Degradation of Antenna Chlorophyll-Binding Protein CP43 during Photoinhibition of Photosystem II†

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ABSTRACT: When photosystem II (PS II) membranes from spinach were treated with Tris (0.8 M, pH 9.0) and illuminated with white light (5000 μE m^{-2} s^{-1}) under aerobic conditions at 25 °C, not only were the reaction center-forming D1 and D2 proteins degraded but the antenna chlorophyll-binding protein CP43 was also degraded. Three products of the degradation of CP43, with molecular masses of 17.0, 15.5, and 14 kDa, respectively, were identified by sodium dodecyl sulfate/urea polyacrylamide gel electrophoresis and Western blotting with a specific antibody. Degradation products of another antenna chlorophyll-binding protein of PS II, CP47, were not detected under the same conditions. Concomitant with the damage to the D1 and D2 proteins and CP43, cross-linked products of the D1 protein, CP43, and CP47 were formed. These products were identified as slow-moving smeared bands in the higher molecular weight range of the gel during electrophoresis. Both the degradation and the cross-linking of these proteins were prevented by the addition of electron donors to PS II, a result that suggests that these processes were caused by the donor-side mechanism of photoinhibition. The photoinduced degradation of CP43 and the cross-linking among the D1 protein, CP43, and CP47 were less obvious in the PS II membranes that had been treated with hydroxylamine rather than Tris and in the membranes that had been treated with Tris and reconstituted by addition of an extrinsic 33-kDa protein (OEC33). These results indicate that removal of OEC33, which is closely associated with CP43, from the PS II complex accelerates the degradation and cross-linking of CP43 during photoinhibition. It is suggested that OEC33 is involved in the stabilization of the antenna chlorophyll-binding proteins in PS II during photoinhibition.

In higher plants, algae, and cyanobacteria, strong illumination results in the photoinhibition of photosynthesis (Powles, 1984). Photosystem II (PS II)[1] is the target of such photoinhibition, and specific degradation of the reaction center-forming D1 protein, as well as inhibition of electron transport in PS II, occurs *in vitro* with isolated thylakoids, PS II membranes, and PS II reaction center complexes under these conditions (Ohad et al., 1985; Mattoo et al., 1989; Barbato et al., 1991a; Barber & Andersson, 1992; Aro et al., 1993).

Two mechanisms of photoinhibition have been proposed from studies *in vitro*. The first one involves the overreduction of the acceptor side of PS II, which is caused by the illumination of PS II with strong light under aerobic conditions. At the binding site of plastoquinone (Q_B) in the D1 protein, reaction of oxygen with Q_B^{2-} results in production of oxygen radicals which might damage the D1 protein (Kyle et al., 1984). More recently, it was postulated that double reduction of Q_A might occur during strong illumination (Styring et al., 1990), and that the triplets of chlorophyll formed by the charge recombination between $P680^+$ and $pheophytin^-$ under these conditions produce toxic oxygen species, which subsequently damage the D1 protein via an unknown mechanism at a hydrophilic loop that is exposed to the stroma (Telfer et al., 1990; Vass et al., 1992; Barber & Andersson, 1992). From the results of experiments with core particles of PS II, it was suggested that a serine-type protease associated with CP43 might be involved in the degradation of the D1 protein (Salter et al., 1992).

The second mechanism of photoinhibition involves the accumulation of oxidizing radicals on the donor side of PS II during strong illumination (Theg et al., 1986; Jegerschöld et al., 1990; Blubaugh et al., 1991). The oxidized forms of the primary electron donor P680 and the secondary electron donor Tyr_Z that are produced under these conditions are strong oxidants and induce degradation of the D1 protein. It is proposed that the hydrophilic loops on the lumenal side of the D1 protein are susceptible to damage during photoinhibition (Barbato et al., 1991b, 1992a). Proteolysis in this case was suggested to be mediated by a serine-type protease (Virgin et al., 1991; Misra et al., 1991) that is tightly bound to the reaction center complex of PS II (Shipton & Barber, 1991).

After degradation, the damaged D1 proteins have to be replaced by newly synthesized proteins via a repair process *in vivo*. The first step in such repair is removal of the

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[1] Abbreviations: PS II, photosystem II; D1 and D2, the reaction center-forming proteins of PS II; Q_A, bound plastoquinone in the D2 protein functioning as the acceptor of electron from the primary electron acceptor $pheophytin^-$; Q_B, bound plastoquinone in the D1 protein functioning as the acceptor of electron from Q_A^-; CP43 and CP47, the antenna chlorophyll−protein complexes of PS II with apparent molecular masses of 43 and 47 kDa, respectively; OEC33, OEC24, and OEC18, the extrinsic proteins of PS II with molecular masses of 33, 24, and 18 kDa, respectively; P680, the primary electron donor of PS II; Tyr_Z, tyrosine residue functioning as the electron donor to P680; PBS, phosphate-buffered saline; SDS−PAGE, SDS−polyacrylamide gel electrophoresis; PVDF, poly(vinylidene difluoride); ECL, enhanced chemiluminescence; LHC II, light-harvesting chlorophyll-protein complex of PS II; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DPC, 1,5-diphenyl carbazide.

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damaged D1 proteins from the impaired PS II complexes. The release of Mn and OEC proteins from PSII has been observed in parallel with the degradation of the D1 protein with thylakoids membranes and inside-out thylakoid vesicles (Virgin et al., 1988; Hundal et al., 1990). It is proposed that the photodamaged PS II complexes located in the stacked regions of thylakoids move to the unstacked regions, where the functional PS II complexes are reorganized by incorporation of newly synthesized D1 proteins (Mattoo et al., 1989). The observation that there is a significant difference in the distribution of the proteins in PS II between the stacked and the unstacked regions of thylakoids, before and after the photoinhibition, supports the hypothesis that photoinhibition induces disintegration of the damaged PS II complexes at the stacked regions and subsequent lateral movement of the constituents to the unstacked regions (Hundal et al., 1990; Barbato et al., 1992b).

In spite of the accumulation of considerable amounts of data related to the degradation of the D1 protein, the details of the repair of PS II complexes, in particular of the steps at which degraded D1 proteins are removed from the PS II complexes, remain to be elucidated. We do not know exactly how PS II complexes are broken up into their constituent proteins after the degradation of the D1 protein and are then reassembled again to form new and active PS II complexes. It was shown that the D2 protein is also damaged during photoinhibition, and several degradation products were identified (Schuster et al., 1988; Barbato et al., 1992d; Virgin et al., 1990). However, we do not know whether other protein components in PS II are similarly sensitive to strong illumination.

In the present study, we found that CP43, as well as the D1 and D2 proteins, was degraded as a result of illumination with strong white light (5000 μE m^{-2} s^{-1}) of the PS II membranes that had been pretreated with alkaline Tris. Three degradation products of CP43 were formed under the present conditions. Extensive cross-linking among CP43, CP47, and the D1 protein was also observed in parallel with the degradation of CP43. The cross-linking among these proteins was detected recently with the PS II membranes that had been treated with Tris and illuminated with weak light (20 μE m^{-2} s^{-1}) (Mori et al., 1995). The cross-linking of the PS II polypeptides did not affect the yields of the degradation products of the D1 protein. By contrast, the cross-linking among CP43, CP47, and the D1 protein and degradation of CP43 seemed to be closely associated processes. Both the photoinduced degradation of CP43 and the formation of cross-links among the D1 protein, CP43, and CP47 were not obvious in the presence of the extrinsic 33-kDa protein in PS II membranes (OEC33). OEC33 has been assigned to be the Mn-stabilizing protein in oxygen-evolving system of PS II (Yamamoto, 1988b), but it was shown lately that the protein is also crucial for stabilization of the the core polypeptides of PS II (Mayfield et al., 1987; Hiramatsu et al., 1991). On the basis of the results obtained in the present study, we discuss meanings of the degradation and cross-linking of CP43 in PS II and a possible role of OEC33 in the turnover of the PS II complexes during the photoinhibition.

MATERIALS AND METHODS

Preparation of PS II Membranes and PS II Complexes. PS II membranes were prepared from spinach by the method of Kuwabara and Murata (1982). The PS II membranes were suspended in a solution that contained 0.4 M sucrose, 15 mM NaCl, 5 mM $MgCl_2$, 30% (v/v) ethylene glycol, and 20 mM Mes-NaOH (pH 6.0) and stored at −80 °C prior to use. PS II complexes were prepared from the PS II membranes by the procedure of Ghanotakis et al. (1987).

Treatment of the PS II Membranes with Tris, NaCl, and NH_2OH. For treatment with Tris, PS II membranes were suspended in a solution of 0.8 M Tris-HCl and 3 mM EDTA (pH 9.0), and the suspension was stirred for 15 min on ice. The membranes were collected by centrifugation at 35000*g* for 15 min and washed twice with a solution that contained 0.4 M sucrose, 40 mM Mes-NaOH, and 10 mM NaCl (pH 6.5) (SMN solution) with centrifugation at 35000*g* for 15 min. For treatments with NaCl and with NH_2OH, PS II membranes were suspended in a solution that contained 0.4 M sucrose, 1.0 M NaCl, and 40 mM Mes-NaOH (pH 6.5) and in a solution that contained 0.4 M sucrose, 10 mM NaCl, 3 mM NH_2OH, 3 mM EDTA, and 40 mM Mes-NaOH (pH 6.5), respectively, and stirred for 15 min on ice. All the procedures were carried out in darkness.

Illumination of PS II Membranes and PS II Complexes with Strong Light. PS II membranes and PS II complexes were suspended in SMN solution at 500 μg of chlorophyll mL^{-1}. Microtubes containing 100 μL each of the suspension were set in a thermostatically controlled water bath and illuminated with white light from a 750 W projector lamp with a heat-absorbing filter HA-50 (Hoya, Japan). The intensity of light at the surface of the microtubes, measured with a quantum sensor QSPAR (Hansatech, U.K.), was 5000 μE m^{-2} s^{-1}. Anaerobic conditions were achieved as described previously (Mori et al., 1995). Lowering the tension of oxygen to the zero level was monitored by an oxygen electrode (Rank Brothers, U.K.).

Isolation and Reconstitution of OEC33. Isolation of the extrinsic 33-kDa protein of PS II (OEC33) was carried out as follows. The PS II membranes that had been stored at −80 °C were thawed on ice and washed twice with SMN solution with centrifugation at 35000*g* for 15 min. To remove the extrinsic 24- and 18-kDa proteins (OEC24 and OEC18), the membranes were treated twice with 1 M NaCl as described above. OEC33, which was retained in the PS II membranes after the treatment with 1 M NaCl, was liberated by treatment of the membranes with a solution of 1 M $CaCl_2$ and 40 mM Mes-NaOH (pH 6.5) for 30 min at 4 °C. The suspension was then centrifuged at 17500*g* for 15 min to pellet the PS II membranes, and the supernatant was dialyzed overnight against 10 mM Mes-NaOH (pH 6.5) at 4 °C. OEC33 was purified from the supernatant by ion-exchange chromatography on a column (2.5 cm i.d. × 10 cm) of a CM-Toyopearl 650 S (Tosoh, Japan) and concentrated with a Centri cell 20 (Funakoshi, Japan). OEC33 was quantitated by measuring absorption at 276 nm. OEC33 was reconstituted to the PS II membranes that had been treated with Tris by addition of purified OEC33, in darkness, to Tris-treated PS II membranes which were suspended in SMN solution at 500 μg of chlorophyll/ml, with subsequent incubation for 30 min on ice. The suspension was centrifuged at 30000*g* for 15 min, and the pellet, after washing once with SMN solution, was resuspended in SMN solution.

Preparation and Purification of Antibodies. Antibodies against proteins of PS II were raised by immunizing rabbits as described previously (Yamamoto, 1988a). The antigenic

proteins, D1, D2, CP43, and CP47, were obtained by preparative SDS−polyacrylamide gel electrophoresis of the PS II complex from spinach. The antibodies against the α-subunit of cyt *b*559 was a gift from Dr. Bertil Andersson, University of Stockholm. The antibodies were affinity purified with antigens immobilized on nitrocellulose filters, as described by Sambrook et al. (1989). The titers of the antibodies, estimated by the reactivity of the antibodies with the cross-linked products among D1, D2, CP43, CP47, and cyt *b*559 in Western blotting, were adjusted almost to the same level by appropriate dilution with PBS.

Gel Electrophoresis and Immunoblotting. SDS−polyacrylamide gel electrophoresis (SDS−PAGE) was carried out by the method of Laemmli (1970) with modifications. As the stacking gel and separation gel, 4.5% and 10−20% polyacrylamide gels containing 6.0 M urea were used, respectively. The concentration of Tris in the separation gel was increased to 0.6 M as described by Ikeuchi and Inoue (1988). Western blotting was carried out as described by Towbin et al. (1979) with a semi-dry blotting apparatus (Sartorius, Germany). Proteins that had been transferred to PVDF membranes (Millipore) were detected with an enhanced chemiluminescence (ECL) Western blotting detection system (Amersham, Japan). The intensities of bands of proteins were determined with a Scanning Imager A-300 (Molecular Dynamics). Concentrations of chlorophyll were determined from the absorption coefficients reported by Mackinney (1941). When various artificial electron donors of PS II and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) were added to the suspension of PS II membranes, these reagents were solubilized in ethanol, and final concentrations of ethanol in the raction mixtures were less than 1%.

RESULTS

When the PSII membranes were treated with Tris (0.8 M, pH 9.0) in darkness and then illuminated with strong white light at 5000 μE m^{-2} s^{-1} under aerobic conditions at 25 °C, a significant decrease in the amount of CP43 was detected by the SDS/urea-PAGE (Figure 1). The level of CP47 was also reduced under the same conditions. By contrast, the effects of strong illumination on CP43 and CP47 were not obvious when PS II membranes had been treated with NH_2-OH. It is known that the treatment of PS II membranes with Tris removes all the extrinsic proteins and Mn, thereby inhibiting electron transport on the oxidizing side of PS II (Yamamoto et al., 1981). Treatment with NH_2OH of PS II membranes also inhibits PS II by removing Mn from the membranes, but no significant release of the extrinsic proteins occurs (Yamamoto & Nishimura, 1983). These results suggest that the extrinsic proteins of PS II are effective in preventing the decrease in amounts of CP43 and CP47 in PS II under strong illumination. In the PS II membranes that had been treated with Tris and illuminated, the extent of the decrease in the amount of CP43 was about twice that in the amount of CP47 under the present conditions (Figure 2).

After Western blotting with a sensitive fluorescence-detection system, we observed degradation fragments of the D1 protein, with molecular masses of 24.0 and 17.0 kDa, after illumination of Tris-treated PS II membranes with strong white light (Figure 3). The 24- and 17-kDa fragments were previously identified as C-terminal fragments of the D1

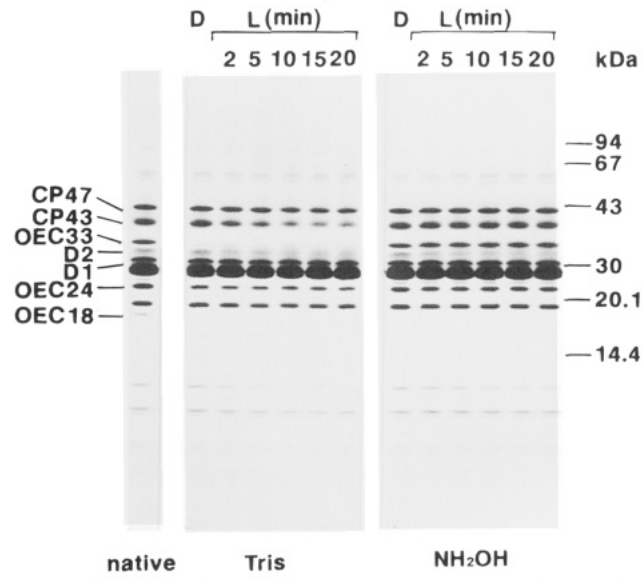

FIGURE 1: Effects of illumination with strong light on the proteins of PS II membranes that were pretreated with either Tris or hydroxylamine. PS II membranes that had been pretreated with 0.8 M Tris (pH 9.0) or 3 mM hydroxylamine (pH 6.5) were irradiated with white light (5000 μE m^{-2} s^{-1}) at 25 °C for 2, 5, 10, 15, and 20 min under aerobic conditions. After the illumination, the PS II membranes were collected by centrifugation and subjected to SDS/urea-PAGE. The proteins on the gel were stained with Coomassie brilliant blue. D represents the PS II membranes that had been kept in darkness (dark control), and L represents the PS II membranes that had been illuminated for a given period of time. On the left, the polypeptide pattern obtained from the PS II membranes is shown (indicated as "native" at the bottom). The treatments of the PS II membranes (Tris or NH_2OH) are also indicated at the bottom. At the far right, the molecular masses of marker proteins are shown.

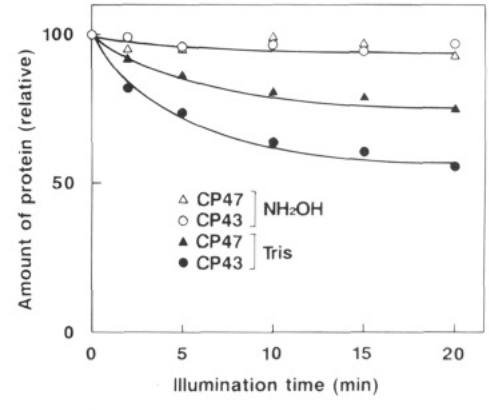

FIGURE 2: Typical kinetics of the decreases in the amounts of CP43 and CP47 in the PS II membranes that had been pretreated with Tris or hydroxylamine during strong illumination. The relative amounts of the proteins were estimated from densitograms of the gels shown in Figure 1.

protein (Barbato et al., 1991b, 1992a). In the case of the D2 protein, a 29-kDa fragment was observed, which is probably the same fragment as the 28-kDa fragment that was reported previously with the reaction center complex of PS II (Barbato et al., 1992d).

Under the same photoinhibitory conditions, we detected three degradation products of CP43. The molecular masses of the fragments were 17.0, 15.5, and 14.0 kDa. Since the amounts of the various fragments of CP43 were smaller than those of the D1 protein, detection of these fragments was rather difficult, even though the titer of the antibody against CP43 was almost the same level as that against the D1

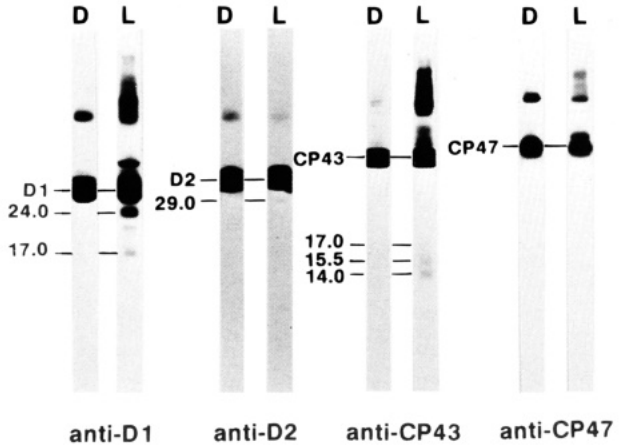

FIGURE 3: Cross-linking and degradation of D1, D2, CP43, and CP47 in the PS II membranes that had been pretreated with Tris and illuminated with white light for 20 min. The other conditions for illumination were the same as those described in the legend to Figure 1. The products of cross-linking and degradation were detected by SDS/urea-PAGE, Western blotting with specific antibodies, and fluorography with enhanced chemiluminescence. The letters D and L at the top of the gels indicate that the PS II membranes that had been pretreated with Tris were kept in darkness (D) or illuminated (L), respectively. The numbers on the left side of each gel show the molecular masses (in kDa) of the degradation fragments. The antibodies used for the Western blotting are specified at the bottom of the gels.

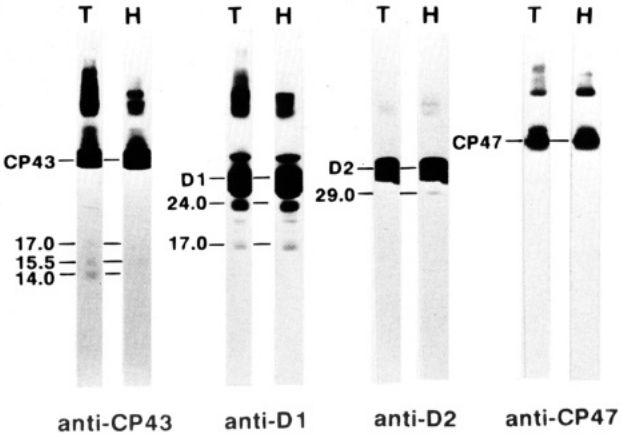

FIGURE 4: Cross-linking and degradation of D1, D2, CP43, and CP47 in PS II membranes that had been pretreated with Tris or hydroxylamine and illuminated with strong light. T, PS II membranes after treatment with 0.8 M Tris (pH 9.0). H, PS II membranes after treatment with 3 mM hydroxylamine. The membranes were illuminated for 20 min under the conditions described in the legend to Figure 1. The products of cross-linking and degradation were detected as described in the legend to Figure 3. The numbers on the left of each gel indicate the molecular masses (in kDa) of the degradation fragments. The antibodies used for Western blotting are shown at the bottom of each gels.

protein (see Materials and Methods). Furthermore, we failed to find any degradation products of CP47 in the gel, even though the amount of CP47 in the membranes was apparently decreased by the illumination.

Another prominent feature associated with the degradation of CP43 was the formation of cross-links between CP43 and the D1 protein (Figure 3). With regard to the cross-linked products generated from the proteins in PS II, a 41-kDa product of the D1 protein and the α-subunit of cyt *b*559 was reported by Barbato et al. (1992c). We detected a similar cross-linked product with a molecular mass of 38.0 kDa by Western blotting with an antibody against the D1 protein (the results of Western blotting with the antibody against the α-subunit of cyt *b*559 are not shown). In the region of the gel between the top of the gel and the band of the D1−D2 heterodimer, which cross-reacted with the antibodies against the D1 and the D2 proteins, we observed smeared bands that cross-reacted with the antibody against the D1 protein. The bands were not seen after staining of the gel with Coomassie blue but became apparent upon Western blotting with the antibodies against both CP43 and the D1 protein and subsequent fluorography with ECL. The antibody against CP47 also reacted with smeared bands near to the top of the gel. By contrast, when we use an antibody against the D2 protein, positive reaction was only observed with the D2 protein itself and the D1−D2 heterodimer.

The photoinduced degradation of the D1 and D2 proteins was also observed when PS II membranes were pretreated with NH_2OH (Figure 4). The molecular masses of the degradation fragments were the same as those of fragments detected after illumination of the PS II membranes that had been treated with Tris. By contrast, no degradation of CP43 was apparent in the PS II membranes that had been treated with NH_2OH, and only a small amount of the fragments of CP43 was detected after illumination. In addition, the amounts of the products of cross-linking among the D1 protein, CP43, and CP47 were smaller in the case of membranes treated with NH_2OH than in the case of those treated with Tris. Once again, degradation of CP47 was not observed. In NH_2OH-treated membranes, Mn is removed from PS II and oxygen-evolving activity is lost but the extrinsic OEC proteins in PS II are retained (Yamamoto & Nishimura 1983). Thus, the results obtained here indicate that these extrinsic proteins in PS II play a regulatory role in the degradation of CP43 under strong illumination.

To examine more directly the role of one of the extrinsic proteins, OEC33, in the degradation of the PS II proteins, we added purified OEC33 to the PS II membranes that had been treated with Tris and studied the effects of reconstitution of OEC33 on the light-induced decrease in the amount of CP43 in the membranes (Figure 5A). With the addition of increasing amounts of OEC33, the protein became bound to the depleted membranes. In parallel with the binding of OEC33, we observed an increase in the amount of CP43 after photoinhibitory illumination. These results show that the decrease in the amount of CP43 in the Tris-washed PS II membranes, induced by the illumination with strong light, was prevented to a significant extent by the presence of OEC33. By contrast, no remarkable changes in the amount of CP43 were observed upon illumination of the PS II membranes that had been treated with hydroxylamine, as shown in Figures 1 and 2. Western-blotting with the antibodies against the D1 protein and CP43 revealed the effect of added OEC33 more clearly (Figure 5B,C). The reconstitution of OEC33 prevented the formation of cross-linked products among CP43, CP47, and the D1 protein, which were otherwise detected as smeared bands close to the origin after SDS/urea-PAGE. The degradation of the D1 protein was unaffected by the reconstitution of OEC33, while the degradation of CP43 was significantly blocked in the presence of added OEC33. These results, as well as the results obtained with the PS II membranes that had been treated with NH_2OH (Figure 4), demonstrate clearly that OEC33 protects CP43 from damage during strong illumination.

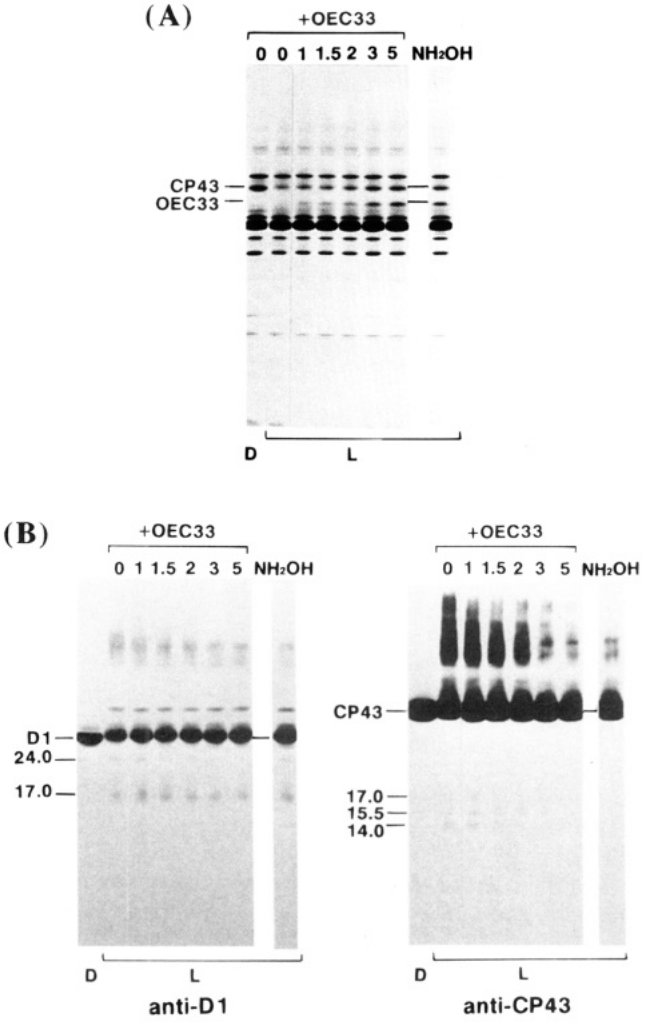

FIGURE 5: (A) Effects of reconstitution of OEC33 to the PS II membranes that had been treated with Tris on the profile of the proteins in PS II detected by SDS/urea-PAGE. The PS II membranes that had been treated with 0.8 M Tris (pH 9.0) and reconstituted with OEC33 were subjected to SDS/urea-PAGE, and proteins were stained with Coomassie brilliant blue. The amounts of OEC33 added are shown at the top of the gels (+OEC33) as relative amounts. The number "1" corresponds to 1 molecule of OEC33 added per 200 molecules of chlorophyll. D and L at the bottom of the gel indicate that the PS II membranes were kept in darkness (D) or illuminated (L). The illumination conditions were the same as those described in the legend to Figure 1. The far right lane shows the pattern of proteins in the PS II membranes that had been treated with 3 mM hydroxylamine (pH 6.5). (B) Effects of reconstitution of OEC33 to the PS II membranes that had been treated with Tris on the cross-linking and degradation of the D1 protein and CP43. The experimental conditions are the same as those described in panel A. The proteins were detected by Western blotting with the antibodies against the D1 protein (the left gel) and against CP43 (the right gel), respectively.

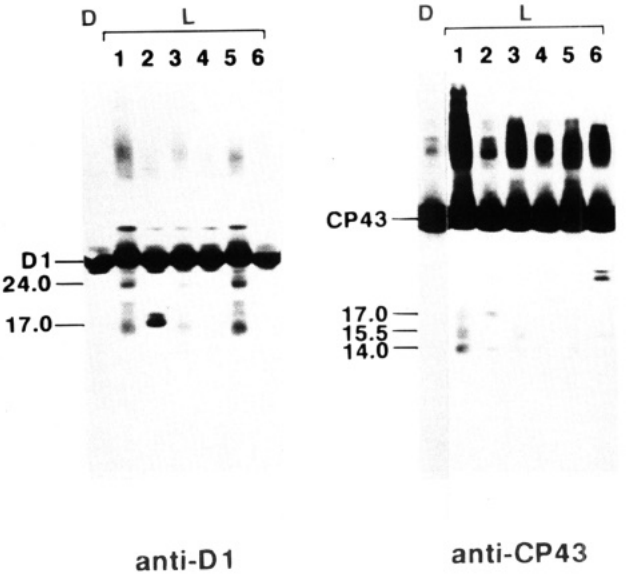

FIGURE 6: Effects of various donors of electron to PS II and of the electron- transport inhibitor DCMU on the photoinduced cross-linking and degradation of the proteins in PS II membranes. D and L indicate that PS II membranes were kept in darkness or illuminated with white light for 20 min. Other conditions were the same as those described in the legend to Figure 1. 1, No addition; 2, +0.5 mM DPC; 3, +0.5 mM hydroxylamine; 4, +0.5 mM ascorbate; 5, +0.5 mM potassium ferrocyanide; 6, +40 μM DCMU. The numbers of the right of the gels show the molecular masses (in kDa) of the degradation products of the D1 protein and CP43. The antibodies used for Western blotting are shown at the bottom of the gels.

To determine whether the degradation of the D1 protein and CP43 was induced by the donor-side mechanism of photoinhibition in PS II under the present conditions, the effects of various artificial donors of electron to PS II were examined (Figure 6). With the addition of these electron donors, the extent of the degradation of the D1 protein and of CP43 and the formation of cross-links among the D1 protein, CP43, and 47 were reduced significantly. These results indicate that inhibition of PS II on the donor side, caused by the treatment with Tris, was responsible for the damage to CP43 during the photoinhibitory illumination of PS II. The pattern of degradation of both the D1 protein and CP43 varied slightly depending on the electron donor used (Figure 6, lanes 2−5), but the reason for this variation remains to be determined. It is possible that the pattern of degradation is also dependent on the concentrations of the electron donors employed here. The addition of DCMU, an electron-transfer inhibitor, prevented both the degradation of the D1 protein and the cross-linking of the D1 protein with CP43 and CP47 (Figure 6, lane 6). Upon illumination of Tris-treated PS II membranes in the presence of DCMU, the 17-, 15.5-, and 14-kDa fragments of CP43 no longer appeared, and weak bands of polypeptides of 27 and 25 kDa appeared. The former products of the degradation of CP43 were probably induced by the electron transport in PS II. The mechanism for induction of formation of the 27- and 25-kDa bands in the presence of DCMU is unknown.

The presence of oxygen is critical for the generation of the degradation products of PS II complexes under particular photoinhibitory conditions. We examined the effects of oxygen on the degradation and cross-linking of CP43 and the D1 protein in PS II membranes during strong illumination (Figure 7). The production of the 24- and 17-kDa fragments of the D1 protein was dependent on the presence of oxygen, and, under anaerobic conditions, no products of the degradation of the D1 protein were detected. By contrast, the cross-linked products of the D1 protein, CP43, and CP47 were observed both under aerobic and anaerobic conditions. The degradation of CP43 was also observed both under aerobic and anaerobic conditions.

DISCUSSION

The D1 and D2 proteins that form the reaction center of PS II are the targets of photoinhibition, and the degradation

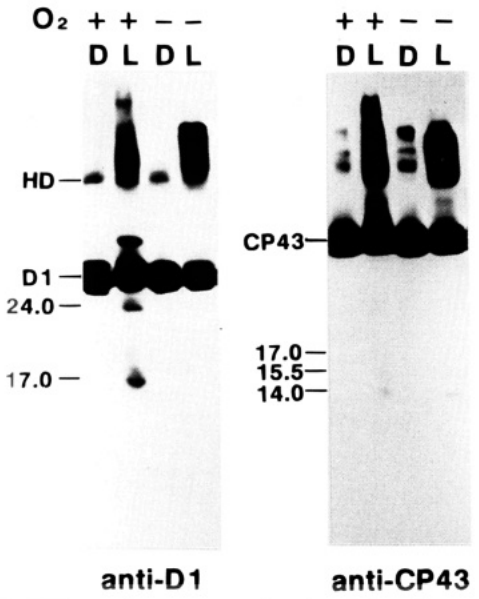

FIGURE 7: Effects of oxygen on the photoinduced cross-linking and degradation of the proteins in PS II membranes. The PS II membranes that had been treated with 0.8 M Tris were illuminated with white light for 20 min (L) or kept in darkness (D) in the presence ($+O_2$) or absence ($-O_2$) of oxygen. The cross-linking and degradation of the proteins were examined by SDS-urea/PAGE and Western blotting with specific antibodies against the D1 protein and CP43. The numbers on the left of the gels show the molecular masses (in kDa) of the degradation products. HD indicates the heterodimer of the D1 and D2 proteins. The antibodies used are specified at the bottom of the gels.

of these proteins during illumination with strong light has been well documented (Greenberg et al., 1987; Virgin et al., 1990; Barbato et al., 1991a, 1992a,d; Shipton & Barber, 1991; Barber & Andersson, 1992). Degradation of the proteins other than the D1 and D2 proteins in PS II has been suggested (Mori & Yamamoto, 1992; Miyao, 1994), but no definite degradation products were identified. In the present study, we detected for the first time the products of the degradation of CP43 in PS II membranes and PS II complexes that had been treated with Tris or NH_2OH, using Western blotting with purified specific antibodies and a sensitive detection system. In these preparations of PS II , the highly oxidizing species, namely, $P680^+$ and Tyr_Z^+ , that are produced in the reaction center of PS II under strong illumination damage the surrounding polypeptides (Jegerschöld et al., 1990; Blubaugh et al., 1991). With these preparations, not only the D1 protein but also CP43 and CP47, which are closely associated with the D1 protein, may be damaged during strong illumination. The origin of the degradation products of CP43 with molecular masses of 17.0, 15.5, and 14.0 kDa remains to be determined. From hydropathy plots, six membrane-spanning regions (helices I−VI) have been predicted for CP43 (Bricker, 1990). In particular, a large hydrophilic loop, connecting helices V and VI and exposed to the lumenal side, has been suggested to be important for the function and stabilization of the polypeptide on the basis of results of site-directed mutagenesis in cyanobacteria (Kuhn & Vermaas, 1993). Under the same illumination conditions and using PS II membranes, we detected a 24- and a 17-kDa fragment of the D1 protein (Figure 3). These fragments probably correspond to the C-terminal 24- and 16-kDa fragments of the D1 protein, which were reported by Barbato et al. (1991a,b, 1992a). According to them, these fragments of the D1 protein are produced by cleavage of the lumen-exposed loop that connects helices I and II and of the loop between helices III and IV, respectively, after donor side inactivation of PS II. As the degradation of CP43 detected under the present conditions was due to the donor-side mechanism of photoinhibition, it is likely that the fragments of CP43 observed here were also produced by the cleavage of loops on the lumenal side that connect the transmembrane helices.

As reported recently (Mori et al., 1995) and confirmed in the present study, another significant event that is induced by illumination of PS II membranes, which are devoid of the donor side of PS II, is cross-linking among the D1 protein, CP43 and CP47. The exact stoichiometry of the polypeptides involved in the cross-linking is not yet known, but the quantum efficiency of the reaction seems to be high because even weak light (20 μE m^{-2} s^{-1}) caused extensive cross-linking in PS II membranes that had been treated with Tris. The apparent molecular masses of the cross-linking products induced by the weak light were almost the same as those induced by the strong light employed in the present study (Mori et al., 1995). Cross-linking of the proteins in PS II was also noticed during acceptor-side photoinhibition of PS II by strong illumination (Miyao, 1994). However, the cross-linking reaction was more prominent during donor-side photoinhibition. It has been proposed that active oxygen species are responsible for the cross-linking of the proteins (Miyao, 1994), but our results indicate that efficient cross-linking occurs even in the absence of oxygen (Figure 7). Since the D1 protein is cleaved at different sites in donor- and acceptor-side photoinhibition, the cross-linking may depend on the amino acid sequence around the sites of cleavage of the D1 protein as well as on the relative orientation of the fragments of the D1 protein and the polypeptides located close to them in the PS II membranes.

The exact relationship between the cross-linking and the degradation of the polypeptides in PS II observed here is unknown. Apparently, the cross-linking of the polypeptides did not affect the efficiency of production of the degradation fragments of the D1 protein under the present conditions (Figures 4 and 5B). In the case of CP43, however, an increase in the extent of degradation of CP43 seemed to be accompanied by an increase in the cross-linking of the PS II proteins (Figures 4 and 5C). In a previous study, where PS II membranes that had been treated with Tris were illuminated with weak light (20 μE m^{-2} s^{-1}), we observed a rather close relationship between the cross-linking of the proteins in PS II and production of a 38-kDa cross-linking form and a 16-kDa degradation fragment of the D1 protein (Mori et al., 1995). The production of the degradation fragments of the D1 protein under the weak illumination was not so significant as that observed under strong illumination, while low incident intensity was saturating for the cross-linking of the polypeptides (Mori & Yamamoto, 1992). It is possible that cross-linking of the D1 protein, CP43, and CP47 is a process which affects the early step of photo-induced degradation of the D1 protein but does not affect the degradation when the degradation is extensive.

It was reported that the addition of cations regulates the formation of cross-linked products in PS II membranes (Mori et al., 1995): the cross-linking is prevented in the presence of cations, and divalent cations are more effective in this regard than monovalent cations. In general, the addition of salts enhances the aggregation of proteins by shielding their

surface charges. Thus, the cross-linking observed here might not be due to the nonspecific aggregation of proteins in PS II. The distribution of local charges around the cleavage sites of the D1 protein and/or CP43 is probably closely related to the efficiency of formation of cross-linked products. The loops, which connect the α-helices of the D1 protein spanning the thylakoid membranes, have been proposed to be the sites of cleavage in the case of donor-side photoinhibition (Barbato et al., 1991b, 1992a). Charged amino acid residues are abundant in the loops in the D1 protein and CP43 (Trebst, 1985; Sayre et al., 1986; Bricker, 1990), and the conformation of these loops might be very susceptible to cations. In PS II complexes that were devoid of LHC II, no extensive cross-linking of the proteins in PS II was detected after strong illumination (data not shown). It is possible that the regulation by cations of cross-linking in the PS II membranes is mediated by LHC II, or, alternatively, that the presence of LHC II is necessary for maintaining the conformation of PS II complexes, that allows the cross-linking of the polypeptides in PS II.

The degradation of CP43 and the formation of cross-links between the D1 protein and CP43 were prevented by readdition of OEC33 to the PS II membranes that had been treated with Tris at pH 9 (Figure 5). OEC33 has been shown to bind to the core complexes of PS II. Data showing the association of OEC33 with CP43 and the regulation of stability of the PS II complex were reported previously (Isogai et al., 1985; Mayfield et al., 1987; Hiramatsu et al. 1991). OEC33, located close to CP43, might protect CP43 from aggregation with the photodamaged D1 protein by maintaining a certain conformation of both the D1 protein and CP43, or it might protect CP43 from toxic oxygen molecules and from a protease by steric hindrance. Since the effects of OEC33 that we observed were similar to the recently reported effects of salts added to PS II membranes (Mori et al., 1995), it seems likely that OEC33 might function as a polycation and regulate the cross-linking and degradation of CP43 electrostatically. There is also a possibility that, in PS II depleted of OEC33, degradation of the D1 protein under strong illumination induces destabilization of CP43 and renders CP43 susceptible to degradation. Once CP43 has been degraded in the PS II complexes, the entire PS II complex becomes unstable and disorganization of the complex might be accelerated. CP43 was shown to have a critical role in the stabilization and function of PS II from results of experiments with mutants of *Chlamydomonas reinhardtii* with site-directed mutations in or deletion of *psb* C (Rochaix et al., 1989) and with similar mutants of *Synechocystis* 6803 (Carpenter et al., 1990; Rögnor et al., 1991; Kuhn & Vermaas, 1993). Consequently, even the partial deterioration of CP43 might be sufficient to cause the disintegration of the PS II complex. For efficient turnover of the D1 protein that has been damaged by irradiation, replacement of the D1 protein without damage to other surrounding proteins may be essential. The release of OEC proteins from inside-out thylakoid vesicles upon strong illumination has been reported (Hundal et al., 1990). However, under our present experimental conditions, the extent of release of OEC proteins from PS II membranes upon strong illumination was not very significant (data not shown). If OEC33 is removed completely from PS II, destabilization of CP43 might occur, with resultant disintegration of PS II. We suggest that, during the repair of the damaged D1 proteins in PS II membranes, OEC33 is not eliminated from the membranes but is retained on the surface of the PS II complex to stabilize the remaining polypeptides of PSII. To characterize the processes that follow the degradation of the D1 protein in greater detail, more extensive studies are required both *in vitro* and *in vivo*.

Oxygen is involved in the photoinduced damage of PS II and the degradation of the D1 protein (Macpherson et al., 1993; Hideg et al., 1994). According to the proposed mechanism of acceptor-side photoinhibition of PS II, accumulation of reducing equivalents on the acceptor side of PS II during strong illumination accelerates the charge recombination between $P680^+$ and pheophytin$^-$ and the subsequent formation of a triplet of P680. The reaction between the triplet of P680 and oxygen molecules produces singlet oxygen molecules, which damage the D1 protein. In the present study, the effects of oxygen on the D1 protein were clearly observed with respect to degradation of the D1 protein but not with respect to the cross-linking with CP43 and CP47 (Figure 7). It is possible, therefore, that the cross-linking is the primary event in the photoinhibition, and that cross-linking occurs irrespective of the presence or absence of oxygen. Under aerobic conditions, the degradation of the D1 protein may be triggered when a certain level of active oxygen species accumulates around the D1 protein during illumination. It is possible that oxygen plays a role as an electron acceptor from Q_B when the PS II membranes are illuminated with strong light. It was shown previously that photoinduced production of fragments of the D1 protein in PS II reaction centers depends on the presence of an electron acceptor (Shipton & Barber, 1991). With an electron acceptor, the oxdized form of the primary electron donor ($P680^+$) was stabilized, and the donor-side photoinhibition became prominent. In our present conditions, accumulation of $P680^+$ and Tyr_Z^+ under the illumination may induce the donor-side photoinhibition of PS II, as the primary event. Electrons transferred to oxgen may then produce active oxgen species at the reducing side of PS II, which may exaggerate the damage in PS II. The production of active oxygen species in PS II that has been depleted of the oxygen-evolving system was also reported (Ananyev et al., 1992; Chen et al., 1992). It has been proposed that a protease is involved in the degradation of the D1 protein (Barber & Andersson, 1992), but it has also been suggested that it is mainly active oxygen species that participate in the degradation process (Miyao, 1994). We noticed that the extent of degradation of CP43 was considerably small, and we speculate that one of the reasons for this difference is a low concentration of active oxygen species around CP43. It is possible that the active oxygen species produced in the core region of the D1 protein do not diffuse efficiently to the surrounding polypeptides and, therefore, are unable to degrade CP43 even in the presence of oxygen.

The D2 protein seems not to be involved in cross-linking, although the degradation product of the D2 protein, with a molecular mass of 29.0 kDa, was detected reproducibly (Figures 3 and 4). A 41-kDa cross-linked product between the D1 protein and the α-subunit of cyt *b*559 has been reported (Barbato et al., 1992c). Cross-linking of the polypeptides in PS II, as well as degradation of D1, D2, and CP43, may be critical steps in the degradation and repair of PS II complexes during photoinhibition, and further studies

on the relationship between these processes are now in progress.

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