Specific Degradation of the D1 Protein of Photosystem II by Treatment with Hydrogen Peroxide in Darkness: Implications for the Mechanism of Degradation of the D1 Protein under Illumination†

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ABSTRACT: The D1 protein of the photosystem II (PSII) reaction center has a rapid turnover and is specifically degraded under illumination *in vivo*. When isolated PSII membranes were treated in darkness with 10 mM hydrogen peroxide (H_2O_2), an active form of oxygen that is generated at the acceptor side of PSII under illumination, proteins of the PSII reaction center were specifically damaged in almost the same way as observed under illumination with strong light. The D1 protein and, to a lesser extent, the D2 protein were degraded to specific fragments, and cross-linked products (the covalently linked adduct of the D1 protein and the α subunit of cytochrome b_{559} and the heterodimer of the D1 and D2 proteins) were generated concomitantly. The site of cleavage of the D1 protein that gave rise to a major fragment of 22 kDa was located in the loop that connects membrane-spanning helixes IV and V. Treatment with H_2O_2 caused the same damage to proteins in isolated thylakoids and in core complexes that contained the non-heme iron at the acceptor side, but not in isolated reaction centers depleted of the iron. From these observations and the effects of reagents that are known to interact with the non-heme iron, it is suggested that the damage to proteins is caused by oxygen radicals generated by the non-heme iron in the Fe(II) state in a reaction with H_2O_2. It is proposed, moreover, that a similar mechanism is operative during the selective and specific degradation of the D1 protein under illumination.

Photosystem II (PSII)[1] of oxygenic photosynthetic organisms is a supramolecular complex that consists of more than 20 different protein components and is embedded in the thylakoid membrane (Vermass & Ikeuchi, 1991). Among the various proteins, the D1 and D2 proteins bind all the redox components involved in the photochemical reaction and the subsequent electron transport reactions (Trebst, 1986; Michel & Deisenhofer, 1988), and they form the reaction center complex, the central part of PSII, probably in association with cytochrome b_{559} and the *psbI* product (Nanba & Satoh, 1987).

The D1 protein is known as a rapid turnover protein that is specifically degraded under illumination *in vivo* (Mattoo et al., 1981, 1984). Under growth light conditions, the effects of degradation of the D1 protein are counteracted by a repair system that includes synthesis of the protein *de novo* (Prášil et al., 1992). By contrast, under strong illumination that causes photoinhibition of PSII, the rate of degradation exceeds that of repair so that the amount of D1 protein decreases (Prášil et al., 1992). Unlike the D1 protein, the D2 protein, which has a structure homologous to the D1 protein, is not degraded under growth light conditions. The degradation of the D2 protein occurs only under strong photoinhibitory illumination, but degradation still proceeds more slowly than that of the D1 protein (Prášil et al., 1992). Degradation of other intrinsic proteins occurs much more slowly than that of the D1 and D2 proteins even under photoinhibitory illumination *in vivo* (Schuster et al., 1988). The light-dependent degradation of the D1 protein can also be observed in isolated thylakoids and PSII preparations under photoinhibitory illumination (Aro et al., 1993).

Under growth light conditions *in vivo*, degradation of the D1 protein gives rise to a fragment of 23.5 kDa, and the cleavage site is located in the loop that connects the membrane-spanning helixes IV and V on the stromal side of the thylakoid membrane (Greenberg et al., 1987). By contrast, under photoinhibitory illumination, various fragments can be detected, and a fragment of 22−24 kDa is considered to be a primary product of degradation both *in vivo* (Shipton & Barber, 1994) and *in vitro* (Aro et al., 1993). The site of the primary cleavage under photoinhibitory illumination is also localized in the loop that connects helixes IV and V (De Las Rivas et al., 1992; Salter et al., 1992; Shipton & Barber, 1994).

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[1] Abbreviations: anti-D1, anti-$D1_C$, and anti-D2, antibodies raised against the entire D1 protein, the C-terminal region of the D1 protein, and the entire D2 protein, respectively; BQ, 1,4-benzoquinone; Chl, chlorophyll; DABCO, 1,4-diazabicyclo[2.2.2]octane; DCIP, 2,6-dichlorophenolindophenol; DPC, 1,5-diphenylcarbazide; Mes, 2-(*N*-morpholino)ethanesulfonic acid; P680, primary electron donor of photosystem II; PAGE, polyacrylamide gel electrophoresis; PSII, photosystem II; Q_A and Q_B, primary and secondary quinone acceptors of photosystem II, respectively; SDS, sodium dodecyl sulfate.

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Two possible mechanisms have been proposed for the specific degradation of the D1 protein. One mechanism involves a protease(s) specific to the D1 protein. This mechanism has long been implicated, and recent studies *in vitro* suggest that a component of PSII itself has the activity of a serine-type protease and participates in the degradation (Aro et al., 1993). According to this model, singlet oxygen ($^1O_2$) generated by the triplet state of P680 under illumination alters the conformation of the D1 protein and renders it susceptible to degradation by the putative protease. This possibility is inferred from the observation that inhibitors of serine-type proteases suppress degradation of the D1 protein in isolated PSII subcomplexes under photoinhibitory illumination (Aro et al., 1993). As demonstrated previously (Miyao, 1994), however, such protease inhibitors do not necessarily suppress the degradation of the D1 protein: the inhibitors slightly prevent the decrease in the level of the intact D1 protein of 32 kDa, but they do not substantially suppress the cleavage of the D1 protein to specific fragments (Miyao, 1994).

The other possible mechanism involves active oxygen species generated in PSII under illumination. Sopory et al. (1990) investigated the effects of scavengers of active oxygen species on the half-life of the D1 protein *in vivo*, and they proposed that some oxygen radical other than $^1O_2$ might participate in the degradation. In isolated PSII subcomplexes under photoinhibitory illumination, not only $^1O_2$ but also other active oxygen species appear to be involved in the degradation (Mishra et al., 1994; Miyao, 1994).

Hydrogen peroxide (H_2O_2) is one of the active oxygen species generated in PSII under illumination (Schröder & Åkerlund, 1990; Ananyev et al., 1992). In general, H_2O_2 itself cannot directly damage proteins, but it does damage them indirectly by generating toxic hydroxyl radicals (•OH) in the presence of metal ions, such as Fe(II), Cu(I), and Mn(II), via the Fenton reaction:

$$H_2O_2 + Fe(II) \rightarrow \cdot OH + OH^- + Fe(III)$$

(Halliwell & Gutteridge, 1984; Stadtman, 1993). In this report, we demonstrate that treatment of PSII membranes with H_2O_2 in darkness results in degradation of the D1 protein in the same way as observed under photoinhibitory illumination. It is proposed that the non-heme iron at the acceptor side of PSII generates toxic oxygen radicals in a reaction with H_2O_2 and these radicals damage the D1 protein. The possible involvement of H_2O_2 in the degradation of the D1 protein under illumination is discussed.

MATERIALS AND METHODS

Preparation of Thylakoids, PSII Membranes, and PSII Subcomplexes. Thylakoids were prepared from rice seedlings by differential centrifugation according to Ono et al. (1986) with slight modifications, and suspended in 5 mM $MgCl_2$, 10 mM NaCl, 0.4 M sucrose, and 50 mM Mes−NaOH (pH 6.5) and stored in liquid nitrogen. PSII membranes were prepared from seedlings of rice and wheat with Triton X-100 and stored in liquid nitrogen in the presence of 30% (v/v) ethylene glycol (Miyao, 1994). Before use, the PSII membranes were thawed and washed 3 times with medium A [10 mM NaCl, 0.4 M sucrose, and 50 mM Mes−NaOH (pH 6.5)] by centrifugation and resuspension. When indicated, the membranes were washed with medium A that contained 2.0 mM EDTA and then with medium A that had been treated with the chelating ion-exchange resin Chelex 100 (Bio-Rad), and finally suspended in the same medium. PSII membranes depleted of the Mn cluster were prepared by treating the membranes with 1.0 mM NH_2OH in darkness (Miyao & Inoue, 1991).

Core complexes were prepared from intact PSII membranes by treatment with *n*-octyl β-D-glucopyranoside followed by sucrose density gradient centrifugation (Ikeuchi & Inoue, 1988b). Reaction center complexes were prepared from the PSII membranes with Triton X-100 by a method based on that of Nanba and Satoh (1987) with the modifications described previously (Miyao, 1994). 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 by the method of Arnon (1949).

Treatments with H_2O_2 and with Photoinhibitory Light. The sample was suspended in medium A that contained 2.0 mM EDTA and allowed to stand in darkness at 25 °C for 10 min. When indicated, medium A that had been treated with Chelex 100 was used instead of medium A plus EDTA. Final Chl concentrations were 0.3 mg/mL for the thylakoids and the PSII membranes, 0.1 mg/mL for the core complexes, and 20 μg/mL for the reaction center complexes. In the case of the core and reaction center complexes, 2 mM *n*-dodecyl β-D-maltoside was included in the suspension. For treatment with H_2O_2, the suspension was supplemented with 0.05 volume of a solution of H_2O_2 to give the designated concentrations of H_2O_2 and incubated at 25 °C for 30 min in darkness or under illumination from white fluorescent lamps at 100 μE/(m^2•s). Then, catalase from bovine liver (Sigma) was added to the suspension, to remove H_2O_2, at a final concentration of 0.1 mg/mL. Control treatment was performed as above using water instead of a solution of H_2O_2. For photoinhibitory light treatment, the suspension was illuminated with white light at 8 mE/(m^2•s) and 10 °C using a setup described previously (Miyao, 1994). After treatment, the suspension was divided into aliquots, frozen in liquid nitrogen, and stored at −80 °C.

Analytical Procedures. Digestion by lysyl endopeptidase of the PSII membranes was performed as follows. Twenty microliters of a membrane suspension at 0.3 mg of Chl/mL was supplemented with 1.0 μL of 1.0 M Tris (pH not adjusted), 0.3 μL of 0.2 M EDTA, 3.0 μL of 1% SDS, and 5.0 μL of lysyl endopeptidase from *Achromobacter* (Wako) dissolved in medium A at 4 units/mL. The final pH of the reaction mixture was 8.6. After incubation at 30 °C for 1 h, the suspension was subjected to SDS−PAGE.

SDS−PAGE and subsequent immunoblotting were performed as described previously (Miyao, 1994). For immunoblotting, three different antisera were used: anti-D1 raised against the entire D1 protein of spinach purified by SDS−PAGE, anti-$D1_C$ raised against a synthetic oligopeptide that corresponded to residues 326−333 of the D1 protein of spinach, and anti-D2 raised against the entire D2 protein of spinach purified by SDS−PAGE. For detection of fragments of the D1 and D2 proteins, a sample of about 20−40 times as much as the amount optimum for quantification of the intact protein band was applied for SDS−PAGE. The intensities of immunoreacted bands were quantified in terms

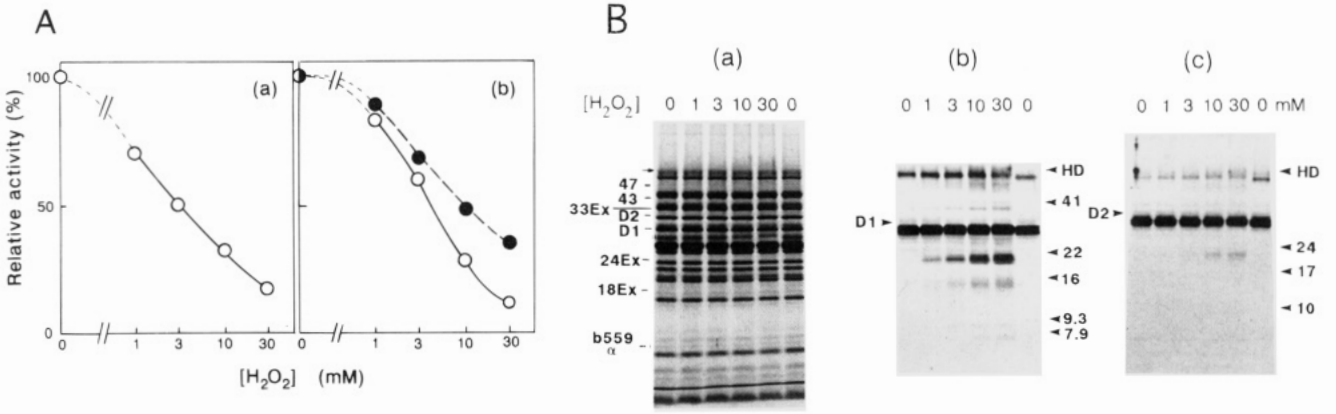

FIGURE 1: Effects of treatment with H_2O_2 in darkness on the activities and proteins of rice PSII membranes. Rice PSII membranes that had been washed with 2.0 mM EDTA were suspended at 0.3 mg of Chl/mL in medium A which had been depleted of metal ions by treatment with Chelex 100. After standing in darkness at 25 °C for 10 min, the suspension was supplemented with H_2O_2 to give the designated concentrations of H_2O_2 and incubated in darkness at 25 °C for 30 min. Then the suspension was supplemented with catalase to remove H_2O_2 and subjected to assays of PSII activities and SDS−PAGE. For assays of oxygen-evolving activity, PSII membranes, treated as described above, were washed twice with medium A to remove catalase. (A) PSII activities. (a) Oxygen-evolving activity measured with phenyl-BQ; (b) DCIP reduction activity measured with (●) or without (○) DPC. Activities of the control sample were 610 μmol of O_2/[(mg of Chl)·h], and 140 and 120 μmol of DCIP/[(mg of Chl)·h] with and without DPC, respectively. (B) Polypeptide and immunoblot profiles. (a) Polypeptide profiles after staining with Coomassie blue; (b) immunoblot profiles with anti-D1; (c) immunoblot profiles with anti-D2. Apparent molecular masses of fragments were estimated from their mobilities on the gel with intrinsic proteins of PSII taken as molecular mass markers. An arrow indicates the position of a band of catalase, and HD denotes the heterodimer of the D1 and D2 proteins.

of peak areas on densitograms recorded with a TLC scanner (CS-930, Shimadzu).

PSII activities were measured in medium A that contained 2.0 mM EDTA and 5 mM $CaCl_2$ at 25 °C: oxygen evolution was measured with 0.8 mM phenyl-BQ as an artificial electron acceptor using a Clark-type oxygen electrode, and reduction of DCIP was determined spectrophotometrically from the absorbance change at 600 nm in the presence of 50 μM DCIP and, when indicated, 1.0 mM DPC as an electron donor.

RESULTS

Figure 1 shows the effects on PSII activities and proteins of treatment in darkness of the PSII membranes from rice with various concentrations of H_2O_2. To prevent adventitious formation of hydroxyl radicals by the reaction between H_2O_2 and contaminating free metal ions, the PSII membranes and the medium used in this experiment were first depleted of free metal ions by washing with EDTA and treatment with Chelex 100, respectively.

Electron transport activity from water to exogenous acceptors, measured in terms of either the oxygen evolution or the reduction of DCIP, decreased with increasing concentrations of H_2O_2 (Figure 1A). DPC, which donates electrons directly to the reaction center, partially restored the capacity of DCIP reduction, an indication that the treatment impaired both the donor and acceptor sides of PSII.

The treatment also damaged proteins of the PSII reaction center (Figure 1B). As seen from the Coomassie-stained gel, bands of the D1 and D2 proteins specifically became smeared, and the positions on the gel of these bands were slightly shifted toward the origin, while bands of other proteins remained totally unaffected. Immunoblots revealed that the D1 protein was degraded to specific fragments of 22, 16, 9.3, and 7.9 kDa. The D2 protein was also degraded to specific fragments, albeit to a lesser extent than the D1 protein: at 10 mM H_2O_2, the amount of 22-kDa fragment of the D1 protein was equivalent to 7−8% of that of the intact protein in the control sample, while the 24-kDa fragment of the D2 protein was equivalent to about 3%. In these immunoblots, changes in the bands of the intact proteins were not clearly seen, since the gels were overloaded to detect bands of the fragments (see Materials and Methods). Quantification under optimum conditions indicated that the 32-kDa band of the D1 protein and the 34-kDa band of the D2 protein decreased by 8−12% and 2−4%, respectively, at 10 mM H_2O_2. Immunoblots also revealed that H_2O_2 caused cross-linking reactions between the D1 protein and other proteins of the reaction center. A distinct band of 41 kDa, possibly a covalent adduct of the D1 protein and the α subunit of cytochrome b_{559} (Barbato et al., 1992), was generated, and the amount of a heterodimer of the D1 and D2 proteins increased slightly.

It is unlikely that such damage to proteins was caused by hydroxyl radicals generated by the reaction of H_2O_2 with a trace of contaminating metal ions, since chelators (EDTA up to 8 mM and diethylenetriaminepentaacetic acid at 1 mM) did not prevent the damage (data not shown). In the experiments described below, treatment with H_2O_2 was performed in the presence of 2.0 mM EDTA rather than with materials and media that had been depleted of free metal ions. Identical results were obtained under both sets of experimental conditions.

The damage to proteins was completely suppressed by catalase but not at all by either superoxide dismutase or scavengers of $^1O_2$ (histidine and DABCO; Foote, 1976; Figure 2a). *n*-Propyl gallate, a scavenger of hydroxyl and alkoxyl radicals (Bors et al., 1989), had a suppressive effect: the amounts of 22- and 16-kDa fragments of the D1 protein and the heterodimer decreased by about 40, 35, and 30%, respectively, in the presence of 2 mM *n*-propyl gallate. These observations suggest that hydroxyl and/or alkoxyl radicals were generated during treatment with H_2O_2 and these then damaged proteins. Since free metal ions did not participate in damaging proteins, it is suggested that some component(s) of PSII react(s) with H_2O_2 and generate(s) toxic

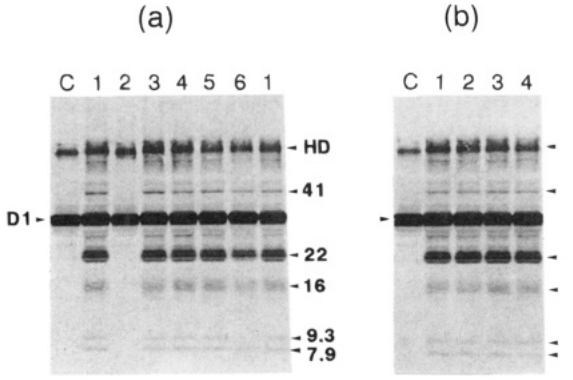

FIGURE 2: Effects of scavengers of active oxygen species and inhibitors of serine-type proteases on damage to the D1 protein by treatment with H_2O_2. Rice PSII membranes were suspended in medium A that contained 2.0 mM EDTA, incubated with designated additives in darkness at 25 °C for 10 min, and then treated with 10 mM H_2O_2 at 25 °C for 30 min in darkness. Immunoblots with anti-D1 are shown. (a) Effects of scavengers. (1) No addition; (2) 0.1 mg/mL catalase from bovine liver; (3) 0.1 mg/mL superoxide dismutase from bovine erythrocytes; (4) 10 mM histidine; (5) 5 mM DABCO; (6) 2 mM *n*-propyl gallate. (b) Effects of protease inhibitors. (1) No addition; (2) 0.2 mM (4-amidinophenyl)-methanesulfonyl fluoride (APMSF); (3) 2 mM phenylmethanesulfonyl fluoride (PMSF); (4) 0.2 mM N^{α}-tosyl-L-phenylalanine chloromethyl ketone (TPCK). C denotes control sample that was treated in the same way but in the absence of H_2O_2 and additives.

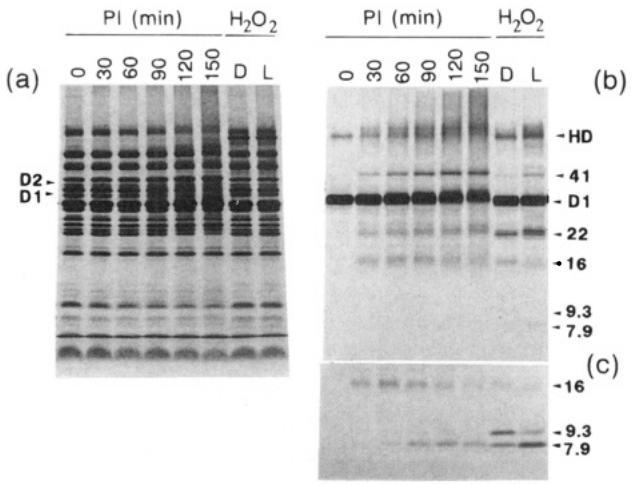

FIGURE 3: Comparison of damage to the D1 protein by photoinhibitory illumination and treatment with H_2O_2. Rice PSII membranes were suspended in medium A that contained 2.0 mM EDTA and illuminated with white light [8 mE/(m^2·s)] for the designated times (PI), or treated with 10 mM H_2O_2 for 30 min in darkness (D) or under illumination with weak light [100 μE/(m^2·s); L]. (a) Polypeptide profiles; (b and c) immunoblot profiles with anti-D1 and with anti-$D1_C$, respectively.

oxygen radicals. Irreversible inhibitors of serine-type proteases did not suppress the damage (Figure 2b).

The damage to proteins caused by treatment with H_2O_2 was similar to that observed under photoinhibitory illumination (Figure 3). The pattern of fragmentation of the D1 protein was almost the same, although the yield of fragments was lower in the case of photoinhibitory illumination. High-molecular-mass aggregates were detectable as a smear in the upper part of the gel, and intrinsic proteins other than the D1 and D2 proteins were also damaged under photoinhibitory illumination.

Treatment with H_2O_2 was effective even in complete darkness, but illumination with weak light during the

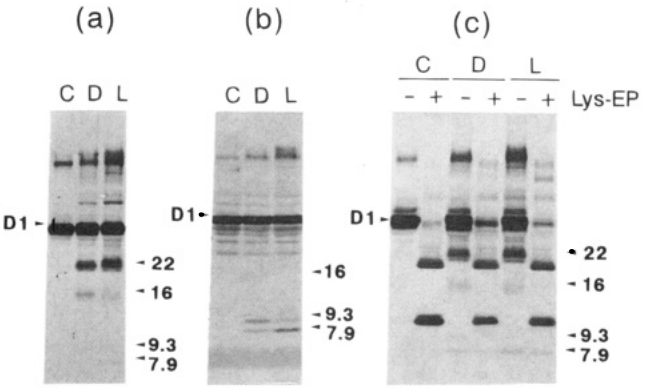

FIGURE 4: Identification of fragments of the D1 protein generated by treatment with H_2O_2. (a and b) Rice PSII membranes were treated with 10 mM H_2O_2 in the presence of 2.0 mM EDTA in darkness or under weak-light illumination for 30 min, and then they were analyzed by immunoblotting with anti-D1 (a) and anti-$D1_C$ (b). (c) Wheat PSII membranes were treated with H_2O_2 as above, digested with lysyl endopeptidase (Lys-EP), and analyzed by immunoblotting with anti-D1. C, D, and L denote control samples, samples treated with H_2O_2 in darkness, and samples treated with H_2O_2 under illumination, respectively.

treatment slightly enhanced the damage (Figure 3, lanes D and L). A remarkable effect of the illumination was the change in the relative amounts of the small fragments of 9.3 and 7.9 kDa: the fragment of 9.3 kDa was much more abundant after treatment in darkness, while that of 7.9 kDa became dominant after treatment in the light. In addition, the positions on the gel of the 22- and 16-kDa fragments slightly differed between the light- and dark-treated samples. As discussed below, these changes reflected a shift in the cleavage site of the D1 protein that resulted from illumination. It is noted that these changes were observed only when samples were illuminated during treatment with H_2O_2. Illumination prior to treatment was totally ineffective (data not shown).

The origins of fragments of the D1 protein were examined with an antibody specific to the C-terminal region of the protein (residues 326−333; anti-$D1_C$), and an endopeptidase specific to lysine residues (Figure 4). The D1 protein of wheat contains only one lysine residue at position 238 [see Ikeuchi and Inoue (1988a)], in the middle of the loop that connects helixes IV and V. Therefore, the presence of this residue in fragments can be easily examined with the D1 protein of wheat and lysyl endopeptidase. Since the D1 protein of rice does not have such a residue, this particular experiment shown in Figure 4c was done using PSII membranes from wheat. It is noted that the features of degradation by treatment with H_2O_2 were identical in the D1 proteins of wheat and rice.

As shown in Figure 4, the 22-kDa fragments generated by treatments with H_2O_2 in the light and dark both did not cross-react with anti-$D1_C$ (Figure 4b) but were digested by the peptidase (Figure 4c). By contrast, two small fragments of 9.3 and 7.9 kDa cross-reacted with anti-$D1_C$ but were not digested by the peptidase. These results suggest that the cleavage occurred on the C-terminal side of residue 238 both in the light and in the dark. As judged from the apparent molecular masses of the fragments, it seems likely that the 22-kDa fragments and the small fragments were N-terminal and C-terminal fragments, respectively, of the cleavage, and that the cleavage occurred between residues 250 and 280. The presence of two different small fragments of C-terminal

origin implies that there were two different cleavage sites in this region which were separated by as many as 10 amino acid residues. This hypothesis is supported by the results shown in Figure 3 that in the case of treatment under illumination, after which the 7.9-kDa fragment was much more abundant than the 9.3-kDa fragment, the 22-kDa fragment of N-terminal origin was slightly larger than that generated by treatment in darkness. This observation also indicates that the site of cleavage varies depending on the light conditions during the treatment; one site is predominantly subject to cleavage in darkness, while the other is more susceptible in the light.

The 16-kDa fragments generated by the treatments in the light and dark both cross-reacted with anti-$D1_C$ (Figure 4b; also see Figure 3) and were also digested by lysyl endopeptidase (Figure 4c). These results suggest that the cleavage occurs on the N-terminal side of residue 238. As judged from the differences in migration distance in the gel between the 16-kDa fragment and the larger fragment of the lysyl endopeptidase digestion, we propose that the cleavage site is located at a position about 30−50 amino acid residues from residue 238 toward the N-terminus. In this case also, the cleavage occurred at two different sites, separated by several amino acid residues, depending on the light conditions.

As above, the cleavage sites of the D1 protein were localized to two different regions; one was on the C-terminal side of residue 238 (region I), and the other was on the N-terminal side of the residue (region II). As judged from the relative amounts of fragments, the cleavage occurred predominantly in region I. The location of the cleavage sites in the D1 protein is identical to that observed under photoinhibitory illumination (De Las Rivas et al., 1992; Salter et al., 1992). In the case of samples exposed to photoinhibitory illumination, the presence of two different cleavage sites in close proximity has not previously been reported. As shown in Figure 3, however, photoinhibitory illumination also gave rise to two small fragments that cross-reacted with anti-$D1_C$ and that were the same size as fragments produced by treatment with H_2O_2. Therefore, it seems likely that, under photoinhibitory illumination, cleavage occurs at two different sites on the C-terminal side of residue 238 as it does in the case of treatment with H_2O_2.

Figure 5 shows the effects of treatment with H_2O_2 on the D1 protein in five different preparations. In thylakoids and in PSII membranes with and without the Mn cluster, H_2O_2 caused the same damage to the D1 protein. The extent of damage, however, decreased slightly in the following order: thylakoids, PSII membranes with the Mn cluster, and PSII membranes without the Mn cluster. In the case of thylakoids, the presence of KCN, an inhibitor of catalase, was necessary if damage was to be observed. Another catalase inhibitor, 3-amino-1*H*-1,2,4-triazole, that specifically binds to catalase in the presence of H_2O_2 showed a similar effect (data not shown). Since catalases are not present in chloroplasts (Asada, 1994), this result must have been a consequence of contamination of the thylakoids by catalases from other organelles. The treatment also damaged the D1 protein in the core complexes. In this case, the 9.3-kDa fragment was hardly detected. By contrast, no damage at all was observed in the reaction center complexes.

From the above observations, we can deduce the component(s) that react(s) with H_2O_2 and generate(s) toxic oxygen

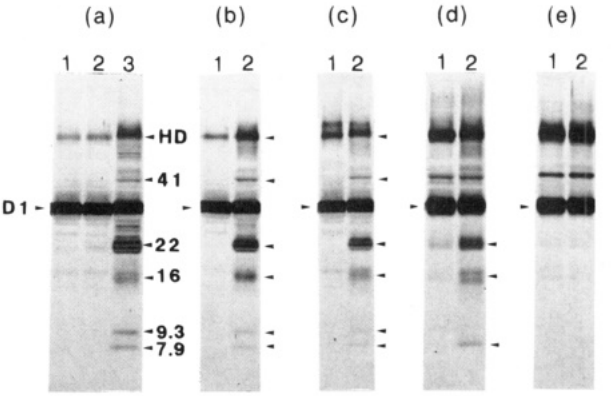

FIGURE 5: Effects of treatment with H_2O_2 on the D1 protein in various preparations from rice. The various preparations were treated with 10 mM H_2O_2 in the presence of 2.0 mM EDTA in darkness for 30 min. Immunoblots with anti-D1 are shown. (a) Thylakoids; (b) intact PSII membranes; (c) PSII membranes depleted of the Mn cluster; (d) core complexes; (e) reaction center complexes. (1) Control sample; (2) sample treated with H_2O_2; (3) sample treated with H_2O_2 in the presence of 4.0 mM KCN. Amounts of sample subjected to SDS−PAGE were adjusted so that the intensity of the 32-kDa band of the intact D1 protein in the control sample was the same for all preparations.

radicals. P680 and pheophytin can both be excluded, since they are present in isolated reaction center complexes in which no damage was observed. The Mn cluster and Q_B are unlikely candidates, since the damage was observed in PSII membranes depleted of the Mn cluster and the core complexes depleted of Q_B (Ikeuchi & Inoue, 1986). Cytochrome b_{559} is unlikely also, since the composition of its redox forms was almost the same in the core and the reaction center complexes (data not shown). Thus, the remaining candidates are Q_A and the non-heme iron at the acceptor side of PSII. To identify the actual component(s), we investigated the effects of various reagents that interact with Q_A and/or non-heme iron (Figure 6).

Q_A is in the oxidized form in darkness and is reduced under illumination, while non-heme iron remains in the Fe(II) state both in the light and in the dark. When ferricyanide is added in darkness, Q_A remains unaffected, but the non-heme iron is oxidized to the Fe(III) state (Diner & Petrouleas, 1987). As shown in Figure 6a, ferricyanide suppressed both the fragmentation of the D1 protein and the cross-linking reactions by treatment with H_2O_2 in darkness: the amounts of 22- and 16-kDa fragments of the D1 protein were reduced by 65 and 55%, respectively, and the increase in the heterodimer was suppressed by 60%. When the iron that had been oxidized by ferricyanide was reduced again by illumination (Diner & Petrouleas, 1987), ferricyanide did not have any suppressive effects. These results suggest the participation of the non-heme iron in the Fe(II) state.

If Q_A were to remain in the reduced form (Q_A^-) in darkness for some reason, it would be oxidized by ferricyanide in darkness and re-reduced under illumination as is the non-heme iron. The participation of Q_A^- was examined in experiments in which Q_A^- was oxidized by exogenous benzoquinones (BQs) prior to treatment with H_2O_2 (Figure 6b). Among three benzoquinones tested, phenyl- and dichloro-BQs specifically suppressed the formation of the 16-kDa fragment but only slightly suppressed the formation of the 22-kDa fragment and the cross-linking reactions. Thus, it is unlikely that Q_A^- plays a central role in the generation of oxygen radicals. The slight suppression by phenyl- and

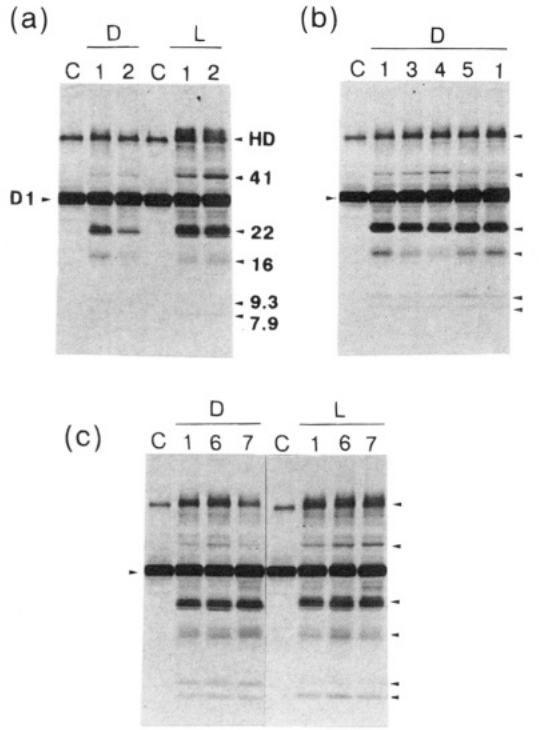

FIGURE 6: Effects of various additives on damage to the D1 protein by treatment with H_2O_2. Rice PSII membranes were suspended in medium A that contained 2.0 mM EDTA, incubated with designated additives in darkness at 25 °C for 10 min, and then treated with 10 mM H_2O_2 at 25 °C for 30 min in darkness or under illumination with weak light. Immunoblots with anti-D1 are shown. (a) Effects of ferricyanide; (b) effects of 1,4-benzoquinones; (c) effects of formate and bicarbonate. In the experiments in (c), the incubation and treatment were performed at pH 6.0 instead of pH 6.5 in order to reduce the level of bicarbonate in the medium and enhance binding of formate to non-heme iron (e.g., Stemler & Govindjee, 1973). (1) No addition; (2) 5 mM potassium ferricyanide; (3) 1 mM phenyl-BQ; (4) 1 mM 2,6-dichloro-BQ; (5) 1 mM tetramethyl-BQ; (6) 50 mM sodium formate; (7) 20 mM sodium bicarbonate. C, D, and L denote the same as those in Figure 4.

dichloro-BQs might be ascribable to oxidation of the non-heme iron in a reduction-induced oxidation mechanism that occurs with these quinones but not with tetramethyl-BQ (Diner & Petrouleas, 1987).

From the above observations, we conclude that the non-heme iron in the Fe(II) state plays a central role in the generation of toxic oxygen radicals in a reaction with H_2O_2. This conclusion agrees with the observation in Figure 5 that treatment with H_2O_2 did not cause any damage in the reaction center complexes that lack the non-heme iron (Satoh, 1992). The differences in the extent of damage in thylakoids and PSII membranes with and without the Mn cluster (Figure 5) might reflect the intactness of the acceptor side around the iron.

Figure 6c shows the effects of bicarbonate and formate, which are known to coordinate to the non-heme iron and to modify its coordination state and midpoint potential (Diner & Petrouleas, 1987, 1990). Both bicarbonate and formate did not substantially affect the overall pattern of damage to the D1 protein but slightly altered it in a complicated manner. During treatment with H_2O_2 in darkness, formate was basically ineffective, while bicarbonate suppressed the cross-linking reactions and concomitantly increased the levels of the fragments of 22 and 16 kDa. When the treatment was performed under illumination, formate slightly enhanced the fragmentation while bicarbonate was ineffective. These observations cannot be fully explained at present, but they indicate that the modification of non-heme iron by bicarbonate and formate does not affect the generation of oxygen radicals but alters the accessibility of the radicals to individual target sites.

DISCUSSION

Damage to Proteins by Treatment with H_2O_2. We have previously demonstrated that the damage to the D1 protein in isolated PSII subcomplexes under photoinhibitory illumination is caused by the direct action of active oxygen species generated in PSII under illumination (Miyao, 1994). The present study showed that treatment of PSII membranes with exogenous H_2O_2 in darkness damages proteins in almost the same way as photoinhibitory illumination (Figures 1, 3). The D1 protein and, to a lesser extent, the D2 protein were degraded to specific fragments, and the cross-linked products were generated. A marked difference from photoinhibitory illumination was that damage was restricted to proteins of the PSII reaction center in the case of treatment with H_2O_2.

The cleavage of the D1 protein caused by treatment with H_2O_2 was also observed in isolated core complexes (Figure 5) in which the putative serine-type protease that degrades the D1 protein is absent (Miyao, 1994). In addition, irreversible inhibitors of serine-type proteases did not suppress the cleavage of the D1 protein in PSII membranes (Figure 2b). Therefore, it is likely that protease activity does not participate in the cleavage by treatment with H_2O_2.

It is suggested that the damage to proteins was caused by toxic oxygen radicals generated by non-heme iron in the Fe(II) state in a reaction with H_2O_2. The involvement of the iron in generation of oxygen radicals and degradation of the D1 protein under illumination has been proposed by Sopory et al. (1990). In the case of treatment with H_2O_2, concentrations of H_2O_2 in the millimolar range were required to cause detectable damage in PSII membranes (Figure 1). Such a high concentration of H_2O_2 does not accumulate in the stroma under physiological conditions (Asada, 1994). However, the critical factor probably is the local concentration of H_2O_2 around the non-heme iron. The damage could occur *in vivo*, if the local concentration of H_2O_2 around the iron increases sufficiently.

The non-heme iron is known to be oxidized by H_2O_2 from the Fe(II) to the Fe(III) state (Diner & Petrouleas, 1987). Therefore, it is likely that the oxygen radicals are generated by a mechanism similar to the Fenton reaction. Damage to proteins caused by H_2O_2 in the presence of free metal ions has been well documented (Stadtman, 1993). According to Stadtman (1993), metal ions are bound to proteins and generate hydroxyl radicals via the Fenton reaction. These radicals preferentially oxidize amino acid residues at metal-binding sites, such as His, Arg, and Pro residues, and lead to cleavage of peptide bonds or cross-linking reactions. If this mechanism corresponds to the mechanism of damage to proteins observed in this study, it would be expected that the damage would occur in close proximity to the non-heme iron.

From the homology between PSII and the reaction center of purple photosynthetic bacteria, the non-heme iron is considered to be coordinated to four His residues, His215 and His272 of the D1 protein and His215 and His269 of the

D2 protein (Trebst, 1986; Michel & Deisenhofer, 1988). In addition, the residues that participate in binding of Q_B, namely, Phe255 and Ser264 of the D1 protein, are suggested to be located in the vicinity of the iron (e.g., Diner et al., 1991). Cleavage region I of the D1 protein includes His272, which participates in the binding of iron, and Phe255 and Ser264, which participate in the binding of Q_B, and these residues are the most probable candidates for the cleavage sites. In region II, cleavage seems to occur in close proximity to the Q_B-binding site, since phenyl- and dichloro-BQs, which bind to this site, specifically suppressed the cleavage in this region (Figure 6b). Accordingly, the most probable target might be His215, which participates in binding of Q_B and non-heme iron. Further studies are required to identify the cleavage sites precisely.

The shift in the cleavage site of the D1 protein caused by illumination during treatment with H_2O_2 is of particular interest. Each of the two cleavage regions contained 2 different cleavage sites that were separated by several to 10 amino acid residues. One site was preferentially cleaved in darkness and another site under illumination. This effect of illumination might reflect some interaction of the iron with Q_A^- and/or Q_B^-, or the occupancy of the Q_B-binding site by a plastoquinone molecule.

Participation of H_2O_2 in Degradation of the D1 Protein under Illumination. From the effects of catalase, H_2O_2 has been proposed to participate in photoinhibition of PSII in both thylakoids (Barényi & Krause, 1985; Bradley et al., 1991) and PSII membranes (Šetlík et al., 1990) and also in the degradation of the D1 protein in isolated PSII subcomplexes under photoinhibitory illumination (Miyao, 1994).

H_2O_2 is produced during illumination by autooxidation at the acceptor side of PSII (Schröder & Åkerlund, 1990). Although not yet proven, it has been proposed that a plastosemiquinone radical in the Q_B-binding site could generate H_2O_2 via a superoxide anion (Kyle, 1987). H_2O_2 can also be produced from water at the donor side (Ananyev et al., 1992). In this case, however, H_2O_2 remains bound to the donor side unless the environment of the Mn cluster is perturbed (Ananyev et al., 1992), and it is unlikely that this bound H_2O_2 reacts with the non-heme iron at the acceptor side. With respect to the H_2O_2 at the acceptor side, the yield appears to be high enough to cause detectable damage to the D1 protein under photoinhibitory illumination. According to Schröder and Åkerlund (1990), strong illumination of PSII membranes for 10 min yields about 80 molecules of H_2O_2 per PSII. Under illumination with weak light, H_2O_2 is likely to be produced at the acceptor side, since Klimov et al. (1993) reported that illumination with 10 saturating flashes led to production of H_2O_2 at a yield of about 0.01 molecule per PSII per flash. The yield of H_2O_2 under illumination with weak light would be very low. Even so, it is quite possible that H_2O_2 reacts with the non-heme iron and generates oxygen radicals, so far as H_2O_2 is produced specifically in the immediate vicinity of the iron. From these considerations, we suggest that H_2O_2 participates in the degradation of the D1 protein during illumination not only with strong photoinhibitory light but also with weak light. If H_2O_2 is produced in the Q_B-binding site, as proposed by Kyle (1987), it would selectively and specifically degrade the D1 protein.

It has been proposed that $^1O_2$ produced by the reaction of the triplet state of P680 plays an essential role in degrading the D1 protein under illumination (Vass et al., 1992; Aro et al., 1993; Hideg et al., 1994). In fact, scavengers of $^1O_2$ dramatically suppress the degradation in PSII subcomplexes under photoinhibitory illumination (Mishra et al., 1994; Miyao, 1994), and exposure of PSII subcomplexes to $^1O_2$, generated by photosensitizing reaction of added rose bengal, damages the D1 protein in the same way as observed under photoinhibitory illumination (Mishra & Ghanotakis, 1994). Thus, $^1O_2$ itself can degrade the D1 protein at specific sites, and it undoubtedly participates in the degradation under strong photoinhibitory illumination. However, it is unclear whether $^1O_2$ is also involved under illumination with weak light, since neither scavengers nor stabilizers of $^1O_2$ affect the half-life of the D1 protein under illumination with weak light *in vivo* (Sopory et al., 1990).

The cleavage of the D1 protein under illumination occurs at some distance from P680, the site of generation of $^1O_2$. The cleavage site is located on the outer surface of PSII on the stromal side of the thylakoid membrane, while P680 is on the opposite side. According to Telfer et al. (1994), $^1O_2$ generated by the triplet state of P680 under strong illumination is initially inaccessible to exogenous scavengers and preferentially quenched by components inside the reaction center, but after prolonged illumination, it escapes to the outer aqueous phase and becomes accessible to scavengers and is able to be quenched. Taking these and the suppressive effects of $^1O_2$ scavengers into consideration, we can assume that $^1O_2$ must accumulate to quite a high level to reach the cleavage site under photoinhibitory illumination. The proposed accumulation of a significant amount of $^1O_2$ is supported by the observation that not only the D1 protein but also other intrinsic proteins are damaged under photoinhibitory illumination (Figure 3; Nedbal et al., 1990; Miyao, 1994). On the other hand, under weak-light illumination, $^1O_2$ appears not to accumulate to a level sufficient for degradation of the D1 protein, since a scavenger had no suppressive effects (Sopory et al., 1990). From these considerations, we can postulate that $^1O_2$ does not directly participate in cleavage of the D1 protein under weak-light illumination. At present, it is unclear whether $^1O_2$ can be generated in PSII under weak-light illumination. If generated, $^1O_2$ might be efficiently quenched by β-carotene, as proposed previously (Asada, 1994; Macpherson et al., 1993), or it might participate in the impairment of PSII that precedes degradation of the D1 protein.

Treatment with H_2O_2, as described herein, exposure to $^1O_2$ (Mishra & Ghanotakis, 1994), and photoinhibitory illumination (Aro et al., 1993) all damage the D1 protein in almost the same way. Although the precise cleavage sites have not yet been determined, the same amino acid residues appear to be attacked. In general, $^1O_2$ has high reactivity toward specific amino acid residues such as His, Trp, Tyr, Met, and Cys residues (Foote, 1976), and hydroxyl radical toward Trp, Tyr, and Phe residues (Stadtman, 1993). The D1 protein contains many residues susceptible to attack by active oxygen species, but cleavage occurs only at particular sites. This suggests that the D1 protein has regions that are specifically susceptible to cleavage by active oxygen species. Some characteristic sequences, such as the PEST-like region on the N-terminal side of residue 238 (Greenberg et al., 1987) or the QEEET motif in residues 241−245 (Tyystjärve et al., 1994), might contribute to this susceptibility. These intrinsic features of the D1 protein, together with the site-specific

generation of active oxygen species as occurs in the case of treatment with H_2O_2, could be responsible for the selective and specific degradation of the D1 protein under illumination.

We propose that the D1 protein can be cleaved to specific fragments solely by the action of active oxygen species generated inside PSII under illumination. However, it should be noted that active oxygen by itself cannot degrade proteins to small peptides or amino acids. In the case of treatment with H_2O_2, the fragments of the D1 protein in thylakoids were stable for at least several hours when kept in darkness at 25 °C after treatment (data not shown). We presume that the primary cleavage of the D1 protein is performed by active oxygen species but that the complete degradation requires additional enzymatic processes.

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