Involvement of Active Oxygen Species in Degradation of the D1 Protein under Strong Illumination in Isolated Subcomplexes of Photosystem II†

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ABSTRACT: The effects of strong illumination on the proteins in photosystem II (PSII) were investigated using three different isolated subcomplexes of PSII, namely, the PSII complex depleted of major light-harvesting proteins, the core complex, and the reaction center complex. Under illumination, not only the D1 protein of the reaction center but also other intrinsic proteins sustained some damage in all three subcomplexes: Coomassie blue-stained bands after polyacrylamide gel electrophoresis were smeared, and their migration distances on the gel were reduced with increasing duration of illumination. Such damage occurred first in the D1 and D2 proteins and subsequently in the 43- and 47-kDa proteins of the core antenna and the subunit of cytochrome b_{559}. Immunoblot analysis using an antibody specific to the D1 protein showed that the D1 protein was degraded to major fragments of about 23 and 16 kDa during illumination. The smearing and changes in mobility of protein bands, as well as the fragmentation of the D1 protein, were greatly suppressed by scavengers of active oxygen species. From the effectiveness of scavengers, it appeared that superoxide anions participate in the protein damage in the PSII complex, hydrogen peroxide in the PSII and core complexes, and singlet oxygen, hydroxyl, and alkoxyl radicals in all three subcomplexes. We also found that fragments of the D1 protein of 23 and 16 kDa were formed even when PSII complexes that had been completely solubilized with sodium dodecyl sulfate were illuminated. This fragmentation was also suppressed by active oxygen scavengers. These observations suggest that in isolated PSII subcomplexes under strong illumination the D1 protein is cleaved at specific sites solely by the action of active oxygen, and that the D1 protein has amino acid sequences specifically susceptible to attack by active oxygen.

Strong illumination of oxygenic photosynthetic organisms results in loss of their photosynthetic capacity (Powles, 1984). The primary event in this process is impairment of photosystem II (PSII;[1] Kyle, 1987). The impairment of PSII during illumination (photoinhibition) involves photoinactivation of PSII activity and specific degradation of the D1 protein of the photochemical reaction center (Prášil et al., 1992; Aro et al., 1993). The photoinhibition is considered to proceed by two different mechanisms, so-called acceptor-side and donor-side photoinhibition (Barber & Andersson, 1992; Aro et al., 1993). The acceptor-side photoinhibition occurs in materials in which the donor side of PSII is functional. Illumination initially affects the acceptor side of PSII and blocks the electron flow while the primary photochemical process continues to occur (Vass et al., 1992). Under these conditions, the triplet state of P680 is formed with high probability and generates toxic singlet oxygen ($^1O_2$) in a reaction with oxygen. Donor-side photoinhibition is observed in materials in which the donor side is inactivated. The initial event is impairment of electron transfer from Tyr_Z, the secondary electron donor to PSII, to $P680^+$ (Blubaugh et al., 1991; Eckert et al., 1991), which leads to stabilization of strongly oxidizing species, such as $P680^+$, Chl^+, and Tyr^+. $^1O_2$ and the oxidizing species generated in this way cause irreversible damage to the reaction center and induce the subsequent degradation of the D1 protein.

The D1 protein is known as a rapid turnover protein which is specifically degraded under illumination *in vivo* (Mattoo et al., 1981, 1984). The degradation *in vivo* gives rise to a fragment of 23.5 kDa (Greenberg et al., 1987). Proteolytic mapping of the fragment (Greenberg et al., 1987) revealed that the cleavage site is located in the loop that connects the membrane-spanning helixes IV and V of the folding model of the D1 protein proposed by Trebst (1986). Subsequently, a number of studies were performed *in vitro*, and it was demonstrated that D1 protein degradation occurs even in PSII membranes and PSII subcomplexes under strong illumination (Aro et al., 1993). In PSII subcomplexes, various fragments of the D1 protein can be observed after strong illumination, and a fragment of 23–24 kDa is considered to be the primary degradation product (Aro et al., 1993). The primary cleavage site varies depending on the illumination conditions. When oxygen-evolving subcomplexes are illuminated under conditions that support oxygen evolution (acceptor-side photoinhibition), the D1 protein is cleaved in the loop connecting helixes IV and V on the stromal side of the thylakoid membrane, giving rise to a 23-kDa fragment of N-terminal origin, as occurs *in vivo* (Salter et al., 1992; De Las Rivas et al., 1992). Such cleavage also occurs when isolated RC complex, which lacks oxygen-evolving capacity, is illuminated in the absence of electron acceptors (De Las Rivas et al., 1993). When illuminated in the presence of DBMIB as an acceptor under conditions that do not permit oxygen evolution

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[1] Abbreviations: anti-D1, antibody raised against the D1 protein; APMSF, (4-amidinophenyl)methanesulfonyl fluoride; Chl, chlorophyll; Cyt, cytochrome; DABCO, 1,4-diazabicyclo[2.2.2]octane; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-1,4-benzoquinone; DFP, diisopropyl fluorophosphate; DM, *n*-dodecyl β-D-maltoside; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; LHCII, light-harvesting chlorophyll complex of photosystem II; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; P680, primary electron donor of photosystem II; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; PSII, photosystem II; RC, reaction center; SBTI, soybean trypsin inhibitor; SDS, sodium dodecyl sulfate; SOD, superoxide dismutase; TPCK, N^{α}-tosyl-L-phenylalanine chloromethyl ketone; Tris, tris(hydroxymethyl)aminomethane.

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(donor-side photoinhibition), cleavage occurs in the loop connecting helixes I and II on the lumenal side of the thylakoid membrane, giving rise to a 24-kDa fragment of C-terminal origin (De Las Rivas et al., 1992).

With respect to the mechanism of degradation of the D1 protein, involvement of specific proteases has long been implicated. Recent *in vitro* studies suggest that serine-type protease(s) intrinsically present in PSII, possibly a component of PSII itself, catalyze(s) the degradation (Barber & Andersson, 1992; Aro et al., 1993). This possibility is inferred from the observation that inhibitors of serine-type proteases suppressed D1 protein degradation in isolated PSII subcomplexes. According to this model, $^1O_2$ or oxidizing species generated inside the reaction center alter the conformation of the D1 protein and render it susceptible to degradation by the putative protease(s). Some uncertainty exists in this protease model. The identity of the protease is controversial. From the binding characteristics of DFP, a covalent blocker of the catalytic site of serine-type proteases, Salter et al. (1992) proposed that the 43-kDa protein of the core antenna might be the protease. On the other hand, Barber and co-workers proposed that one of the reaction center components (D1 and D2 proteins, Cyt b_{559}, and the product of *psbI*) has proteolytic activity, since the inhibitors were effective even in isolated RC complexes (Shipton & Barber, 1992; De Las Rivas et al., 1993). The number of putative proteases is also open to question. There are at least two primary cleavage sites of the D1 protein, and they are located on opposite sides of the thylakoid membrane. In addition, another primary cleavage site which gives rise to a fragment of 16 kDa has been proposed (Barbato et al., 1992a). Even if only the primary cleavages are each catalyzed by a protease, there should be at least three different proteolytic activities in PSII.

Another mechanism for D1 protein degradation has been proposed, namely, involvement of active oxygen species generated in PSII under illumination. Bradley et al. (1991) proposed that hydrogen peroxide (H_2O_2) generated at the specific sites of PSII could cause the degradation. Kyle (1987) proposed that a plastosemiquinone radical in the Q_B-binding site would generate superoxide anion (O_2^-) and hydroxyl radical (·OH) in a reaction with oxygen and these damage the D1 protein. Sopory et al. (1990) investigated the effects of scavengers *in vivo* and suggested that not $^1O_2$ but some other oxygen radical is involved in the degradation. Similarly, from the effectiveness of scavengers and antioxidants, various active oxygen species have been proposed to participate in the photoinactivation of PSII and D1 protein degradation in isolated thylakoids (Barényi & Krause, 1985; Richter et al., 1990; Tschiersch & Ohmann, 1993) and PSII membranes (Šetlík et al., 1990; Chen et al., 1992).

In the present study, the possible involvement of active oxygen species in degradation of the D1 protein was investigated with three different PSII subcomplexes, namely, the PSII complex depleted of the major LHCII, the core complex, and the RC complex. We found that, in these isolated subcomplexes, various different species of active oxygen are involved in D1 protein degradation, and that the D1 protein can be cleaved at specific sites solely by the action of active oxygen.

MATERIALS AND METHODS

Preparation of PSII Membranes and Isolation of PSII Subcomplexes. PSII membranes were prepared from 3–5-week-old rice seedlings with Triton X-100 by the method for wheat (Miyao & Inoue, 1991) with slight modifications, and stored in liquid nitrogen in the presence of 30% (v/v) ethylene glycol. Before use, the membranes were thawed and washed 3 times with 10 mM NaCl, 0.4 M sucrose, and 25 mM Mes–NaOH (pH 6.5; medium A) by centrifugation and resuspension. The oxygen-evolving activity of the PSII membranes was around 700 μmol (mg of Chl)$^{-1}$ h^{-1} with 0.8 mM phenyl-1,4-benzoquinone as an electron acceptor.

PSII complexes were prepared from PSII membranes as described by Kashino et al. (1992) with some modifications as follows. The PSII membranes were pelleted by centrifugation at 40000*g* for 10 min and suspended in 20 mM $NaHCO_3$, 6.25 mM $CaCl_2$, 0.5 M NaCl, 1.25 M sucrose, and 20 mM Hepes–NaOH (pH 7.0) at 3 mg of Chl/mL. The suspension was supplemented with 1/100 volume of 4.0% (w/v) Triton X-100, mixed thoroughly, and allowed to stand on ice for 10 min. This mixture was supplemented with 1/4 volume of 10% (w/v) *n*-heptyl β-D-thioglucoside to give a final detergent concentration of 2%. The mixture was mixed thoroughly, allowed to stand on ice for 15 min, and then diluted with 2 volumes of 20 mM $NaHCO_3$, 5 mM $CaCl_2$, 0.4 M sucrose, and 20 mM Hepes–NaOH (pH 7.0). After standing on ice for 5 min, the mixture was centrifuged at 35000*g* for 15 min, and the supernatant at 40000*g* for 10 min. The resultant supernatant was dialyzed against 20 mM $NaHCO_3$, 10 mM NaCl, 0.4 M sucrose, and 40 mM Mes–NaOH (pH 6.0) for 3 h. The dialyzate was centrifuged at 35000*g* for 15 min, and the resultant pellet consisting of the PSII complexes was suspended in medium A. Core complexes were prepared by treatment of the PSII membranes with *n*-octyl β-D-glucopyranoside followed by sucrose density gradient centrifugation according to Ikeuchi and Inoue (1988) and finally suspended in medium A. The oxygen-evolving activity of the PSII complexes was 2000 μmol (mg of Chl)$^{-1}$ h^{-1} with 0.8 mM phenyl-1,4-benzoquinone, and that of the core complexes was 1800 μmol (mg of Chl)$^{-1}$ h^{-1} with 0.8 mM 2,6-dichloro-1,4-benzoquinone.

RC complexes were prepared from PSII membranes with Triton X-100 by a method based on that of Nanba and Satoh (1987) as modified by Chapman et al. (1988), with the exception that alkaline treatment of the PSII membranes prior to the Triton treatment was omitted and the second chromatographic separation was performed in the presence of 2 mM DM instead of 0.2% Triton X-100. The photochemical activity of the RC complexes, measured at 25 °C as the reduction of 0.25 mM silicomolybdate with 1 mM $MnCl_2$ as an electron donor (Shipton & Barber, 1992), was 200–300 μmol (mg of Chl)$^{-1}$ h^{-1}.

The isolated PSII subcomplexes were frozen in liquid nitrogen and kept at –80 °C until use. All procedures were performed under dim light at 0–4 °C. Chl was determined according to Arnon (1949).

Photoinhibitory Light Treatments. The PSII and core complexes were subjected to acceptor-side photoinhibition essentially as described previously (Virgin et al., 1991; De Las Rivas et al., 1992), and the RC complexes were subjected to either acceptor-side (De Las Rivas et al., 1993) or donor-side (Shipton & Barber, 1992) photoinhibition. The PSII and core complexes were suspended in 1 mM DM, 10 mM NaCl, 0.4 M sucrose, and 50 mM Mes–NaOH (pH 6.0) at 100 μg of Chl/mL. The RC complexes (150–200 μg of Chl/mL, 110 mM NaCl, pH 7.2) were diluted to 50 μg of Chl/mL with 2 mM DM and 0.4 M sucrose containing either 50 mM Mes–NaOH (pH 6.0) or 50 mM Tris-HCl (pH 8.5) to give a final pH of 6.3 or 8.0. After standing in darkness at 20 °C for 10 min, the suspension was illuminated in a glass cuvette,

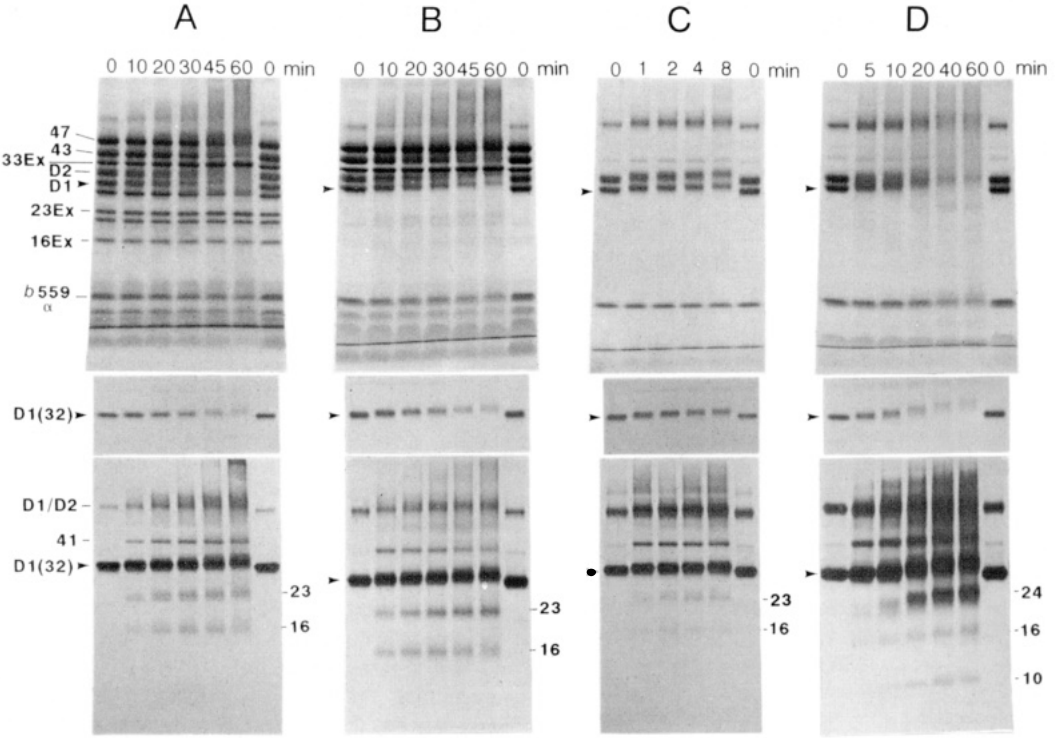

FIGURE 1: Changes in polypeptide and immunoblot profiles of PSII subcomplexes during photoinhibitory illumination. The PSII and core complexes were suspended in 1 mM DM, 10 mM NaCl, 0.4 M sucrose, and 50 mM Mes–NaOH (pH 6.0) at 100 μg of Chl/mL, and the RC complexes were suspended in 2 mM DM and 0.25 M sucrose containing 50 mM Mes–NaOH (pH 6.3) or 50 mM Tris-HCl (pH 8.0) at 50 μg of Chl/mL. After standing in darkness at 20 °C for 10 min, the suspension was illuminated with white light for the designated times in the presence or absence of 0.2 mM DBMIB at 20 °C. (A) PSII complexes illuminated at 8.0 mE/(m²·s). (B) Core complexes illuminated at 8.0 mE/(m²·s). (C) RC complexes illuminated at 2.0 mE/(m²·s) and pH 6.3. (D) RC complexes illuminated at 4.0 mE/(m²·s) and pH 8.0 in the presence of DBMIB. (Upper panels) Polypeptide profiles after staining with Coomassie blue; (middle and lower panels) immunoblot profiles with anti-D1. In the middle panels, the amounts of sample applied for SDS–PAGE were optimized for quantification of the D1 protein band of 32 kDa, while in the lower panels 15–25 times as much sample was applied for the detection of the D1 protein fragments. Arrowheads indicate the position of the 32-kDa D1 protein band.

with gentle stirring, with white light from a projector lamp, which was passed through two layers of heat-reflecting filters and a 10-cm-thick layer of water. The temperature was maintained at 20 °C with circulating water under thermostatic control. The light intensity was reduced by neutral density filters. When indicated, 20 mM DBMIB was added to give a final concentration of 0.2 mM just before illumination. Immediately after illumination, the suspension was divided into aliquots, frozen in liquid nitrogen, and stored at −80 °C.

Polypeptide and Immunoblot Analyses. Samples were solubilized in 50 mM Na_2CO_3, 50 mM dithiothreitol, 2% SDS, and 12% (w/v) sucrose at 25 °C for 30 min and subjected to SDS–PAGE (Miyao & Murata, 1984) using a gel containing 6.5 M urea. The polyacrylamide concentration in the separation gel was 13%. Each lane of the gel was loaded with the equal amount of sample on the Chl basis: in the case of the PSII complexes, the sample amounts loaded were 0.6 μg and 5–100 ng of Chl for Coomassie staining and immunoblotting, respectively. After electrophoresis, the gel was stained with Coomassie brilliant blue R-250 or subjected to immunoblotting as follows. Separated polypeptides were electroblotted onto a nitrocellulose membrane (0.2 μm, Schleicher & Schuell) in the presence of 0.05% SDS according to Towbin et al. (1979) with a semi-dry-type blot apparatus. After being blotted, the nitrocellulose membrane was blocked with 3% (w/v) gelatin and probed with 1000-fold-diluted antiserum raised in rabbit against spinach D1 protein (anti-D1, a generous gift from Dr. M. Ikeuchi). Immunoreacted bands were further immunodecorated with goat antibodies against rabbit IgG conjugated with alkaline phosphatase (Jackson ImmunoResearch) and visualized by reaction with nitroblue tetrazolium and bromochloroindolyl phosphate (BioRad) according to the manufacturer's instructions. The intensities of bands were quantified in terms of peak areas in densitograms recorded with a TLC scanner (CS-930, Shimadzu). The deviation of results was around 5%.

Enzymes and Chemicals. Catalase from bovine liver and SOD from bovine erythrocytes were purchased from Sigma. Active oxygen scavengers and protease inhibitors were all purchased from Sigma, apart from aprotinin and SBTI, which were from Boehringer. All the protease inhibitors except for TPCK effectively inhibited the ability of tyrpsin to hydrolyze a synthetic substrate, N^{α}-benzoyl-D,L-arginine-4-nitroanilide, as assayed by the method of Erlanger et al. (1961).

RESULTS

Figure 1 shows the changes in polypeptide and immunoblot profiles for the three different PSII subcomplexes during photoinhibitory illumination. As seen from the Coomassie-stained gels (upper panels), not only the D1 protein but also other intrinsic proteins were damaged in all the subcomplexes. When the PSII complexes were illuminated at pH 6.0 without electron acceptors (acceptor-side photoinhibition), Coomassie-stained bands of all intrinsic proteins became smeared, and their positions on the gel were shifted closer to the origin with increasing duration of illumination: bands of the D1 and D2 proteins were affected first, and then those of the 43- and 47-kDa proteins of the core antenna and the α subunit of Cyt b_{559} followed. Concomitantly, high-molecular-mass aggregates, seen as a smearing in the upper part of the gel, increased with illumination. By contrast, the stained bands of the three

extrinsic proteins of 33, 23, and 16 kDa were not substantially affected. The smearing and mobility shift of protein bands and aggregate formation were most marked in the RC complexes illuminated at pH 8.0 in the presence of DBMIB (donor-side photoinhibition), and no distinct bands were observed after 40-min illumination. Similar changes in the polypeptide profiles were observed when the intensity of photoinhibitory light was reduced by 75% or when detergent was omitted from the illumination mixture, though the changes occurred more slowly (data not shown).

When proteins were analyzed by immunoblotting using anti-D1 (Figure 1, middle and lower panels), it was clear that the D1 protein was degraded in almost the same way as previously reported for isolated PSII subcomplexes: we observed the gradual disappearance and mobility shift of the 32-kDa D1 protein band (Shipton & Barber, 1992; De Las Rivas et al., 1992, 1993); the appearance of fragments of 23 and 16 kDa in the case of acceptor-side photoinhibition (Salter et al., 1992; De Las Rivas et al., 1993); and the appearance of three major fragments of about 24, 16, and 10 kDa in the case of donor-side photoinhibition (De Las Rivas et al., 1992; Shipton & Barber, 1992). Immunoblots also revealed the appearance of a distinct band of 41 kDa at the onset of illumination, which has been reported to be a covalent adduct of the D1 protein and the α subunit of Cyt b_{559} (Barbato et al., 1992b), as well as the accumulation of high-molecular-mass aggregates during prolonged illumination (e.g., De Las Rivas et al., 1992). Thus, it was evident that the illumination conditions in this study were similar to those in previous studies in terms of damage to the D1 protein. As judged from the densitograms of the immunoblots, the disappearance of the 32-kDa D1 protein band under illumination resulted mainly from the formation of high-molecular-mass aggregates.

Decreased intensity of staining with Coomassie blue and decreased mobility of protein bands in SDS–PAGE are phenomena that are usually observed when proteins are exposed to active oxygen species (Davies, 1987). To examine the possible involvement of active oxygen in protein damage during illumination, the effects of active oxygen scavengers were investigated. In these experiments, the duration of illumination was fixed such that the amount of 23–24-kDa fragment, the putative primary degradation product of the D1 protein, was still increasing. The scavengers used were catalase (for H_2O_2), SOD (for O_2^-), histidine and DABCO (for $^1O_2$; Foote, 1976), and *n*-propyl gallate (for ·OH and alkoxyl radicals; Bors et al., 1989).

In the PSII complexes subjected to acceptor-side photoinhibition, all six scavengers more or less suppressed the protein damage (Figure 2A). As shown by the Coomassie-stained gel, the extent of smearing and shifting of protein bands was reduced by the scavengers, most markedly by catalase, histidine, and *n*-propyl gallate. As seen in the immunoblot profiles, these scavengers also suppressed the decrease in the 32-kDa D1 protein band. Among the scavengers, *n*-propyl gallate was the most effective. Quantification from densitograms indicated that the 32-kDa band decreased to 47% of the dark control value upon 30-min illumination in the absence of scavengers, while more than 80% remained after illumination in the presence of *n*-propyl gallate (Table 1). This value corresponds to an approximately 70% inhibition. Formation of the 23-kDa fragment was also suppressed by catalase, histidine, and *n*-propyl gallate but not by SOD or DABCO. In the RC complexes subjected to donor-side photoinhibition, in which the damage to intrinsic proteins was most marked, histidine and *n*-propyl gallate greatly suppressed the band

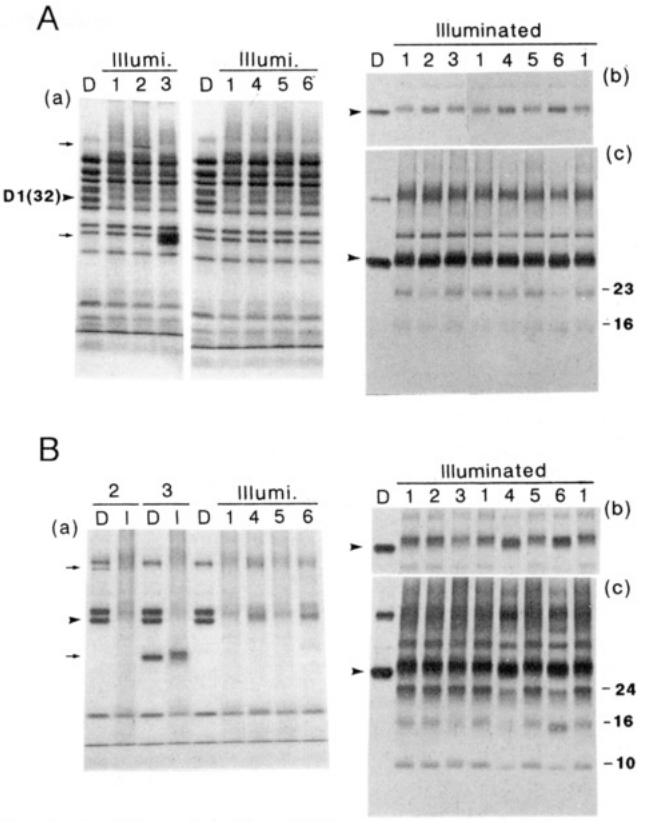

FIGURE 2: Effects of catalase, SOD, and scavengers of active oxygen on protein damage by photoinhibitory illumination. The PSII complexes were suspended in Mes medium (pH 6.0) and the RC complexes in Tris medium (pH 8.0) as described in the legend to Figure 1. After a 5-min incubation in darkness at 20 °C, the suspension was supplemented with the designated additions, incubated for a further 5 min, and then illuminated with white light for 30 min in the presence or absence of 0.2 mM DBMIB. (A) PSII complexes illuminated at 8.0 mE/(m²·s) and pH 6.0. (B) RC complexes illuminated at 4.0 mE/(m²·s) and pH 8.0 in the presence of DBMIB. (a) Polypeptide profiles; (b and c) immunoblot profiles with anti-D1. D and I denote dark control and illuminated samples, respectively. (1) No additions; (2) 100 μg/mL catalase; (3) 100 μg/mL SOD; (4) 10 mM histidine; (5) 1 mM DABCO; (6) 1 mM *n*-propyl gallate. Arrowheads indicate the position of the 32-kDa D1 protein band, and small arrows indicate those of bands of catalase and SOD.

smearing, the aggregation, and the formation of the 24-kDa fragment of the D1 protein (Figure 2B). A striking feature of Figure 2B is the damage to exogenously added proteins (catalase and SOD) under illumination. This was also the case when bovine serum albumin was present during illumination (not shown). This indicates that active oxygen species were even present in the surrounding medium when the RC complexes were illuminated.

Table 1 shows the effects of active oxygen scavengers on the disappearance of the 32-kDa D1 protein and its fragmentation in the three types of PSII subcomplex. With respect to the disappearance of the 32-kDa D1 protein, catalase had suppressive effects in the PSII and core complexes, but SOD was only effective in the PSII complex. By contrast, histidine and *n*-propyl gallate showed remarkable suppression in all three subcomplexes. DABCO showed slight suppression in the PSII complex only. With respect to D1 protein fragmentation, the scavengers that suppressed the disappearance of the 32-kDa D1 protein were also effective in suppressing fragment formation in most cases. One exception was found with *n*-propyl gallate and the core complexes. *n*-Propyl gallate greatly suppressed the decrease in the 32-kDa band, but it increased the amount of 23-kDa fragment, even upon illumination for a shorter time (15 min, data not shown).

Table 1: Effects of Catalase, SOD, and Scavengers of Active Oxygen Species on Degradation of the D1 Protein by Photoinhibitory Illumination of PSII Subcomplexes[a]

type of PSII subcomplex	illumination conditions		relative amounts of D1 protein and its fragments					
			no addition	catalase (0.1 mg/mL)	SOD (0.1 mg/mL)	His (10 mM)	DABCO (1 mM)	*n*-propyl gallate (1 mM)
PSII complex	pH 6.0, 30 min	32-kDa	1.00 (47)	1.31	1.27	1.43	1.10	1.77
		23-kDa	1.00 (4.0)	0.53	1.00	0.82	1.02	0.37
core complex	pH 6.0, 30 min	32-kDa	1.00 (40)	1.18	1.00	1.37	1.01	1.28
		23-kDa	1.00 (9.3)	0.58	0.99	0.83	1.07	1.19
RC complex	pH 6.3, 4 min	32-kDa	1.00 (81)	1.05	1.05	1.10	0.91	1.20
		23-kDa	1.00 (3.4)	1.05	1.03	0.67	1.01	0.78
RC complex	pH 8.0, +DBMIB, 30 min	32-kDa	1.00 (47)	0.99	0.81	1.20	0.91	1.20
		24-kDa	1.00 (13.8)	0.91	0.83	0.60	0.84	0.55

[a] PSII subcomplexes were illuminated with white light in the presence of the designated additions at 20 °C. The experimental conditions were the same as those in the legends to Figures 1 and 2. 32-kDa, 23-kDa, and 24-kDa stand for the intact D1 protein of 32 kDa, the 23-kDa N-terminal fragment, and the 24-kDa C-terminal fragment, respectively, and their amounts were quantified from densitograms of immunoblots with anti-D1. Values in parentheses represent percentages when the amount of intact D1 protein in the dark control sample was taken as 100%.

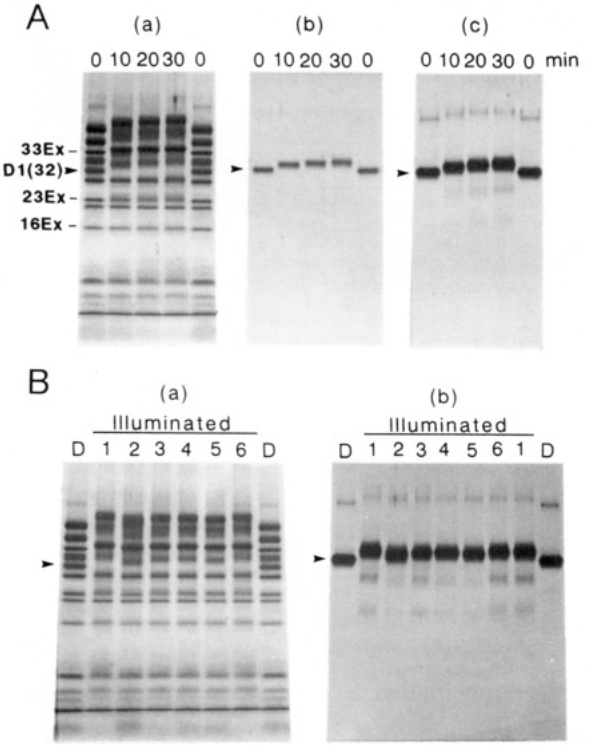

FIGURE 3: Strong illumination of PSII complexes that had been solubilized with SDS. PSII complexes were suspended in 1% SDS, 0.4 M sucrose, and 50 mM Mops–NaOH (pH 7.5) at 100 μg of Chl/mL, incubated at 25 °C in darkness for 10 min, and then illuminated with white light [4.0 mE/(m²·s)] at 20 °C. (A) Time courses. The solubilized sample was illuminated with no additions for the designated times. (B) Effects of active oxygen scavengers. The solubilized sample was illuminated for 30 min with no additions (lane 1), 10 mM histidine (lane 2), 2 mM NaN_3 (lane 3), 2 mM (lane 4) and 5 mM (lane 5) *n*-propyl gallate, and 10 mM D-mannitol (lane 6). D denotes the dark control sample with no additions. (a) Polypeptide profiles; (b and c) immunoblot profiles with anti-D1. Arrowheads indicate the position of the 32-kDa D1 protein band.

Probably, *n*-propyl gallate would retard further degradation of the fragment and allow the fragment to accumulate in this particular case.

Other scavengers tested were 10 mM D-mannitol (·OH), 1 mM dithiothreitol (·OH, O_2^-), and 1 mM NaN_3 ($^1O_2$). They did not have any suppressive effects but tended to stimulate D1 protein degradation in all three PSII subcomplexes (data not shown). This tendency might result from the reducing capacity of these scavengers (the presence of contaminating reducing sugar in the case of D-mannitol), which could cause some additional damage to the intrinsic electron carriers in PSII.

The above observations strongly suggest the participation of active oxygen species not only in the light-induced damage to intrinsic proteins but also in the fragmentation of the D1 protein. They also raise the question of whether active oxygen can cleave the D1 protein directly. To examine this possibility, PSII complexes that had been solubilized with SDS were subjected to strong illumination. Under these conditions, Chl and its derivatives are incorporated into SDS micelles and act as photosensitizers to generate $^1O_2$ from ambient oxygen molecules (Halliwell & Gutteridge, 1984).

Figure 3A shows the changes in the polypeptide and immunoblot profiles of solubilized PSII complexes during illumination. In this experiment, the PSII complexes were solubilized with 1% SDS at pH 7.5 for 10 min and then illuminated with strong light. As seen in the Coomassie-stained gel, illumination caused smearing and mobility shift of protein bands, as observed with the intact complexes (see Figure 1). In this case, the shift was much more marked, and the three extrinsic proteins were also affected. Only the band of a protein of about 22 kDa was totally unaffected. The high-molecular-mass aggregates and the 41-kDa adduct were not formed, suggesting that cross-linking reactions between different proteins do not occur when proteins are completely solubilized. To our surprise, fragments of the D1 protein of about 23 and 16 kDa appeared during illumination. These fragments did not originate from PSII complexes that remained intact in the presence of SDS, since levels of the fragments formed under illumination did not decrease even when the PSII complexes were solubilized with SDS for a longer time (3 h). The pattern of fragment formation changed depending on pH; only the 23-kDa fragment was detected at pH 6.0 (data not shown).

The damage to solubilized proteins under illumination was reduced by active oxygen scavengers (Figure 3B). The smearing and mobility shift of protein bands were greatly suppressed by an $^1O_2$ scavenger, histidine, and the D1 protein fragmentation by histidine and *n*-propyl gallate, a scavenger of ·OH and alkoxyl radicals. In general, $^1O_2$ cannot directly cleave peptide bonds but indirectly by generating alkoxyl radicals via reaction with organic molecules (Elstner, 1982). The suppression by *n*-propyl gallate coincides with this mechanism and suggests the involvement of alkoxyl radicals in the fragmentation of the solubilized D1 protein.

To examine the participation of protease(s) in D1 protein degradation, the effects of inhibitors of serine-type proteases were examined (Table 2). Contrary to previous reports, none of the inhibitors tested had any significant protective effect on D1 protein degradation. APMSF reproducibly but only

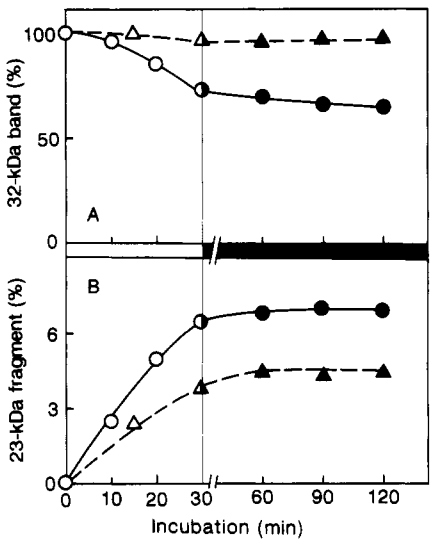

FIGURE 4: Degradation of the D1 protein under photoinhibitory illumination of PSII complexes at low temperature. The PSII complexes were suspended in Mes medium (pH 6.0) as described in the legend to Figure 1 and illuminated with white light [8.0 mE/(m^2·s)] at 20 °C (circles) or 2 °C (triangles) for 30 min. Then, the suspension was transferred to darkness and further incubated at 20 °C. The amounts of 32-kDa D1 protein and of its 23-kDa fragment were quantified from densitograms of immunoblots with anti-D1. 100% corresponds to the amount of intact D1 protein in the dark control sample. (A) Changes in the 32-kDa band. (B) Changes in the 23-kDa fragment.

slightly reduced the extent of the disappearance of the 32-kDa band in all three subcomplexes. Its effects on fragment formation were complex: APMSF reduced the amount of a fragment of 23–24 kDa in the RC complexes but increased it in the PSII complexes. TPCK had small protective effects only in the RC complexes subjected to donor-side photoinhibition. SBTI, a proteinaceous inhibitor of 20 kDa, slightly suppressed fragment formation only in the RC complexes subjected to acceptor-side photoinhibition at a high concentration (2 mg/mL, a molar ratio of SBTI to RC of 10:1). PMSF and aprotinin (a proteinaceous inhibitor of 6.5 kDa) were both ineffective. In the RC complexes subjected to donor-side photoinhibition, proteinaceous inhibitors (SBTI and aprotinin) reduced the amounts of both the 32-kDa band and the 24-kDa fragment. Probably, this resulted from covalent cross-linking of added inhibitors with the D1 protein and its fragment, since the formation of high-molecular-mass aggregates was slightly enhanced in the presence of these inhibitors and a new band of about 40 kDa that cross-reacted with anti-D1 was detected in the presence of aprotinin (data not shown).

We examined various experimental conditions, such as the inhibitor concentration, the preincubation protocol, the illumination time, the intensity of photoinhibitory light, the composition of the illumination mixture (the presence or absence of Ca^{2+}, acceptors, detergent), and so on, but in every case the protective effect was small, if any, being about 10% at most. It should be noted that the inhibitors used in these experiments (apart from TPCK) all effectively inhibited the activity of trypsin, as measured with a synthetic substrate.

The dependence on temperature of D1 protein degradation has been taken as evidence for the participation of a protease (Aro et al., 1993). In thylakoids and PSII membranes, the D1 protein is not degraded upon illumination at 2 °C, but after transfer to 20 °C, degradation occurs even in darkness (Aro et al., 1990; van Wijk et al., 1992). In the PSII subcomplexes used in this study, no such temperature dependence was observed. When the PSII complexes were illuminated at 2 °C, the disappearance of the 32-kDa band was greatly suppressed as compared with at 20 °C, but D1 protein fragmentation did occur, albeit more slowly (Figure 4). Smearing and mobility shift of protein bands were also observed at 2 °C, whereas high-molecular-aggregates were not marked at this temperature (data not shown). After transfer to darkness and 20 °C, the amount of 23-kDa fragment increased slightly for about 30 min and then remained constant.

DISCUSSION

Involvement of Active Oxygen in Light-Induced Protein Damage in Isolated PSII Subcomplexes. We found that photoinhibitory illumination damaged not only the D1 protein but also other intrinsic proteins in all three PSII subcomplexes examined: smearing and mobility shift of Coomassie-stained bands were observed (Figure 1). Similar smearing and shift of bands have been reported for the D1 and D2 proteins in immunoblotting experiments in the core and RC complexes (e.g., Virgin et al., 1991; Shipton & Barber, 1992) and for the Coomassie-stained band of the Cyt b_{559} subunit in the RC complexes (Shipton & Barber, 1991). Therefore, these phenomena seem to be common to PSII subcomplexes under photoinhibitory illumination. Illumination also brought about formation of high-molecular-mass aggregates, indications of cross-linking reactions between proteins. As for the D1 protein, we observed the gradual disappearance of the 32-kDa band, which we ascribed mainly to aggregate formation, and the formation of specific fragments. Among proteins other than the D1 protein, only the D2 protein has been reported to be degraded to specific fragments (Barbato et al., 1992c). At present, it is not clear whether other intrinsic proteins are also cleaved during illumination.

The smearing and mobility shift of intrinsic protein bands, aggregate formation, and also D1 protein fragmentation are considered to be caused by active oxygen species generated during illumination. As judged from the effectiveness of active oxygen scavengers (Table 1), the species that participate in damaging proteins are H_2O_2 in the PSII and core complexes, O_2^- in the PSII complex, and $^1O_2$, ·OH, and alkoxyl radicals in all three subcomplexes.

H_2O_2, O_2^-, and $^1O_2$ are known to be produced in PSII under illumination: H_2O_2 is produced by autoxidation at the acceptor side of PSII (Schröder & Akerlund, 1990) and also at the donor side when the proper functioning of the Mn cluster is disturbed (Ananyev et al., 1992). Production of O_2^- has been demonstrated in PSII membranes depleted of the Mn cluster (Chen et al., 1992). O_2^- can also be produced from H_2O_2 under illumination of Mn-depleted PSII membranes (Mano et al., 1987). $^1O_2$ is formed by the reaction of the triplet state of P680 with oxygen (Aro et al., 1993). $^1O_2$ would also be formed by the reaction of the triplet state of accessory Chl molecules or other chromophores after P680 has been destroyed (Macpherson et al., 1993). ·OH and alkoxyl radicals would be generated by secondary reactions of H_2O_2, O_2^-, and $^1O_2$: ·OH can be generated by conversion of H_2O_2 and O_2^- in the presence of metal ions, while alkoxyl radicals by reactions of $^1O_2$ and ·OH with organic molecules (Elstner, 1982; Halliwell & Gutteridge, 1984). In general, $^1O_2$, ·OH, and alkoxyl radicals can damage proteins directly, and H_2O_2 and O_2^- act indirectly by generating ·OH. Besides the action as active oxygen, H_2O_2 would destroy the Mn cluster (Ghanotakis et al., 1984) and thereby accelerate the photoinactivation of PSII (Callahan et al., 1986; Eckert et al., 1991).

$^1O_2$ and ·OH are known to cause aggregation and fragmentation of proteins. They have high reactivity toward some

Table 2: Effects of Inhibitors of Serine-Type Proteases on Degradation of the D1 Protein by Photoinhibitory Illumination of PSII Subcomplexes[a]

type of PSII subcomplex	illumination conditions		relative amounts of intact D1 protein and its fragments						
			no addition	APMSF (0.1 mM)	PMSF (1 mM)	TPCK (0.1 mM)	aprotinin (0.1 mg/mL)	SBTI (0.1 mg/mL)	SBTI (1 mg/mL)
PSII complex	pH 6.0, 30 min	32-kDa	1.00 (65)	1.06	0.94	1.00	1.00	0.97	
		23-kDa	1.00 (5.1)	1.21	1.22	1.06	1.18	1.09	
core complex	pH 6.0, 30 min	32-kDa	1.00 (52)	1.10	0.98	1.03	0.99	1.03	
		23-kDa	1.00 (8.0)	1.04	1.02	1.10	1.06	0.97	
RC complex	pH 6.3, 4 min	32-kDa	1.00 (81)					1.07[b]	1.02[c]
		23-kDa	1.00 (3.4)					1.07[b]	0.88[c]
RC complex	pH 8.0, +DBMIB, 30 min	32-kDa	1.00 (47)	1.10	1.03	1.10	0.82	0.97	0.72
		24-kDa	1.00 (11)	0.93	0.95	0.90	0.93	0.94	0.90

[a] Experimental conditions were the same as those described in Table 1. [b] 0.2 mg/mL SBTI. [c] 2.0 mg/mL SBTI.

amino acid residues such as aromatic and sulfur-containing residues (Foote, 1976; Stadtman, 1993). When these residues are attacked by $^1O_2$ or ·OH, radicals are generated in their side chains, and these then react with other susceptible residues to form covalent cross-links (Yamamoto, 1977). Presumably, the smearing and mobility shift of intrinsic protein bands resulted from modification of amino acid residues while the high-molecular-mass aggregates from intermolecular cross-linking. With respect to cleavage of peptide bonds, ·OH can act directly to bring about this reaction (Stadtman, 1993), while $^1O_2$ does indirectly, e.g., by generating alkoxyl radicals (Elstner, 1982).

On the basis of the above considerations, it is quite possible that the D1 protein is cleaved by the direct action of active oxygen species. This concept is strongly supported by the observation in Figure 3A that strong illumination of completely solubilized PSII complexes resulted in degradation of the D1 protein to specific fragments. These fragments were similar in size to those formed in the intact complexes. The D1 protein probably includes some amino acid sequences that are specifically susceptible to attack by active oxygen.

Effects of Protease Inhibitors on D1 Protein Degradation in Isolated PSII Subcomplexes. It has been reported that, in spinach core complexes, inhibitors that block the catalytic site of serine-type proteases (DFP, APMSF, and aprotinin) effectively suppressed the disappearance of the 32-kDa D1 protein under illumination (Virgin et al., 1991; Salter et al., 1992). By contrast, in pea RC complexes, SBTI has been reported to almost completely suppress the formation of the D1 protein fragment of 23–24 kDa in both acceptor-side and donor-side photoinhibition (Shipton & Barber, 1992; De Las Rivas et al., 1993). SBTI also suppressed fragmentation of the D2 protein in RC complexes (Barbato et al., 1992c). We reexamined the effects of these inhibitors in terms of suppression of the disappearance of the 32-kDa band and of fragment formation: none of the inhibitors caused marked suppression (Table 2). The inhibitors that gave small but discernible suppression were APMSF with all three subcomplexes and TPCK with the RC complexes. These inhibitors are nucleophilic molecules having an aromatic ring and electron-rich groups, resembling the scavengers histidine and *n*-propyl gallate in this respect. Therefore, their suppressive effects might be ascribable in part to their capacity for scavenging active oxygen. In the case of RC complexes subjected to donor-side photoinhibition, we found that proteinaceous inhibitors could reduce the apparent amount of the 24-kDa fragment band by binding to the fragment. This might be responsible in part for the suppressive effects of SBTI reported previously.

The lack of consistency in the inhibitor effects described above does not result from differences in the plant materials used (spinach and pea *versus* rice) since the inhibitors also failed to suppress D1 protein degradation in spinach PSII complexes (data not shown). The differences would be ascribable to differences in preparations, from the experiment in which two different spinach PSII complexes prepared as in a previous study (Salter et al., 1992) and in a present study were compared (Dr. A. H. Salter, personal communication).

In previous studies, almost complete suppression of D1 protein degradation was reported only with SBTI in the RC complexes in terms of fragment formation (Shipton & Barber, 1992; De Las Rivas et al., 1993). The suppression by other inhibitors was partial, being at most 60% with DFP, APMSF, and aprotinin (Virgin et al., 1991; Salter et al., 1992). Since DFP and APMSF are irreversible inhibitors that bind covalently to the catalytic site, the incomplete suppression by these inhibitors implies that nonenzymatic cleavage of the D1 protein, possibly by the action of active oxygen, occurs simultaneously with enzymatic cleavage. One might consider that the D1 protein is cleaved enzymatically at the early stage of photoinhibition, but after prolonged illumination nonenzymatic cleavage dominates the enzymatic one, so that the inhibitor effects are hardly observed. This is unlikely in the PSII subcomplexes used in this study, since the protease inhibitors did not show marked suppression even when D1 protein degradation was slowed down by reducing the intensity of photoinhibitory light or by omitting detergent from the illumination mixture (data not shown).

More evidence for the participation of a protease is the temperature dependence of D1 protein degradation: suppression of degradation under illumination at 2 °C and subsequent degradation in darkness after transfer to 20 °C (Aro et al., 1993). No such temperature dependence was observed in PSII subcomplexes (Figure 4). Thus, we conclude that the putative protease(s) is (are) absent in all three PSII subcomplexes used in this study. This raises the question of whether the putative protease(s) is (are) a component of PSII.

Comparison with Light-Induced Protein Damage in Vivo. Previous studies have demonstrated that various active oxygen species participate in the photoinactivation of PSII and D1 protein degradation *in vivo* and in isolated thylakoids (see the introduction). The action mechanism of active oxygen *in vivo* has not been elucidated yet. However, the present study suggests the possibility that the D1 protein would be cleaved by the direct action of active oxygen *in vivo*.

The light-induced protein damage in isolated PSII subcomplexes differs from that in more intact systems in some respects. One is damage to intrinsic proteins other than the D1 protein. Under low-light illumination *in vivo*, the D1 protein is specifically degraded (Mattoo et al., 1981, 1984; Greenberg et al., 1989). Under strong photoinhibitory illumination, the D2 protein is also degraded *in vivo* (Schuster

et al., 1988) and in isolated thylakoids (Virgin et al., 1988), albeit much more slowly than the D1 protein. Damage to other PSII proteins and formation of high-molecular-mass aggregates occur only after prolonged illumination for 2–4 h in isolated thylakoids (Roberts et al., 1991; Casano et al., 1994) and PSII membranes (Nedbal et al., 1990). In the case of PSII subcomplexes, damage to other proteins can be observed after photoinhibitory illumination for a short time, and the D1 and D2 proteins seem to be damaged with similar time courses (Figure 1; Barbato et al., 1992c).

This difference can be explained by the lack of membrane structure in PSII subcomplexes. According to Hundal et al. (1990), the hydrophobic core of PSII is partially disassembled during the course of photoinhibition, and separated PSII proteins migrate from the grana to stromal regions of the thylakoid. If PSII is disassembled under illumination, the core antenna and other PSII proteins are separated from the site of formation of active oxygen (probably the reaction center) and can escape attack by active oxygen. If not disassembled, all PSII proteins remain in close proximity to the site of active oxygen formation and are attacked. The latter events occur in the case of PSII subcomplexes that do lack a membrane structure.

Another difference is that the D1 protein is not completely degraded in PSII subcomplexes: the complete degradation to small peptides or amino acids requires a more intact membrane structure (Aro et al., 1993). Generally, active oxygen species cannot degrade a whole protein to amino acids, but modification of proteins by active oxygen is known to increase their susceptibility to proteases in bacteria and mammalian cells (Davies, 1987) and in chloroplasts (Casano et al., 1994). Some reports have proposed that proteases that degrade denatured proteins are present in chloroplasts: a serine-type endopeptidase bound to the thylakoid membrane (Casano et al., 1994) and a protease homologous to the Clp protease of *Escherichia coli* (Vierstra, 1993). We presume that primary cleavage of the D1 protein is performed by active oxygen species generated inside the PSII reaction center but complete degradation can be accomplished by concerted actions of active oxygen and the proteases that digest abnormal proteins.

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