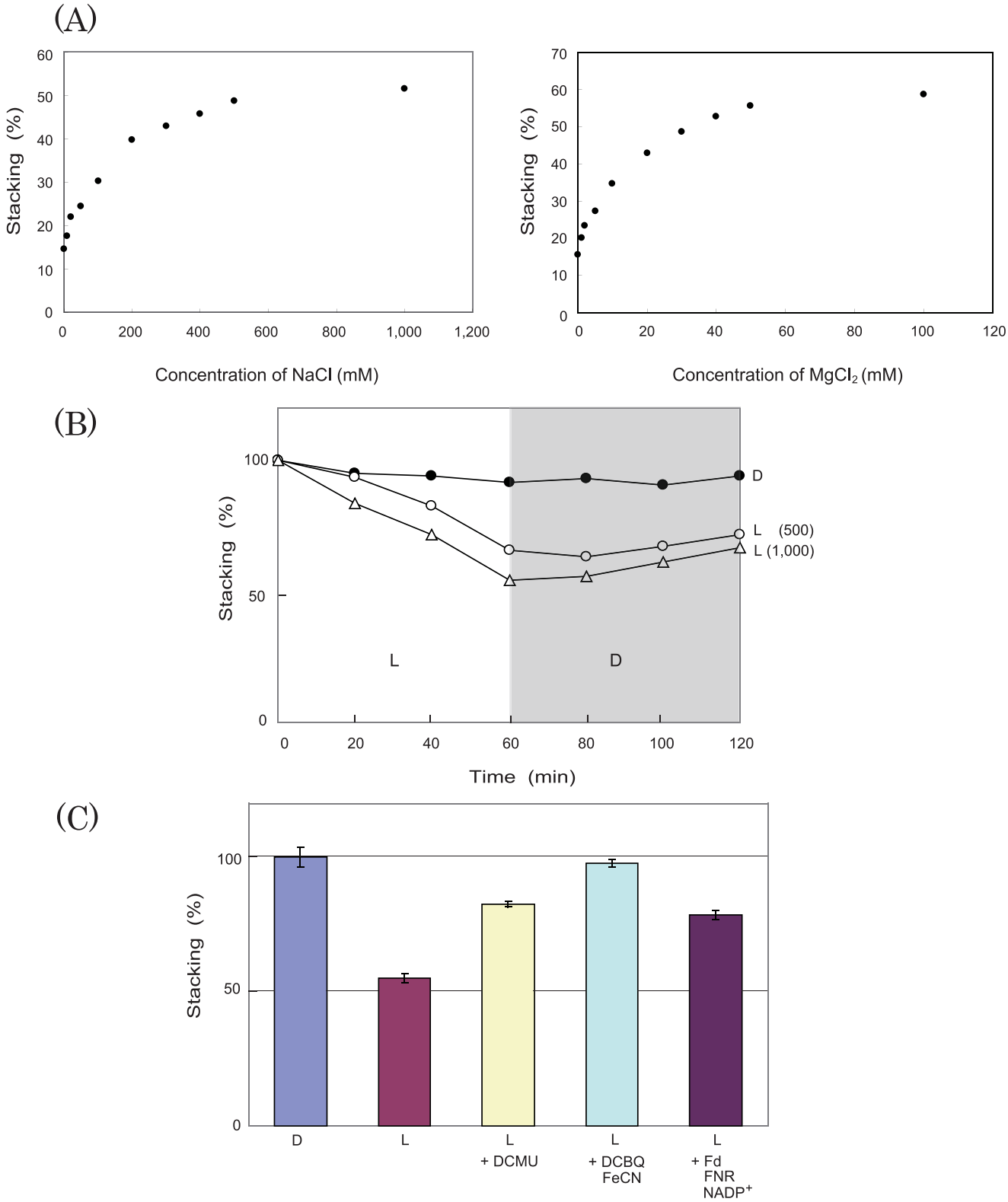


FIGURE 1. **Cation-induced stacking and subsequent light-induced unstacking of thylakoids.** *A*, stacking of isolated spinach thylakoids in the presence of increasing concentrations of NaCl (*left*) and $MgCl_2$ (*right*). NaCl or $MgCl_2$ was added to thylakoids isolated and washed in an NaCl- and $MgCl_2$-free buffer solution containing 0.2 M sucrose and 1.5 mM K_2HPO_4 (pH 7.0). The degree of thylakoid stacking was assayed by the digitonin fractionation method described under "Experimental Procedures" and is shown as a relative value. *B*, time courses of light-induced unstacking of thylakoids. Thylakoids suspended in a buffer solution containing 0.2 M sucrose, 15 mM NaCl, 20 mM $MgCl_2$, and 1.5 mM K_2HPO_4 (pH 7.0) were illuminated with strong white light (intensity: 500 or 1,000 μmol of photons m^{-2} s^{-1}) for 60 min at 25 °C and subsequently incubated in darkness to note the reversibility of unstacking. *D* and *L*, light and dark, respectively. Other conditions were the same as for *A*. *C*, effects of the electron transport inhibitor DCMU and electron acceptors on the strong light-induced unstacking of thylakoid membranes. Thylakoids had been stacked by incubation in the medium described in *B*. DCMU (100 μM) and electron acceptors DCBQ (1 mM) + FecN (1 mM), or Fd (11 μg/ml) + FNR (25 μg/ml) + $NADP^+$ (0.2 mM) were added where indicated. Samples were illuminated with strong light (intensity: 1,000 μmol of photons m^{-2} s^{-1}) for 30 min at 25 °C. Other conditions were the same as for *A*. Data are the mean of three measurements ± S.D.

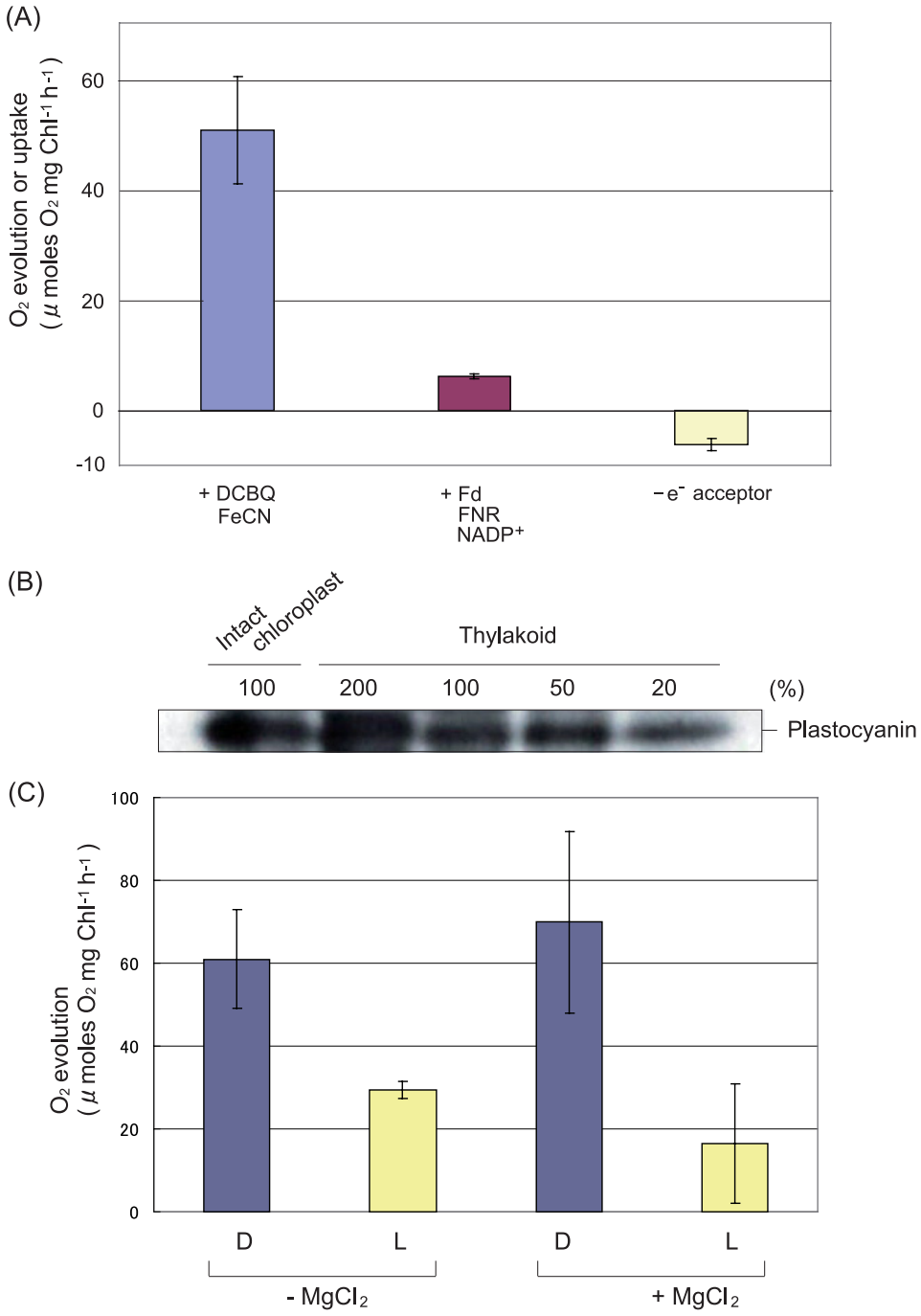


FIGURE 2. **Characterization of thylakoids and effects of strong illumination on the oxygen-evolving activity of stacked and unstacked thylakoids.** *A*, the oxygen-evolving and oxygen consumption activity of thylakoids in the presence and absence of electron acceptors. Reaction mixture contained 0.4 M sucrose, 50 mM Mes (pH 6.5), 10 mM NaCl, 5 mM $MgCl_2$, 10 mM $CaCl_2$, 10 mM NH_4Cl, and thylakoids equivalent to 30 μg of chlorophyll. DCBQ (1 mM) and 1 mM FeCN were used as electron acceptors (*blue bar*). For the measurement of oxygen evolution coupled with $NADP^+$ photoreduction, Fd (11 μg/ml), FNR (25 μg/ml), and 0.2 mM $NADP^+$ were added as electron acceptors (*red bar*). Where indicated, no electron acceptor was added (*yellow bar*). Measurement was carried out with an oxygen electrode at 25 °C. *B*, content of plastocyanin in thylakoids shown by Western blot analysis with the antibody against spinach plastocyanin. As a control, intact chloroplasts that retain plastocyanin in the thylakoid lumen are shown in the *far left lane*. The amounts of thylakoids in each lane are shown on the basis of relative chlorophyll content, and 100% corresponds to 2.5 μg of chlorophyll. *C*, oxygen-evolving activity of stacked and unstacked thylakoids with (*L*) or without (*D*) preillumination with strong light. DCBQ (1 mM) and FeCN (1 mM) were used as electron acceptors. Thylakoids were stacked or unstacked in the presence or absence of 20 mM $MgCl_2$, respectively. For the preillumination, stacked and unstacked thylakoids were irradiated with strong light (intensity: 1,000 μmol of photons m^{-2} s^{-1}) for 10 min at 20 °C. Other conditions were the same as for *A*. Data are the mean of three measurements ± S.D.

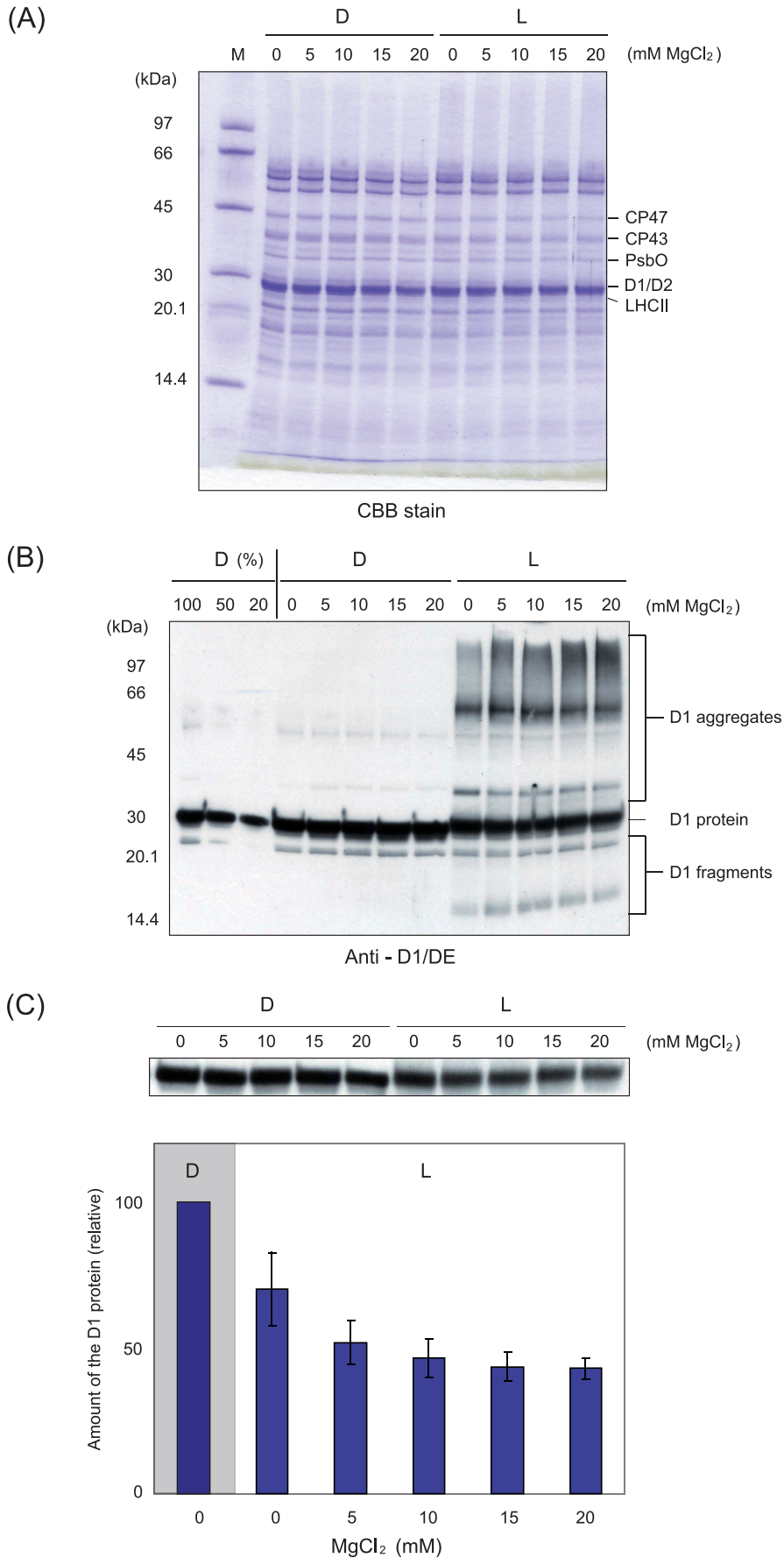
Detection of ROS in PSII in Stacked and Unstacked Thylakoids—To study the involvement of ROS in photodamage to the D1 protein, EPR spin-trapping spectroscopy was used for detection of free radicals generated by PSII. Light-induced production of $HO^•$ was measured using a POBN/ethanol spin-trapping system (23). In this system, the interaction of $HO^•$ with ethanol yields α-hydroxyethyl radical ($CH(CH_3)HO^•$), which reacts with POBN, forming a stable α-hydroxyethyl radical adduct of POBN (POBN-$CH(CH_3)$OH adduct). When thylakoids were illuminated in the presence of POBN, POBN-$CH(CH_3)$OH adduct EPR spectra were detected (Fig. 4*A*), indicating $HO^•$ formation. To avoid contribution of PSI to the overall photogeneration of $HO^•$ in the thylakoid membrane, $HO^•$ production was measured in the presence of DCMU (an *inhibitor* that blocks electron transfer from the primary quinone (Q_A) to the secondary quinone (Q_B) electron acceptor in PSII). Fig. 4*A* shows that the light-induced POBN-$CH(CH_3)$OH adduct EPR signal was significantly lowered by DCMU. This observation indicates that in the thylakoid membrane, $HO^•$ is produced predominantly by PSI, whereas PSII contributes to the overall production of $HO^•$ by about 5–10%. The fact that $HO^•$ production persists in the presence of DCMU reveals that $HO^•$ is partially produced before the Q_B-binding site. An uncoupler NH_4Cl (10 mM) had no effect on the POBN-$CH(CH_3)$OH adduct EPR signal, but the addition of FeCN (1 mM) almost completely suppressed the signal (Fig. 4*B*).

The effect of $MgCl_2$ on photogeneration of $HO^•$ was measured in order to study $HO^•$ production in stacked and unstacked thylakoid membranes. Fig. 4, *C* and *D*, show that the EPR signal of the POBN-$CH(CH_3)$OH adduct was enhanced by increasing concentration of $MgCl_2$ added to the reaction mixture before measurement. These results suggest that $HO^•$ production

is more pronounced in stacked thylakoids compared with unstacked thylakoids.

Effects of Electron Acceptors on the Photodamage to the D1 Protein—EPR measurement clearly showed that HO• is produced around PSII by the strong illumination of stacked thylakoids and that the signal of HO• is suppressed by electron acceptors, so we examined the effects of electron acceptors on the photodamage to the D1 protein in stacked thylakoids (Fig. 5). FeCN (1 mM) suppressed photodamage to the D1 protein, which was represented by a decrease in aggregation and degradation of the D1 protein. The addition of combined DCBQ (1 mM) and FeCN (1 mM) had a more prominent effect on suppressing photodamage. The uncoupler NH_4Cl had no effect on degradation and aggregation in the D1 protein.

LHCII Degradation—In the state transition of chloroplasts of higher plants and algae, the distribution of excitation energy between PSI and PSII is regulated depending on light quality. Phosphorylation and dephosphorylation of LHCII catalyzed by specific kinases and phosphatases have a significant role in the migration of LHC complexes between PSII and PSI (27). Major LHCII complexes that are phosphorylated by overexcitation of PSII relative to PSI move from PSII to PSI to balance the excitation energy between the two photosystems. We wondered if the major LHCII complexes associated with PSII are susceptible to light stress and are degraded in much the same way as the D1 protein or if LHCII migrates from the grana to the stroma thylakoids during light stress. When thylakoids were exposed to strong light, no difference was detected in the amount of LHCII in the Coomassie Blue-stained gel of SDS-PAGE (data not shown). Western blot analysis with the antibody against LHCb1, which accounts for 60% of the total LHCII (28), also indicated little degradation of the major LHCII by strong illumination. Migration of LHCb proteins from the grana to



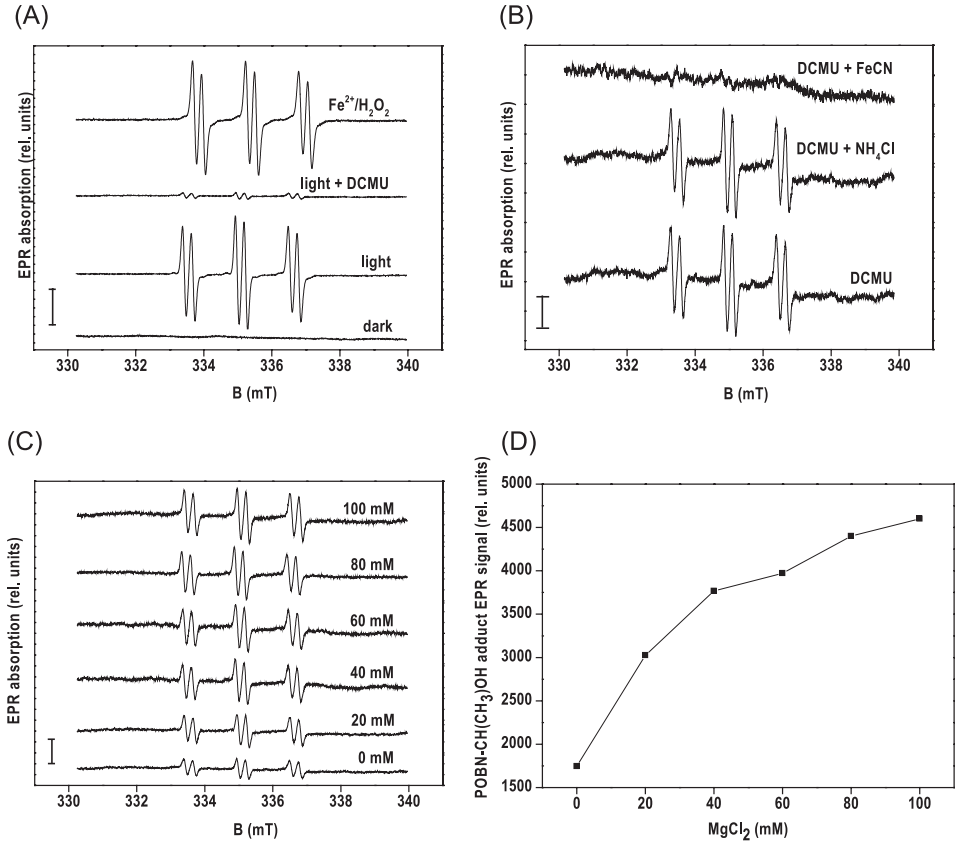


FIGURE 4. **Photogeneration of the hydroxyl radical in thylakoids.** *A*, POBN-CH(CH_3)OH adduct EPR spectra measured in thylakoids in the dark (the *bottom spectrum*) and after illumination in the absence and presence of DCMU (200 μM) (*middle spectra*). The *top spectrum* was obtained by the addition of $FeSO_4$ (100 μM) to H_2O_2 (50 μM). *B*, effect of NH_4Cl (10 mM) and FeCN (1 mM) on POBN-CH(CH_3)OH adduct EPR spectra measured in thylakoids after illumination in the presence of DCMU (200 μM). *C*, effect of $MgCl_2$ on POBN-CH(CH_3)OH adduct EPR spectra measured in thylakoids after illumination in the presence of DCMU (200 μM). Before illumination, $MgCl_2$ at the concentrations indicated in the figure was added to thylakoids. *D*, dependence of POBN-CH(CH_3)OH adduct EPR signal on $MgCl_2$ concentration. The POBN-CH(CH_3)OH adduct EPR signal was determined as the relative height of the central doublet of the first derivative of absorption spectra. In *A–D*, thylakoids (0.30 mg/ml chlorophyll) were illuminated in the presence of 50 mM POBN, 170 mM ethanol, 15 mM NaCl, and 25 mM K_2HPO_4 (pH 7.2) with continuous white light of 1,000 μmol of photons m^{-2} s^{-1}. In *A–C*, the *vertical bars* represent 7,500, 500, and 2,500 relative units, respectively. *mT*, milliteslas.

the stroma thylakoids after strong illumination was not detected (data not shown).

DISCUSSION

We showed that the stacking and unstacking conditions of the thylakoids have a pivotal role in the quality control of PSII during light stress. PSII complexes are abundant in the grana, segregated from PSI complexes. PSII complexes are also separated from phosphatases that dephosphorylate the N-terminal phospho-Thr of the photodamaged D1 protein as well as specific proteases that recognize the damaged D1 protein. These two enzymes are required for the repair of the D1 protein and are probably located in the non-appressed regions of thylakoids: the grana margin and the grana end membranes (29).

Significant unstacking of thylakoids occurred when thylakoids were exposed to excessive light (500–2,000 μmol of photons m^{-2} s^{-1}) at 25 °C. This unstacking should have crucial roles in turnover of the D1 protein. First, thylakoid unstacking may facilitate migration of the PSII complex from the grana to the stroma thylakoids. The distance between two thylakoids under a naturally stacked condition was estimated to be 30–40 Å, whereas electron micrograph tomography showed much closer association of the membranes (30). Surface-to-surface interaction between the two stacked thylakoids via interaction of multiprotein complexes located in the adjacent thylakoids should therefore occur. The thylakoid membranes are crowded (31) because of a high molecular ratio of protein to lipid. In the grana region where PSII complexes are densely packed, movement of protein complexes may be considerably restricted. Under these circumstances, unstacking of thylakoids under light stress should significantly increase diffusion of PSII complexes from the grana to the grana margin and to the stroma thylakoids, which in turn enables efficient repair of damaged PSII complexes (Fig. 6).

The second possible role of membrane unstacking is to dramatically enlarge the area of the grana margin. The grana margin is the domain where the PSII complexes containing the damaged D1 protein migrate from the grana, thereby enabling the encounter of PSII complexes with the specific proteases that recognize and digest the photodamaged D1 protein. Increase in the area of the grana margin is therefore crucial for efficient turnover of the D1 protein during light stress. The ATP-dependent metalloprotease FtsH (filamentation temperature-sensitive H) has been suggested to recognize and degrade

FIGURE 3. **Strong light-induced damage and subsequent degradation and aggregation of the D1 protein in stacked and unstacked thylakoids.** *A*, profile of the proteins in thylakoids shown by SDS/urea-PAGE and Coomassie Blue staining. *D* and *L* at the *top* of the gel indicate that the thylakoids were kept in the dark (*D*) or illuminated with strong light (intensity: 1,000 μmol of photons m^{-2} s^{-1}) for 30 min (*L*). Samples were loaded on the basis of the same chlorophyll (2.5 μg). The concentration of $MgCl_2$ added to the reaction mixture is also shown in millimolar at the *top* of the gel. Molecular mass markers and the major proteins of PSII are shown at the *left* and *right* of the gel, respectively. *B*, Western blot analysis of the D1 protein in thylakoids with the antibody against the DE-loop of the D1 protein. Samples were loaded on the basis of the same chlorophyll (2.5 μg of chlorophyll). *Three lanes* at the *left* of the gel show dilution experiments, and 100% corresponds to 2.5 μg of chlorophyll. Locations of the original band of the D1 protein, D1 fragments, and D1 aggregates are shown on the *right* side of the fluorogram. The x-ray film was overexposed to clearly show the light-induced degradation and aggregation of the D1 protein. *C*, quantification of the D1 protein remained in the position of the original molecular mass. Data are the mean of three measurements ± S.D. The x-ray film was exposed for a shorter time compared with *B* to show the change in the amount of the original D1 band.

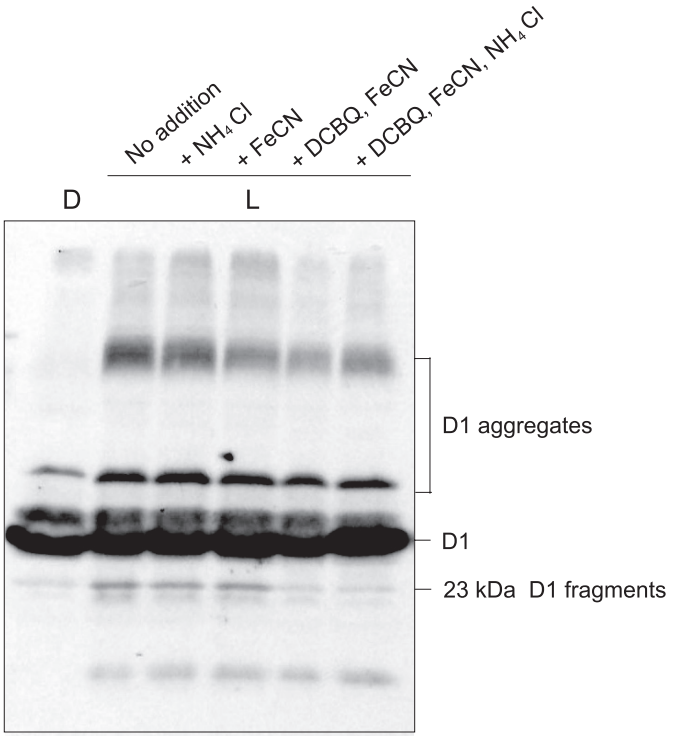


FIGURE 5. **Effects of electron acceptors on photodamage to the D1 protein in stacked thylakoids.** Aggregation and degradation of the D1 protein after strong illumination (1,000 μmol of photons m^{-2} s^{-1} for 30 min) in the presence and absence of electron acceptors and the uncoupler NH_4Cl were examined by Western blot analysis with antibody against the DE-loop of the D1 protein. The samples were loaded on the basis of same chlorophyll (2.5 μg). The chemicals added were 1 mM DCBQ, 1 mM FeCN, and 10 mM NH_4Cl. The bands of the D1 protein and the aggregates and fragments of the D1 protein are shown to the *right* of the fluorogram.

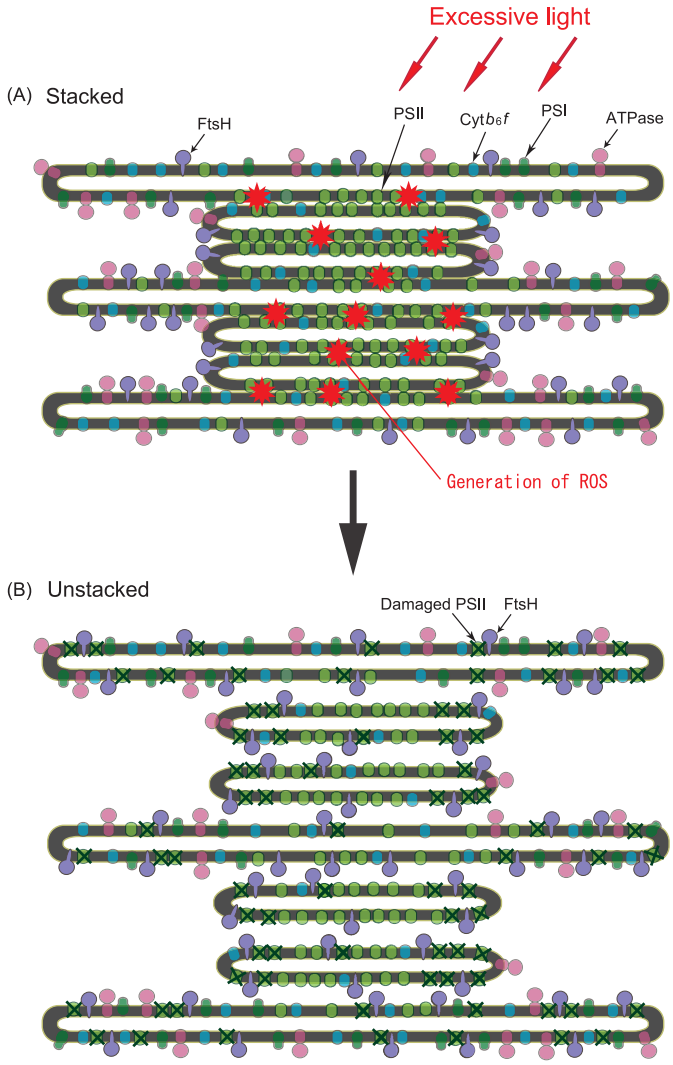


FIGURE 6. **Model showing the generation of reactive oxygen species from PSII located in the stacked thylakoids during excessive illumination and subsequent unstacking of thylakoids.** *A*, the grana portion of the thylakoids is rich in PSII complex, whereas the PSI is abundant in the stroma thylakoids; PSII and PSI are therefore spatially segregated in stacked thylakoids. Strong illumination induces reactive oxygen species near PSII in the grana stack. *B*, after excessive illumination, thylakoids are unstacked, making movement of the photodamaged PSII complex and FtsH proteases easier on the thylakoids. ×, photodamaged PSII complexes. Unstacking of the thylakoids is depicted in an exaggerated manner to show the change in the distribution of the PSII complexes, LHCII, and FtsH.

the photo- or heat-damaged D1 protein in PSII (26, 32–38). The active form of the FtsH protease has a hexameric ring structure, and its diameter is about 120 Å (39). The size of the hexameric protease is comparable with that of the ATP synthase complex, so such a large structure may not be able to enter the appressed region of the grana stack, which is rich in PSII complexes. These lines of evidence support the view that migration of PSII complexes and proteases on thylakoid membranes is crucial for degradation of the damaged D1 protein and that efficient degradation of the D1 protein becomes possible if thylakoids are unstacked (Fig. 6).

The FtsH proteases are relatively unstable and readily degraded in isolated thylakoids even in the dark (data not shown). The intactness of FtsH may be essential for efficient turnover of the damaged D1 protein. The proteases are expected to be more stabilized when they work actively to degrade the damaged D1 protein in unstacked thylakoids. Illumination of the grana produces ROS in PSII as well as PSI, which may cause oxidative damage to various components in the thylakoids, including the FtsH proteases. FtsH may lose its activity if it is damaged. In the presence of $MgCl_2$, photo-induced degradation of the D1 protein was stimulated (Fig. 3*B*). One may argue that this result contradicts the view that ROS produced upon excessive illumination of stroma thylakoids damages the FtsH. We suggest that primary cleavage of the D1 protein is partly due to the action of ROS and partly due to that of proteases; stimulation of the D1 cleavage observed here may be attributed largely to ROS-induced cleavage.

The third possible role of thylakoid unstacking is protection of PSII from damage by ROS. The latter are the inevitable by-products of photosynthesis. In PSII, ROS are formed during excessive illumination in the vicinity of the D1 protein and result in oxidative damage to the protein. One of the ROS produced by the acceptor-side mechanism of photoinhibition of PSII is $^1O_2$ formed via a charge recombination mechanism (40–44). Besides light stress, moderate heat stress has been shown to form $^1O_2$ (45). Singlet oxygen generated under heat stress has been proposed to originate from lipid peroxidation, which eventually produces oxidative damage to the D1 protein (46). The hydroxyl radical has been demonstrated to be another ROS produced on the PSII electron acceptor side by the acceptor-

side mechanism of photoinhibition of PSII (47). The authors proposed that interaction of the superoxide anion radical ($O_2^{\cdot-}$) with non-heme iron results in the formation of bound peroxide, which is reduced to $HO^{\cdot}$. It seems likely that $^1O_2$ and $HO^{\cdot}$ are involved in cleavage of the DE-loop of the D1 protein. ROS may therefore directly participate in the impairment of the D1 protein in stresses caused by light and heat.

Significantly more ROS were produced in stacked thylakoids than in unstacked thylakoids according to EPR spin-trapping spectroscopy (Fig. 4). There are two possible explanations why stacked thylakoids stimulate ROS production under light stress. First, the photochemistry of PSII is more efficient in stacked thylakoids. It is assumed that the grana structure in chloroplasts in higher plants is formed to optimize the capture and usage of excitation energy in PSII. LHCII complexes, working as the peripheral antenna and containing many molecules of chlorophyll *a*, chlorophyll *b*, and carotenoid, are closely associated with the PSII core and contribute to form the grana. PSII core complexes are organized into dimers probably to increase the efficiency of excitation usage. Under these conditions, excessive illumination should facilitate ROS production via overreduction of the acceptor side of PSII. Second, thylakoid stacking leads to segregation of PSII from PSI, which should cause delay of the electron transport between PSI and PSII. Diffusion of plastoquinone in the lipid phase of the thylakoid is known to be one of the rate-limiting steps in the linear electron transport from PSII to PSI (48). Cytochrome b_6/f, which accepts electrons from plastoquinone, is located in the grana and stroma thylakoids equally. When cytochrome b_6/f accepts electrons from plastoquinone at the grana, plastocyanin, which accepted electrons from the cytochrome b_6/f, must diffuse for a long distance in the lumen to PSI. This electron transport step should be another rate-limiting step for the overall electron transport. It has been shown that in unstacked thylakoids, cytochrome *f* and plastocyanin relaxed close to their thermodynamic equilibrium with the primary electron donor of PSI, P700, whereas in stacked thylakoids much lower equilibrium constants (<5) were calculated, indicating restricted long range diffusion of plastocyanin (49). The delay in electron transport at the acceptor side of PSII may inevitably induce ROS production under excessive illumination. To avoid the unfavorable generation of ROS, thylakoids may become unstacked to randomize the distribution of PSI and PSII on the thylakoids (Fig. 6). Illumination of PSI also generates $O_2^{\cdot-}$ at its reducing side through the Mehler reaction. It was suggested that the ROS produced at PSI is highly damaging to PSII (50). In our measurement of EPR spin trapping, DCMU was added to eliminate the contribution of PSI to the overall signals (Fig. 4).

The present study was focused on the effects of PSII-induced ROS on PSII, but it would be interesting to use isolated thylakoid membranes with reconstituted linear electron transfer chain by adding Fd, FNR, and $NADP^+$ as electron acceptors and investigate if stacking and unstacking have the same effect on the quality control of PSII. Our thylakoid sample retained plastocyanin in the lumen (Fig. 2*B*) but not ferredoxin and FNR. The addition of ferredoxin, FNR, and $NADP^+$ supported the whole chain electron transport from water to $NADP^+$ (Fig. 2*A*). In the presence of these electron acceptors, light-induced unstacking of thylakoids was significantly prevented (Fig. 1*C*), indicating that ROS produced during electron transport in PSII and the linear electron transport pathway through PSI and PSII are responsible for the thylakoid unstacking. Because of the low oxygen-evolving activity coupled to $NADP^+$ photoreduction, we could not compare the effects of high light on the oxygen evolution between stacked and unstacked thylakoids.

In PSII, an inner layer of minor antenna proteins connects the peripheral LHCII trimers to the PSII core complex (51, 52). In the long term photosynthetic acclimatization to high growth irradiation, LHCII proteins are known to be degraded to reduce the absorption cross-section. Significant degradation of LHCII complexes was not observed when thylakoids were exposed to strong illumination for a short period (data not shown). We also examined migration of LHCb proteins comprising peripheral LHCII timers from the grana to the stroma thylakoids, but convincing data confirming migration of LHCb proteins have not been obtained. Detachment of the major LHCII protein from the PSII core should decrease the absorption cross-section, reduce the excitation of PSII by excessive light, and eliminate further damage caused by ROS to the D1 protein. In general, it is believed that thylakoid stacking is maintained primarily by van der Waals attractive forces between many LHCP proteins around the PSII core and partly by electrostatic interaction between the components in the thylakoids (4). Moderate heat stress (40 °C) also induced significant thylakoid unstacking, so hydrogen bonding should also be involved in membrane stacking (26). Detachment and migration of LHCII from the grana to stroma thylakoids should be studied further to elucidate the roles of thylakoid unstacking under light stress. Interaction between the PSII·LHCII complexes on the two membrane surfaces in the stacked thylakoids may facilitate trapping and usage of excitation energy (53). Unstacking of thylakoids should also reduce the possibility of this type of interaction and eliminate the overexcitation of PSII through transfer of intermembrane excitation energy. Two-dimensional packing of PSII·LHCII complexes has been suggested to be enhanced in the presence of high concentrations of $MgCl_2$ (>10 mM) (6). This causes quenching of excitation energy by "non-photochemical quenching" in the presence of the xanthophylls cycle, in which charge transfer from chlorophyll *a* to lutein (54) or to zeaxanthin (55) in LHCII may play an important part. In conditions where PSII·LHCII complexes are highly packed in the grana, ROS generation by excessive light is also inevitable, which should eventually damage PSII complexes in stacked thylakoid areas.

Under natural conditions, stacking and unstacking of thylakoids can occur within minutes, and these are reversible processes (4–6). The strong light-induced unstacking of thylakoids observed here was mostly irreversible (Fig. 1*B*). In isolated thylakoid membranes, proteins and other membrane components (*e.g.* lipids) damaged photooxidatively under light stress are not repaired because of the absence of a repair system. This damage may produce irreversible changes to the surface properties of thylakoids. These processes should eventually cause significant unstacking of thylakoids. Despite these limitations in the *in vitro* experiments, our findings clearly indicate the

possible roles of membrane unstacking in the quality control of PSII during light stress.

In conclusion, thylakoid unstacking under excessive illumination is crucial for the protection of PSII from the ROS generated and for efficient degradation of the D1 protein damaged by ROS. This provides a potential structural basis for PSII regulation under light stress.

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