Degradation of the D1 Protein of Photosystem II under Illumination *in Vivo*: Two Different Pathways Involving Cleavage or Intermolecular Cross-Linking†

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ABSTRACT: The D1 protein of the photosystem II reaction center turns over the most rapidly of all the proteins of the thylakoid membrane under illumination *in vivo*. *In vitro*, the D1 protein sustained cleavage in a surface-exposed loop (DE loop) or cross-linking with another reaction center protein, the D2 protein or cytochrome b_{559}, under illumination. We found that the D1 protein was damaged in essentially the same way *in vivo*, although the resultant fragments and cross-linked adducts barely accumulated due to digestion by proteases. *In vitro* studies detected a novel stromal protease(s) that digested the adducts but not the monomeric D1 protein. These observations suggest that, in addition to cleavage, the cross-linking reactions themselves are processes involved in complete degradation of the D1 protein *in vivo*. Peptide mapping experiments located the cross-linking sites with the D2 protein among residues 226−244, which includes the cross-linking site with cytochrome b_{559} [Barbato, R., *et al*. (1995) *J. Biol. Chem. 270*, 24032−24037], in the N-terminal part of the DE loop, while N-terminal amino acid sequencing of the fragment located the cleavage site around residue 260 in the C-terminal part of the loop. We propose a model explaining the occurrence of simultaneous cleavage and cross-linking and discuss the mechanisms of complete degradation of the D1 protein *in vivo*.

Proteins within living cells are subjected to a variety of damage and may be modified covalently or noncovalently. Noncovalent damage, such as alteration of the three-dimensional structure under heat stress conditions, can be reversed by various molecular chaperones (*1*). Covalent damage, which involves oxidation of amino acid residues and the resultant cleavage and cross-linking of proteins, is mediated by active oxygen species under oxidative stress conditions (*2*). Some covalent modifications, such as methionine oxidation and proline isomerization, can be reversed by specific enzymes (*3*). However, severely damaged proteins are selectively degraded by proteolytic systems to prevent these proteins and the resultant large protein aggregates from causing harmful effects (*2*, *4*). The proteasome in eukaryotes (*2*) and ATP-dependent proteases such as Clp, FtsH, Lon, and DegP in bacteria (*5*) participate in proteolysis of damaged proteins.

In plants, such quality control systems for proteins are more essential than in animals and bacteria since solar light energy, a substrate of photosynthesis, can generate active oxygen species as byproducts of photochemical reactions when in excess of demand for photosynthesis (*6*, *7*). The mechanisms for the removal of damaged proteins in the chloroplast, the site of photosynthesis, are not fully understood, although the chloroplast homologues of bacterial Clp, FtsH, and DegP proteases have recently been identified (*8*, *9*). The D1 protein of photosystem II (PSII)[1] has an intriguing feature, in that it undergoes the most rapid turnover under illumination of all of the proteins in the thylakoid membrane of the chloroplast (*10*). Thus, it is expected that there would be a specific quality control system for this protein. The mechanism of its turnover, however, is not completely understood, although it is generally accepted that active oxygen species generated inside PSII trigger the turnover (*11*−*14*).

The D1 protein is an intrinsic membrane protein of 32 kDa that has five membrane-spanning helixes. Together with the homologous D2 protein, it forms the reaction center complex, which is a core part of PSII consisting of more than 25 protein species (*15*). Among other protein complexes in the thylakoid membrane, PSII is the most susceptible to damage under illumination; PSII electron transport is first inactivated, and then the D1 protein, and also the D2 protein to a lesser extent, are selectively degraded, leading to the disassembly of PSII (*12*, *13*, *16*). These events are termed photoinhibition of PSII (*14*). The photoinhibition occurs even

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[1] Abbreviations: Chl, chlorophyll; Cyt b_{559} α, α-subunit of cytochrome b_{559}; DCIP, 2,6-dichlorophenolindophenol; F_0, F_v, and F_m, minimal, variable, and maximal chlorophyll fluorescence, respectively; Lys-EP, lysylendopeptidase; PAGE, polyacrylamide gel electrophoresis; PSII, photosystem II; PVDF, polyvinylidene difluoride; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; SDS, sodium dodecyl sulfate.

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under illumination conditions that are optimal for plant growth, although the level of PSII is kept constant by *de novo* synthesis of the D1 protein and reassembly of PSII (*14*).

The mechanisms of D1 protein degradation have been intensively studied *in vitro*, especially using isolated PSII preparations (*12*, *13*). Under photoinhibitory illumination of PSII preparations, a fraction of the D1 protein is cleaved at specific sites and another fraction is covalently cross-linked to other proteins of PSII. Unless the oxygen-evolving machinery of PSII is impaired, the cleavage occurs in two different regions of the protein, one in the loop that connects membrane-spanning helixes D and E (DE loop) on the stromal side of the thylakoid membrane and the other inside or in the vicinity of helix D (*17*−*19*). The cross-linking of the D1 protein occurs with either the D2 protein or the α-subunit of cytochrome b_{559} (Cyt b_{559} α) to form the D1−D2 heterodimer or the adduct of 41 kDa (*20*, *21*). Whether the D1 protein is cleaved by direct action of active oxygen species (*19*, *22*−*24*) or enzymatically by specific protease(s) (*8*, *25*) is still subject to controversy. In contrast, cross-linking reactions are considered chemical processes induced by active oxygen species (*21*).

Although studies with PSII preparations have expanded our understanding of how the initial damage to the D1 protein occurs, the mechanisms of complete D1 protein degradation remain uncertain since fragments and cross-linked adducts are not further degraded in PSII preparations. In addition, the question of whether the initial damage that occurs *in vivo* would be the same as that observed *in vitro* has arisen (*26*).

In this study, damage to the D1 protein during photoinhibitory illumination was compared in three materials, namely, thylakoids, intact chloroplasts, and leaf disks. We found that initial damage to the D1 protein under illumination was essentially the same in the three materials, whereas further degradation of fragments and adducts occurred only in intact systems (chloroplasts and leaf disks) but not in thylakoids. We also found that the cross-linking reaction of the D1 protein is a process involved in the complete degradation of the D1 protein *in vivo*. By comparing the sites of initial damage in the D1 protein, we propose a model explaining simultaneous cleavage and cross-linking.

EXPERIMENTAL PROCEDURES

Sample Preparations. Unless stated otherwise, samples were prepared from spinach plants, which had been purchased from a local market and kept in darkness at 4 °C for more than 12 h. Intact chloroplasts were isolated from the spinach leaves by Percoll density gradient centrifugation, treated with thermolysin, and then repurified by Percoll density gradient centrifugation (*27*). Thylakoids and stromal proteins were isolated from the intact chloroplasts by hypotonic rupture followed by differential centrifugation (*27*). The thylakoids were illuminated with weak light in the presence of ATP to phosphorylate thylakoid proteins (*27*). This treatment causes phosphorylation of ∼90% of the D1 protein (*27*). The stromal proteins were concentrated with a Centriprep-10 (Amicon, MA) and dialyzed against 10 mM Hepes-NaOH (pH 7.8) for 4 h. PSII complexes depleted of the major light-harvesting complexes and PSII core complexes were prepared from spinach and wheat as described previously (*23*). All procedures were performed under dim light at 0−4 °C. The intact chloroplast preparations were used for experiments within 30 min of isolation, whereas other preparations were kept frozen in liquid nitrogen until they were used. Chl was determined by the method of Arnon (*28*).

Treatments with Photoinhibitory Light and with Active Oxygen Species. Thylakoids were suspended in 10 mM NaCl, 5 mM $MgCl_2$, 0.4 M sucrose, and 50 mM Hepes-NaOH (pH 7.8) at 0.2 mg of Chl/mL. Intact chloroplasts were suspended in 1 mM $MnCl_2$, 1 mM $MgCl_2$, 1 mM EDTA, 0.33 M sorbitol, and 30 mM Hepes-KOH (pH 7.8) at 0.2 mg of Chl/mL. After incubation in darkness at 25 °C for 5 min, the suspension was illuminated with white light at 25 °C (*23*). The light intensities were 1000 and 2500 μE m^{-2} s^{-1} for thylakoids and chloroplasts, respectively. After illumination, a portion of each suspension was withdrawn and either subjected to an assay of PSII activity or prepared for SDS−PAGE as follows. The suspension of illuminated thylakoids was supplemented with $^1/_4$ volume of 10% (w/v) SDS, 25% (v/v) 2-mercaptoethanol, and 312.5 mM Tris-HCl (pH 6.8; 5× solubilization buffer) and immediately frozen in liquid nitrogen. The suspension of illuminated chloroplasts was passed through 40% (v/v) Percoll containing 0.33 M sorbitol and 30 mM Hepes-KOH (pH 7.8) by centrifugation at 17000*g* for 10 s. The resultant pellet, consisting of chloroplasts that retained their envelopes, was washed once with and resuspended in the medium used for photoinhibitory illumination, solubilized for SDS−PAGE as outlined above, and frozen in liquid nitrogen. PSII complexes were illuminated at 100 μE m^{-2} s^{-1} and 25 °C (*23*).

Photoinhibitory illumination of leaf disks was performed essentially as described by Rintamäki *et al*. (*29*). The petiole of a mature spinach leaf was immersed in either water or 2 mM lincomycin (Sigma, St. Louis, MO) and incubated at room temperature under dim light and a gentle air stream in a fume hood for more than 4 h. Disks (2.5 cm in diameter) were cut out from the leaf, floated on distilled water in a Petri dish placed in a water bath, and illuminated from above with white light at 2500 μE m^{-2} s^{-1}. Unless stated otherwise, the leaf temperature was kept at 25 °C. After illumination, the leaf disks were subjected to measurement of Chl fluorescence or immediately frozen in liquid nitrogen. For SDS−PAGE, thylakoids were isolated from the frozen leaf disks as follows. The disks were first broken into small pieces in liquid nitrogen and then thoroughly homogenized on ice with a mortar and pestle in a medium containing 10 μM leupeptin (Sigma), 10 mM ascorbic acid, 10 mM NaF, 10% (w/v) glycerol, 1% (w/v) polyvinylpyrrolidone (average molecular weight of 40 000; Sigma), and 50 mM Hepes-NaOH (pH 7.4). The homogenate was subjected to a brief centrifugation, which was stopped when the speed reached 680*g*. The resultant supernatant was centrifuged at 17000*g* for 5 min, and thylakoids obtained as a pellet were washed once with 10 mM NaF, 10 mM NaCl, 5 mM $MgCl_2$, 0.1 M sucrose, and 50 mM Hepes-NaOH (pH 7.8), resuspended in 10 mM NaCl, 5 mM $MgCl_2$, 0.8 M sucrose, and 50 mM Hepes-NaOH (pH 7.8), solubilized for SDS−PAGE as described above, and immediately frozen in liquid nitrogen.

For exposure to active oxygen species, samples were incubated with designated concentrations of H_2O_2 in darkness at 25 °C for 30 min, or exposed to $^1O_2$ by illumination with

green light in the presence of 10 μM rose bengal at 25 °C for 30 min, essentially as described previously (*19*, *24*).

Assay of PSII Activity. The photoreduction of DCIP was assessed spectrophotometrically (*19*). Activities of DCIP photoreduction of thylakoids and chloroplasts before illumination were 220−230 μmol of DCIP reduced (mg of Chl)$^{-1}$ h^{-1}. Chl fluorescence was measured at 25 °C using a modulated fluorometer (PAM101/102/103, Walz). Before measurements were taken, leaf samples were kept in darkness at 25 °C for 30 min. F_0 was measured with modulated weak red light (1.6 kHz, 650 nm), and F_m was induced with a saturating white light pulse (6500 μE m^{-2} s^{-1}, 0.3 s). F_v was equal to $F_m - F_0$. The F_v/F_m values of thylakoids and leaf disks before illumination were 0.70 and 0.80, respectively.

Protein Analyses. SDS−PAGE and subsequent immunoblotting were performed as described previously (*27*), with the exception that the pH of the separation gel buffer was changed from 8.85 to 8.95 to improve the resolution of small peptides. For detection of cross-linked adducts and fragments of the D1 protein by immunoblotting, thylakoid and chloroplast samples corresponding to 2 μg of Chl were used for SDS−PAGE. For detection of the D1 protein of 32 kDa, the sample amounts were decreased to $^1/_{10}$. Seven different antisera were used: anti-D1$_{Total}$ raised against the entire D1 protein of spinach; anti-D1$_{AB}$, anti-D1$_{DE}$, anti-D1$_{DE1}$, anti-D1$_{DE2}$, and anti-D1$_C$ raised against synthetic polypeptides that corresponded to residues 57−76, 225−249, 227−235, 239−247, and 333−344 of the D1 protein of spinach, respectively; and anti-D2$_{DE}$ raised against a synthetic polypeptide that corresponded to residues 220−242 of the D2 protein of spinach. Anti-D1$_{Total}$ was a generous gift of M. Ikeuchi (The University of Tokyo, Tokyo, Japan). Anti-D1$_{DE1}$, anti-D1$_{DE2}$, and anti-D1$_C$ were generous gifts of T. Ono (The Institute of Physical and Chemical Research). The intensities of immunoreacted bands were quantified in terms of peak areas on densitograms recorded with a TLC scanner (CS-9300PC, Shimadzu). For N-terminal amino acid sequencing, polypeptides were separated by SDS−PAGE using the separation gel containing 18% polyacrylamide and 7 M urea, electroblotted onto a PVDF membrane (Millipore), and stained with Coomassie brilliant blue R-250. The stained bands were cut out and subjected to sequencing using a gas-phase protein sequencer (477A, Applied Biosystems, Foster City, CA).

Protein Isolation and Peptide Mapping. The D1 and D2 proteins and the heterodimer were isolated from thylakoids or PSII complexes by SDS−PAGE as follows. Samples were subjected to SDS−PAGE, and the gel was stained with 0.15% (w/v) Coomassie brilliant blue R-250 and 40% (v/v) methanol for 30 min and destained with 3 mM Na_2CO_3, 10 mM $NaHCO_3$, and 20% (v/v) methanol for 30 min and subsequently with distilled water for 2 h. The protein bands were cut out from the gel and incubated with 0.1% (w/v) SDS, 1 mM EDTA, and 62.5 mM Tris-HCl (pH 6.8) at 4 °C for 8 h.

To examine proteolysis by the stromal proteins, the gel slices containing proteins were subjected to the second SDS−PAGE together with 30 μg of the stromal proteins. Electrophoresis was stopped for 30 min so that samples were reacted with the stromal proteins inside the stacking gel. The D1 proteins used in these experiments were isolated from phosphorylated thylakoids before and after illumination at 1000 μE m^{-2} s^{-1} for 30 min and the heterodimer from PSII complexes after illumination at 100 μE m^{-2} s^{-1} for 30 min.

For peptide mapping, proteins isolated from wheat PSII complexes, which had been illuminated at 100 μE m^{-2} s^{-1} for 30 min, were electroeluted from the gel slices into 0.1% (w/v) SDS, 192 mM glycine, and 25 mM Tris at 4 °C and 200 V for 2 h using a gel eluter (Centrilutor; Amicon), concentrated with a Centricon-10 (Amicon), and finally dissolved in 0.1% (w/v) SDS, 1 mM EDTA, and 62.5 mM Tris-HCl (pH 7.0) at concentrations that corresponded to 0.6 and 1.2 mg of Chl/mL of the original PSII complexes for the D1 and D2 proteins and the heterodimer, respectively. They were supplemented with $^1/_{10}$ volume of either lysyl-endopeptidase (Lys-EP) from *Achromobacter lyticus* (10 units/mL, Wako) or V8 protease from *Staphylococcus aureus* (20 units/mL, Roche) and incubated at 30 °C for 30 min. Digestion was terminated by sequential addition of $^1/_{100}$ volume of 10 mM 4-amidinophenylmethanesulfonyl fluoride (APMSF, Sigma) and $^1/_4$ volume of 5× solubilization buffer. Samples were analyzed by SDS−PAGE and immunoblotting.

RESULTS

Comparison of Damage to the D1 Protein under Photoinhibitory Illumination in Vivo and in Vitro. Figure 1 compares damage to the D1 protein under photoinhibitiory illumination in thylakoids, intact chloroplasts, and leaf disks from spinach. We ensured that the thylakoid and intact chloroplast samples used in this study were free of contamination by proteases from other organelles by using a thermolysin treatment during purification procedures. Thylakoids were subjected to a phosphorylation treatment prior to illumination to phosphorylate the D1 protein, as occurs under photoinhibitory illumination *in vivo* (*29*). Leaf disks were obtained from leaves that had been incubated with lincomycin, an inhibitor of protein synthesis in the chloroplast, to enhance inactivation of PSII (Figure 1A; *26*). They were illuminated with strong white light at 25 °C. Under our illumination conditions, all proteins except the D1 and D2 proteins were totally unaffected even when PSII activity was decreased to ∼20% of original levels in all three samples, as determined from polypeptide profiles after staining with Coomassie blue (data not shown).

Damage to the D1 protein was compared under conditions in which the extent of inactivation of PSII was comparable in the thylakoid, chloroplast, and leaf disk samples (Figure 1B,C). One major difference between *in vivo* and *in vitro* was observed in the level of the D1 protein of 32 kDa (upper panels of Figure 1B). When PSII activity had decreased to 20% of its value prior to illumination, the level of the D1 protein (D1 + D1*) was decreased to ∼25% in leaf disks, but only to 80−90% in thylakoids and chloroplasts. Immunoblotting for detection of fragments and cross-linked adducts (lower panels of part B and part C of Figure 1) indicated that the fragments and cross-linked adducts of the D1 protein were generated in almost the same way in all three materials, although their levels were highest in thylakoids and greatly decreased in chloroplasts and leaf disks. Fragments of the D1 protein derived from cleavage in the DE loop (the 22 kDa N-terminal fragment and the <10 kDa C-terminal fragments) and those derived from cleavage inside

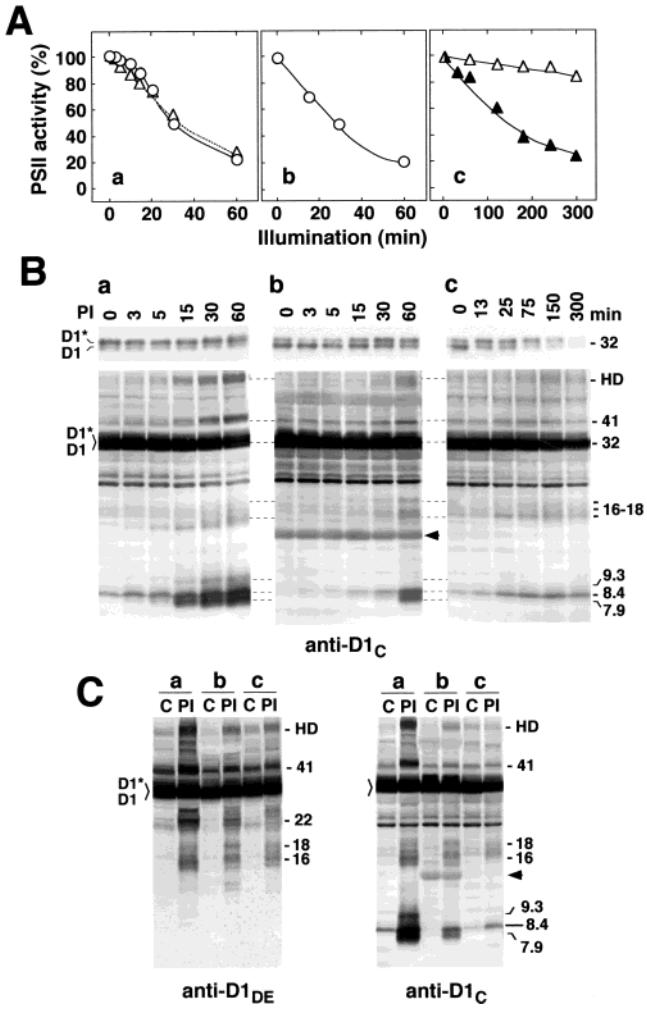

FIGURE 1: Inactivation of PSII and damage to the D1 protein under photoinhibitory illumination. Phosphorylated thylakoids (a), intact chloroplasts (b), and leaf disks (c) from spinach were illuminated at 25 °C with white light at 1000, 2500, and 2500 μE m^{-2} s^{-1}, respectively. (A) Inactivation of PSII. PSII activity was determined as the activity of DCIP photoreduction (○) or the F_v/F_m value of Chl fluorescence (△ and ▲). Leaf disks were obtained from leaves which had been incubated with (▲) or without (△) 2 mM lincomycin. (B and C) Damage to the D1 protein. In these experiments, leaf disks from lincomycin-treated leaves were used. In part C, control samples kept in darkness (C) and illuminated samples (PI), in which PSII activities were decreased to ~20% of the control, were compared. Sample amounts used for SDS−PAGE were optimized for detection of fragments and cross-linked adducts, except for the immunoblots shown in the upper panels of part B, which were optimized for quantification of the D1 protein band of 32 kDa. D1, D1*, and HD denote the unphosphorylated and phosphorylated forms of the D1 protein and the D1−D2 heterodimer, respectively. Arrowheads indicate a band of the small subunit of Rubisco, which cross-reacted with anti-D1$_C$. Molecular masses of fragments were estimated from their mobilities on the gel, with the intrinsic proteins of PSII used as molecular mass markers.

or in the vicinity of helix D (the 16−18 kDa fragments) were detected, as were two different cross-linked adducts (the heterodimer and the 41 kDa adduct). These results indicate that the initial damage to the D1 protein occurs in almost the same way *in vivo* and *in vitro*.

It has been suggested previously that the initial cleavage of the D1 protein *in vivo* occurs on the N-terminal side of the DE loop, probably in the CD loop, to generate a 20 kDa

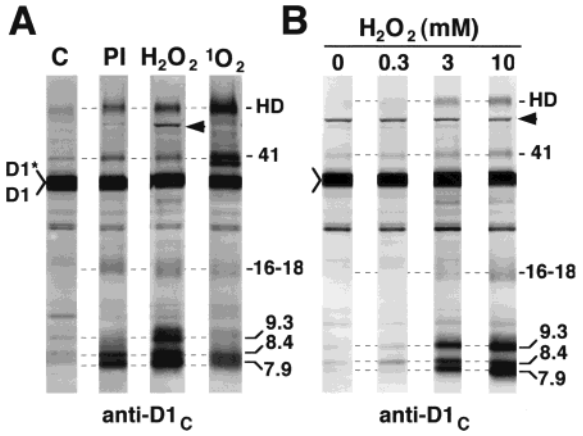

FIGURE 2: Comparison of damage to the D1 protein caused by photoinhibitory illumination and exposure to active oxygen species in phosphorylated thylakoids. (A) Samples were kept in darkness for 60 min (C), illuminated at 1000 μE m^{-2} s^{-1} for 60 min (PI), incubated with 10 mM H_2O_2 in darkness for 30 min (H_2O_2), or exposed to $^1O_2$ generated by the photosensitizing reaction of 10 μM rose bengal ($^1O_2$). (B) Samples were incubated with the designated concentrations of H_2O_2 in darkness for 30 min. Arrowheads indicate a band of catalase, which cross-reacted with anti-D1$_C$, used to stop the reaction of H_2O_2.

C-terminal fragment, and that cleavage in the DE loop occurs only under illumination with extremely strong light (*26*). Although the leaf disks were exposed to photoinhibitory light (2500 μE m^{-2} s^{-1}) that was stronger than full sunlight, we found that the composition of fragments generated in leaf disks was unchanged when they were illuminated at 1000 μE m^{-2} s^{-1} (data not shown). We consider that the 20 kDa fragment reported in the previous study corresponds to one of the 16−18 kDa fragments and that the discrepancy in cleavage sites *in vivo* might be able to be ascribed to the difference in cross-reactivity of antibodies used to detect fragments of the D1 protein.

We observed differences in relative levels of the small C-terminal fragments of the D1 protein between *in vivo* and *in vitro* (Figure 1). In leaf disks, only the 8.4 kDa fragment was generated, whereas 9.3 and 7.9 kDa fragments were also generated in thylakoids and chloroplasts. The 8.4 kDa fragment was more abundant in more intact systems, and even *in vitro*, it was generated predominantly in the early stage of illumination. It is thus likely that cleavage in the DE loop occurs essentially at a single site both *in vivo* and *in vitro*. It is noted that a slight modification of the SDS−PAGE conditions enabled us to detect the 8.4 kDa fragment, which could not be separated from the 9.3 kDa fragment in our previous study with thylakoids (*27*). In PSII preparations, in contrast, only the 9.3 and 7.9 kDa fragments were detected under the SDS−PAGE conditions in this study (data not shown).

It has been demonstrated using PSII preparations that exposure to H_2O_2 or $^1O_2$, each of which is generated inside PSII under illumination (*30*, *31*), damages the D1 protein in almost the same way as photoinhibitory illumination (*19*, *22*, *24*). We found that this is also the case in thylakoids (Figure 2). The 8.4 kDa fragment specific to the intact system was also generated. This fragment was generated predominantly at 0.3 mM H_2O_2, whereas the 9.3 and 7.9 kDa fragments accumulated significantly at higher concentrations of H_2O_2.

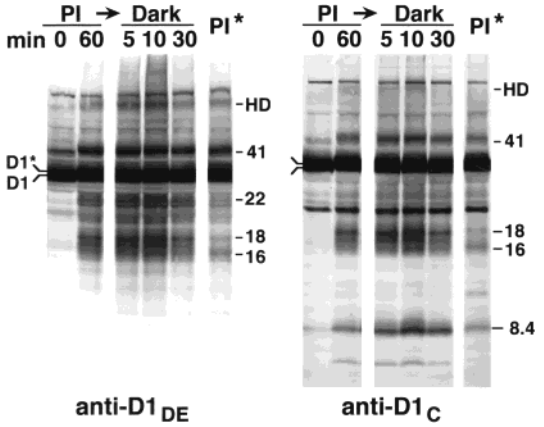

FIGURE 3: Damage to the D1 protein after photoinhibitory illumination at 2 °C and subsequent incubation in darkness at 25 °C. Leaf disks from lincomycin-treated leaves were illuminated at 2500 μE m^{-2} s^{-1} and 2 °C for 60 min (PI), then transferred to darkness and 25 °C, and further incubated for 5, 10, and 30 min (Dark). PI* denotes a sample illuminated at 25 °C for 60 min for comparison. The F_v/F_m values after illumination at 2 and 25 °C were both ~70% of the control.

The very low level of fragments and adducts of the D1 protein observed in chloroplasts and leaf disks (Figure 1) can be explained in two ways. (1) Cleavage and cross-linking reactions rarely occurred in these materials. (2) Cleavage and cross-linking reactions did occur, but the resultant fragments and adducts were rapidly degraded and so were not able to accumulate. To clarify this point, leaf disks were subjected to photoinhibitory illumination at 2 °C, a temperature at which protease activities were greatly suppressed. As seen in Figure 3, both fragments and adducts did accumulate in leaf disks even under illumination at 2 °C. They were identical in size to those observed at 25 °C, and their levels were even higher than at 25 °C. When leaf disks illuminated at 2 °C were incubated in darkness at 25 °C, the levels of fragments and adducts increased slightly within 10 min and then subsequently decreased. They finally disappeared after prolonged incubation for 1−2 h (data not shown). These results support the latter possibility and suggest that the fragments and adducts were generated at 25 °C *in vivo* but subsequently disappeared, probably through digestion by proteases.

Digestion of the Cross-Linked Adducts in Darkness. Since the levels of fragments and adducts were much lower in chloroplasts than in thylakoids (Figure 1), it is quite possible that the stromal protease(s) participates in further degradation of the fragments and adducts. This hypothesis was examined by incubating illuminated thylakoids with the stromal proteins in darkness (Figure 4A). As reported previously (*27*), dark incubation of illuminated thylakoids led to an increase in the levels of small C-terminal fragments of the D1 protein, even in the absence of the stromal proteins. The presence of stromal proteins did not affect the levels of fragments during incubation. In contrast, the levels of both the heterodimer and the 41 kDa adduct decreased with increasing amounts of the stromal proteins. Since the presence of bovine serum albumin had no effect on the levels of adducts, it is likely that a protease(s) present in the stroma participates in digestion of the adducts. The 32 kDa D1 protein could not be a substrate of this protease, since the level of the 32 kDa band of the protein was not significantly affected by incubation with the stromal proteins for up to 4 h.

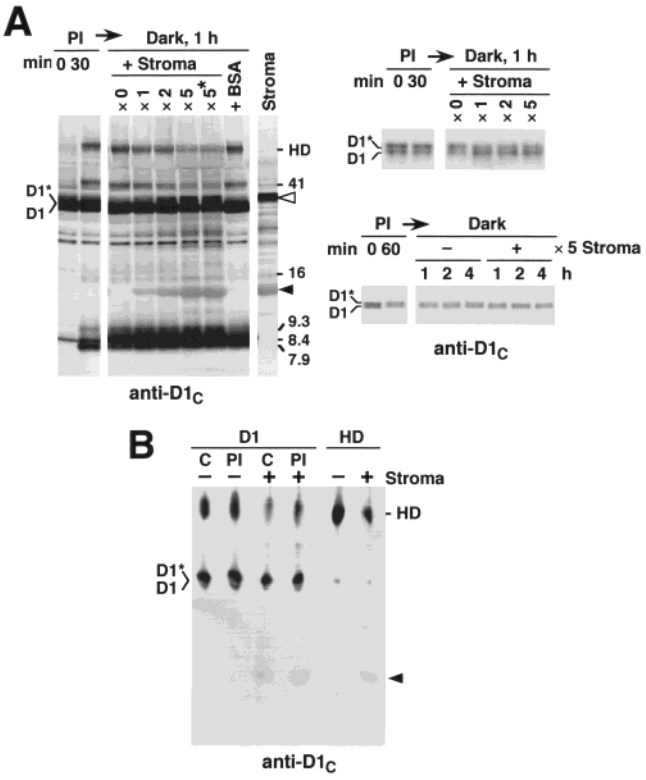

FIGURE 4: Degradation in darkness of the cross-linked adducts by the stromal proteins. (A) Illuminated thylakoids. Phosphorylated thylakoids were illuminated at 1000 μE m^{-2} s^{-1} and 25 °C for 30 or 60 min (PI) and then incubated in darkness at 25 °C for the designated times (Dark) in the presence or absence of the stromal proteins (+Stroma) or bovine serum albumin (+BSA, 1.8 mg/mL). ×1 corresponds to the amount of the stromal proteins equivalent to that in intact chloroplasts. An asterisk indicates that 5 mM ATP was included during the incubation. Stroma denotes isolated stromal proteins. Sample amounts used for SDS−PAGE were optimized for detection of fragments and cross-linked adducts in the left panel and for quantification of the 32 kDa D1 protein band in the right panels. (B) Isolated D1 protein and the heterodimer. The proteins were isolated by SDS−PAGE and incubated with the stromal proteins at 25 °C for 30 min inside the stacking gel of the second SDS−PAGE. The D1 proteins (D1) were obtained from phosphorylated thylakoids before (C) and after (PI) illumination, and the heterodimer (HD) was obtained from illuminated PSII complexes, in which no bands comigrated with the heterodimer during SDS−PAGE. Empty and filled arrowheads indicate an unidentified protein and the small subunit of Rubisco, respectively, which cross-reacted with anti-D1$_C$.

The presence of either ATP (Figure 4A, lane ×5*) or apyrase, which hydrolyzes ATP (not shown), did not affect the digestion of the adducts by the stromal proteins, an indication that the digestion does not require ATP. The effects of various protease inhibitors were also examined. We found that, even in control thylakoid samples, the presence of some serine-type protease inhibitors (Pefablock and phenylmethanesulfonyl fluoride) and a metalloprotease inhibitor (1,10-phenanthroline) selectively increased the level of the heterodimer in darkness (data not shown). Leupeptin and APMSF (serine-type), E64 (cysteine-type), and EDTA (metallo-type), which did not have such effects, did not affect the digestion of the adducts (data not shown). Thus, the protease(s) that digests cross-linked adducts would be novel.

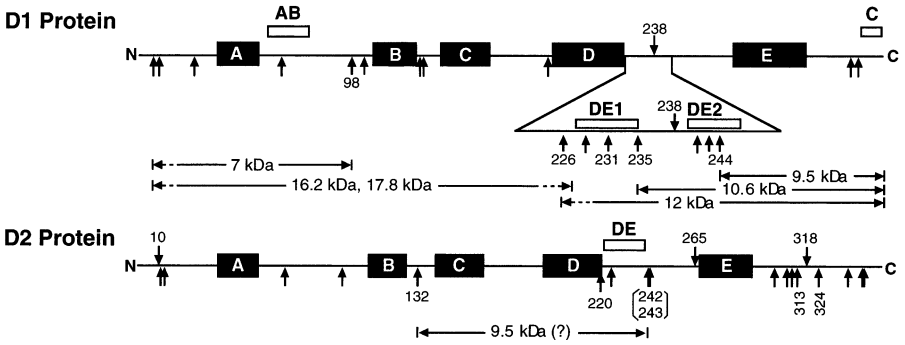

FIGURE 5: Location within the D1 and D2 proteins from wheat of the cleavage sites of proteases and the recognition sites of antibodies used for peptide mapping. The amino acid sequences of wheat proteins are from M. D. C. Barros and T. A. Dyer (unpublished data; see ref *32*). Filled boxes represent membrane-spanning helixes (*38*). Downward and upward arrows indicate the positions of lysine and glutamic acid residues, which are cleaved by *Achromobacter* Lys-EP and *Staphylococcus* V8 protease, respectively. Empty horizontal bars indicate the positions of the oligopeptides used for production of antibodies. The locations of fragments generated by digestion with V8 protease are also shown.

The digestion by the stromal proteins was also examined using the purified heterodimer (Figure 4B). In the absence of the stromal proteins, a small fraction of the D1 protein dissociated from the heterodimer. When the stromal proteins were present, the level of the heterodimer was reduced to $\sim^1/_3$ without any detectable degradation products, whereas the level of the dissociated D1 protein decreased only slightly. These results suggest that a protease(s) present in the stroma selectively digested the heterodimer but not the free D1 protein. This hypothesis was confirmed in experiments using the isolated D1 protein as a substrate. In the D1 protein preparation, a smeared band of 60−66 kDa was generated due to artificial cross-linking of two molecules of the D1 protein (the homodimer), which is often observed in the isolated D1 protein (*21*). Incubation of the D1 protein preparation, from either control or illuminated samples, with the stromal proteins greatly reduced the level of the homodimer. However, the level of the free D1 protein was reduced only slightly. It is thus likely that the protease(s) has a high reactivity toward cross-linked adducts. It is expected that the 41 kDa adduct could also be a substrate of this protease(s). We could not confirm this hypothesis because of a low yield of the 41 kDa adduct. This protease(s) remained active even in the presence of 0.1% SDS, as shown in Figure 4B.

Sites of Initial Damage in the D1 Protein. Since the initial damage to the D1 protein under photoinhibitory illumination was essentially identical both *in vivo* and *in vitro*, the sites of cross-linking and cleavage in the D1 protein were determined using isolated PSII preparations, which consist of a small number of protein components but retain the active oxygen-evolving machinery.

The cross-linking site between the D1 and D2 proteins in the heterodimer was investigated by peptide mapping. Proteases that were used were *Achromobacter* Lys-EP and *Staphylococcus* V8 protease, which specifically cleave a peptide bond on the C-terminal side of lysine and glutamic acid residues, respectively. Products of limited proteolysis with these proteases were analyzed by immunoblotting with six different antibodies. In these experiments, the heterodimer and the D1 and D2 proteins were isolated from illuminated PSII complexes from wheat, since the wheat D1 protein contains a unique lysine residue at position 238 (*32*). The location of the lysine and glutamic acid residues and the recognition sites of antibodies in the wheat D1 and D2 proteins are shown in Figure 5.

Results of peptide mapping with Lys-EP are shown in Figure 6A. Digestion of the isolated D1 protein gave rise to two fragments of 20 and 10.5 kDa, which are the N- and C-terminal fragments, respectively, of cleavage at K238. Digestion of the isolated D2 protein gave rise to fragments of 30, 23, and <10 kDa. Larger fragments originating from the D1 protein homodimer and the D2 protein homodimer were also generated. It is expected that digestion of the heterodimer gives rise to novel bands consisting of fragments of the D1 and D2 proteins cross-linked to each other. Five such conjugates were generated from the heterodimer (Figure 6A, HD). Cross-reactivities with antibodies and assignments of these conjugates are summarized in Table 1. It was found that both the N- and C-terminal fragments of cleavage at K238 of the D1 protein could be detected as conjugates. This result indicates that cross-linking with the D2 protein occurs on both the N- and C-terminal sides of K238 of the D1 protein.

To locate the cross-linking sites in more detail, peptide mapping with V8 protease was carried out. Since the D1 and D2 proteins contain many glutamic acid residues (Figure 5), fragments from the D1 and D2 proteins generated by digestion were first identified. Eight fragments ranging from 7 to 17.8 kDa were generated from the isolated D1 protein (Figure 6B, D1). Of these, a fragment of 9.5 kDa and two fragments of 10.6 and 12 kDa, which cross-reacted with anti-$D1_C$, likely correspond to the C-terminal fragments that have been designated as Sa8 and Sa10 doublet, respectively, in a previous study (*33*). It has been demonstrated by N-terminal amino acid sequencing that Sa8 is derived from cleavage at E244 (*21*). It has also been shown that three (E98, E235, and E244) of 19 glutamic acid residues of the wheat D1 protein are preferentially cleaved by V8 protease (*34*). On the basis of their molecular masses and cross-reactivity with antibodies, it is thus likely that the fragments of 7, 9.5, 10.6, and 12 kDa are derived from cleavage at E98, E244, E235, and E226−231, respectively, and that the two fragments of 16−18 kDa correspond to the N-terminal fragments of cleavage of the DE loop (Figure 5). As for the D2 protein, four major and two minor fragments were generated, all of which cross-reacted with anti-$D2_{DE}$ (Figure 6B, D2). It is possible that the 9.5 kDa fragment corresponds to a fragment

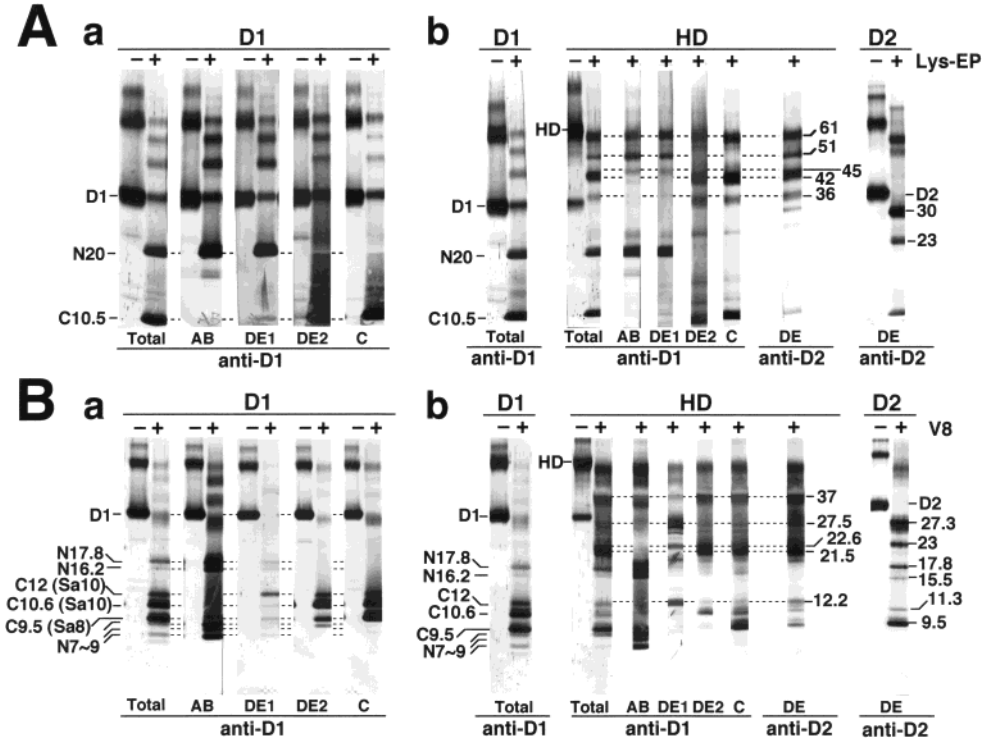

FIGURE 6: Peptide mapping of the D1 and D2 proteins and the heterodimer from wheat. The D1 and D2 proteins and the heterodimer (HD) were isolated from illuminated wheat PSII complexes by SDS−PAGE and electroelution, digested by Lys-EP (A) or V8 protease (B) at 30 °C for 30 min, and then subjected to SDS−PAGE and immunoblotting with the designated antibodies. Panels a and b each show immunoblots from identical gels. Relative sample amounts applied for SDS−PAGE were one, one, and two for the D1 and D2 proteins and the heterodimer, respectively, on the protein basis.

Table 1: Identification of Conjugates Generated by Peptide Mapping of the Heterodimer with Lys-EP and V8 Protease[a]

	cross-reactivity with antibody						fragment contained in conjugate	
	anti-D1					anti-D2		
conjugate	Total	AB	DE1	DE2	C	DE	D1	D2
Lys-EP								
61 kDa	+	+	+	+	+	+	32	30
51 kDa	+	+	+	−	−	+	N20	30
45 kDa	+	+	+	−	−	+	N20	23
42 kDa	+	−	−	+	+	+	C10.5	30
36 kDa	+	−	−	+	+	+	C10.5	23
V8 protease								
37 kDa	+	−	−	+	+	+	C10.6	27.3
27.5 kDa	?	−	+	−	−	+	?	?
22.6 kDa	+	−	+	+	+	+	C12	9.5?
21.5 kDa	+	−	−	+	+	+	C10.6	9.5?
12.2 kDa	−	−	+	−	−	+	?	?

[a] The cross-reactivities with six different antibodies of conjugates containing the fragments of the D1 and D2 proteins (Figure 6) are listed. Fragments of the D1 protein contained in the conjugates were identified from the cross-reactivity with antibodies, while those of the D2 protein were determined from the apparent molecular masses of the conjugates.

of double cleavage at E132 and E242 or E243, since preferential cleavage by V8 protease of the D2 protein at residues 242 and 243 has been suggested previously (*35*).

Digestion of the heterodimer with V8 protease generated five different conjugates of fragments of the D1 and D2 proteins (Figure 6B, HD; also see Table 1). No conjugates cross-reacted with anti-$D1_{AB}$. In addition, the levels of the N-terminal fragments of the D1 protein of 7−9, 16.2, and 17.8 kDa were almost the same in the heterodimer and the isolated D1 protein (compare immunoblots with anti-$D1_{Total}$). These results suggest that the N-terminal side of residue 226 of the D1 protein does not participate in cross-linking with the D2 protein. Conjugates of 37, 22.6, and 21.5 kDa cross-reacted with anti-$D1_C$ and thus contained the C-terminus of the D1 protein. Among them, only the 22.6 kDa conjugate cross-reacted with anti-$D1_{DE1}$, an indication that this conjugate contained the 12 kDa fragment of the D1 protein and that the other two contained either the 10.6 or 9.5 kDa fragment. Since the level of the 10.6 kDa fragment was much lower in the heterodimer than in the isolated D1 protein while that of the 9.5 kDa fragment was comparable, it is likely that the 10.6 kDa but not the 9.5 kDa fragment was included in the conjugates and, therefore, that cross-linking occurred on the N-terminal side of residue 244. Thus, peptide mapping with V8 protease located the cross-linking sites among residues 226−244 in the DE loop of the D1 protein. It is interesting to note that cross-linking with Cyt b_{559} α occurs among residues 239−244 of the D1 protein (*21*).

As for the D2 protein, it seems likely that the cross-linking also occurs inside or in the vicinity of the DE loop of the protein. The level of the 9.5 kDa fragment of V8 protease digestion of the D2 protein, which likely contains the N-terminal part of the DE loop, was much lower in the heterodimer than in the isolated D2 protein (Figure 6B), an indication that this fragment was contained in conjugates (Table 1).

The cleavage sites of the D1 protein were investigated by N-terminal amino acid sequencing of the C-terminal fragments (Figure 7). Samples that were used were spinach PSII core complexes which do not have protein components of 10−30 kDa, and the D1 protein was cleaved by exposure to H_2O_2, which yields higher levels of fragments but less damage once generated fragments than photoinhibitory

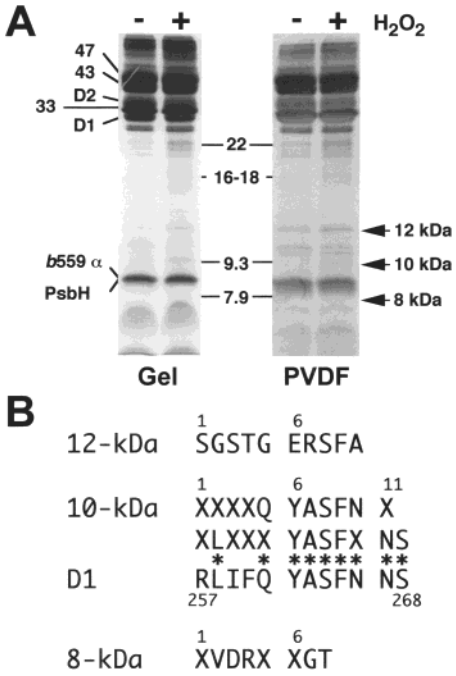

FIGURE 7: N-Terminal amino acid sequencing of fragments of the D1 protein. (A) Separation of the fragments by SDS−PAGE. Spinach PSII core complexes before and after exposure to 10 mM H_2O_2 were subjected to SDS−PAGE (Gel), and separated polypeptides were blotted onto a PVDF membrane (PVDF) for sequencing. Polypeptides were stained with Coomassie blue. The SDS−PAGE conditions were modified so that bands of the 9.3 and 7.9 kDa fragments of the D1 protein were separated from those of other proteins. To visualize fragments generated by exposure to H_2O_2, samples corresponding to 8 μg of Chl, eight times as mush as the optimum level, were used for SDS−PAGE. Horizontal bars indicate the positions of fragments of the D1 protein. (B) N-Terminal amino acid sequences of bands indicated by arrows in panel A. X denotes an unidentified residue. For the 10 kDa band, results of two independent experiments, together with the sequence of residues 257−268 of the spinach D1 protein, are aligned.

illumination (*19*). The yield of fragments, however, was still very low, and fragments were detected as smeared faint bands after staining with Coomassie blue. A sharp band of 12 kDa, which was present in both intact and exposed samples, was successfully sequenced. The obtained sequence perfectly matched with the N-terminal sequence of spinach Cyt b_{559} α (*36*), the molecular mass of which is 9.4 kDa. This band did not cross-react with anti-$D1_{Total}$ (not shown), and it is likely that Cyt b_{559} α was cross-linked with some small protein of PSII during the isolation of PSII core complexes. Sequencing of the bands at the positions of the 9.3 and 7.9 kDa fragments of the D1 protein was troublesome, and many residues remained unidentified because of very low signal intensities and/or modification of amino acid residues. When identified residues were compared, the sequence of a relatively sharp band of 10 kDa, at the position of the 9.3 kDa fragment, matched with the sequence of residues 257−268 of the spinach D1 protein (Figure 7B) (*37*, *38*). This result suggests that the 9.3 kDa fragment is derived from cleavage between residues 256 and 257. The sequence of a smeared faint band of 8 kDa at the position of the 7.9 kDa fragment, by contrast, was not at all similar to the sequences of PSII proteins.

DISCUSSION

Initial Damage to the D1 Protein under Photoinhibitory Illumination in Vivo. In this study, we have clearly shown that the initial damage to the D1 protein under photoinhibitory illumination was essentially identical *in vivo* and *in vitro*. A fraction of the D1 protein was cleaved either in the DE loop or in the vicinity of helix D, and another fraction was cross-linked with either the D2 protein or Cyt b_{559} α, although the resultant fragments and cross-linked adducts barely accumulated at 25 °C *in vivo* through digestion by proteases (Figure 1). As judged from the levels of fragments, cleavage of the D1 protein mainly occurred in the DE loop. This result coincides with a pioneering work of the turnover of the D1 protein under non-photoinhibitory illumination *in vivo* (*39*), which first located the cleavage site in the DE loop.

We found that the cleavage in the DE loop occurs essentially at a single site to give rise to the 8.4 kDa C-terminal fragment both *in vivo* and *in vitro* (Figure 1). The 9.3 and 7.9 kDa C-terminal fragments, which are generated in isolated PSII preparations under photoinhibitory illumination (*19*), were detected only after prolonged illumination *in vitro*. Also, when thylakoids were exposed to H_2O_2, the 8.4 kDa fragment was generated predominantly at 0.3 mM H_2O_2, whereas the 9.3 and 7.9 kDa fragments predominated at higher H_2O_2 concentrations (Figure 2). These observations imply that the cleavage in the DE loop occurs at multiple sites only when the level of active oxygen species around PSII is high. *In vivo*, the level of active oxygen species can be kept low by the action of the active oxygen scavenging systems located in the stroma and on the surface of thylakoid membranes (*7*). This could be the reason only the 8.4 kDa fragment was detected even after prolonged illumination *in vivo*.

N-Terminal sequencing of the 9.3 kDa fragment, generated by exposure of PSII core complexes to H_2O_2, suggests that this fragment is derived from cleavage on the N-terminal side of R257 (Figure 7). On the basis of the reactivity of active oxygen species with amino acid residues, we previously proposed that the 7.9 kDa fragment is derived from cleavage at a residue adjacent to H272 that participates in the binding of the non-heme iron (*19*). As judged from the apparent molecular masses of the fragments, it is likely that the 8.4 kDa fragment is derived from cleavage at a particular residue between residues 257 and 272. This region contains residues involved in the binding of the secondary quinone electron acceptor Q_B (*38*).

Peptide mapping of the heterodimer isolated from illuminated PSII complexes successfully located the sites of cross-linking with the D2 protein among residues 226−244 in the DE loop of the D1 protein (Figure 6 and Table 1). Although the cross-linking with the D2 protein occurred at multiple sites in the PSII complexes, it might occur at a single site *in vivo*, as found in cleavage in the DE loop of the D1 protein. This region includes the site(s) of cross-linking with Cyt b_{559} α (*21*) and overlaps with the PEST-like region, which had previously been proposed to be a possible cleavage site (*39*).

Thus, the sites of initial damage to the D1 protein are located mainly in the DE loop (Figure 8). The sites of cleavage and cross-linking, however, are located apart from each other in the loop: the cleavage occurs close to helix E,

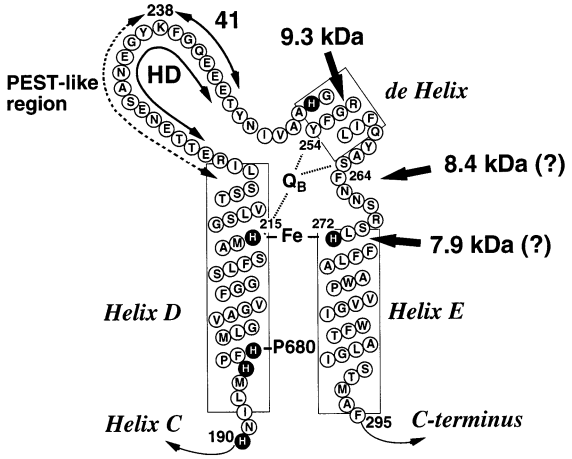

FIGURE 8: Location within the D1 protein of the sites of cleavage and cross-linking caused by photoinhibitory illumination. The region between residues 190 and 295 of the wheat D1 protein is modeled on the folding model of Trebst (*38*). The putative α-helixes are boxed. The residues that are proposed to participate in the binding of Q_B, namely, H215, Y254, F255, S264 (*38*, *48*), and residues 246−249, 266, and 267 (*49*), are positioned close to Q_B. The proposed cleavage sites are indicated with thick arrows, the sites of cross-linking with Cyt b_{559} α (denoted as 41; *21*) and the D2 protein (HD; this study) are indicated with bidirectional arrows, and the PEST-like region (*39*) is indicated with a dashed bidirectional arrow.

whereas the sites of cross-linking are localized to the N-terminal part of the loop. If the structural and functional homology between the D1 and D2 proteins is taken into consideration, it is probable that the initial damage to the D2 protein occurs mainly in its DE loop.

Mechanisms of Initial Damage to the D1 Protein in Vivo. The initial damage to the D1 protein under photoinhibitory illumination *in vivo* (Figure 1) was basically the same as that caused by exposure to active oxygen species of thylakoids (Figure 2) and PSII preparations (*19*, *22*, *24*). In PSII under illumination, active oxygen species are generated close to the sites of initial damage: $^1O_2$ is generated by the reaction of the triplet state of the reaction center chlorophyll P680 with oxygen (*31*), and H_2O_2 is generated on the stromal side of PSII (*30*), probably by the reaction of the reduced form of the quinone electron acceptors (Q_A and/or Q_B) with oxygen, and then converted to a hydroxyl radical by the reaction with the non-heme iron (*19*). It is known that cross-linking reactions of proteins are mediated by oxidation reactions involving active oxygen species (*2*), and this mechanism is accepted for cross-linking of the D1 protein (*21*).

On the other hand, the question of whether the cleavage is mediated solely by the action of active oxygen species or if it involves protease digestion is still controversial. The protease model postulates that active oxygen species alter the conformation of the D1 protein so that the protein can be recognized by specific proteases (*12*). It has recently been demonstrated that the cleavage in the DE loop is catalyzed by the serine-type DegP2 protease, which is loosely associated with the stromal surface of the nonappressed region of the thylakoid membrane (*9*). This finding supports the protease model but cannot explain the cleavage at low temperatures (Figure 3). We consider that cleavage of the D1 protein can occur both with and without the assistance of protease(s) *in vivo*: the cleavage proceeds either enzymatically or chemically at growth temperatures whereas mainly chemically at low temperatures. Even if the cleavage is enzymatic, involved proteases should not necessarily be specific to the D1 protein, since the D2 protein is also cleaved under photoinhibitory illumination *in vivo* (*14*, *16*). The chemical processes have previously been proposed for cleavage in isolated PSII preparations that lacked protease activities (*23*). It seems likely that the chemical cleavage also occurs in thylakoids. As reported previously (*27*), the cleavage in the DE loop of the D1 protein, seen as an increase in the level of the 8.4 kDa fragment in darkness (Figure 4A), was suppressed only partly by a variety of protease inhibitors in thylakoids.

To explain simultaneous cleavage and cross-linking of the D1 protein under illumination, we propose a hypothesis that an amino acid residue(s) of the D1 protein is first oxidized by active oxygen species generated inside PSII under illumination and that the site of oxidation determines the fate of the D1 protein, namely, if it is cleaved or cross-linked. In general, cleavage of proteins by active oxygen species starts with formation of an α-carbon-centered radical in an aliphatic residue or proline oxidation, whereas cross-linking involves the recombination of carbon-centered radicals in side chains of aromatic residues (*2*). As described above, the cross-linking region overlaps with the PEST-like region. The PEST sequence had previously been proposed as a structural signal for rapid degradation of proteins and has a disordered structure (*40*, *41*). It is likely that residues in the PEST-like region of the D1 protein are able to come in contact with other proteins in its proximity to form intermolecular cross-linking when oxidized. In contrast, the C-terminal side of the DE loop containing a short α-helix is expected to be unflexible or tightly packed so that intermolecular reactions barely occur. Consequently, the oxidation of a residue in this region could trigger intramolecular oxidation reactions, resulting in chemical cleavage or intramolecular cross-linking with a near residue. The occurrence of cross-linking within the D1 protein is obscure at present. However, it is quite possible that such cross-linking could act as a signal for protease digestion. The same scenario is applicable to helix D, another site(s) of cleavage of the D1 protein. The active oxygen species have high reactivity toward specific amino acid residues (*42*, *43*). This specificity of oxidation explains well the site-specific damage to the D1 protein.

Mechanism of Complete Degradation of the D1 Protein under Photoinhibitory Illumination in Vivo. It has long been postulated that the fragments and adducts of the D1 protein are digested by proteases. Actually, it has been demonstrated recently that the 22 kDa fragment of cleavage in the DE loop is digested by the FtsH protease, which is anchored to the stromal side of the nonappressed region of the thylakoid membrane (*25*, *44*). We found that the cross-linked adducts also undergo protease digestion *in vivo*.

The adducts were digested by a stromal protease(s) resistant to SDS (Figure 4). This protease selectively digested both the heterodimer and the 41 kDa adduct generated under illumination, and also the artificially generated homodimer of the D1 protein. The D1 protein in its free form, or the fragments of the D1 protein, could not be its substrate even in the presence of SDS. Thus, the putative protease seems to be highly specific to cross-linked adducts and/or aggregates of proteins. Its pH optimum was around 7.8 (not

shown), and it appeared to be active both in the light and in darkness in the chloroplast. It could not be a stromal serine-type Clp protease, since digestion of the adducts was not affected by either inhibitors of serine-type proteases (not shown) or ATP (Figure 4A). The presence of three different SDS-resistant proteases of 14, 30, and 54 kDa in the stroma has been demonstrated (*45*), and it has been proposed that one of these proteases participates in digestion of the heterodimer generated under illumination of PSII membranes (*46*). In addition to the stromal protease, it is likely that a thylakoidal protease(s) also participates in digestion of the adducts. Although the result is preliminary, we found that an ATP-dependent protease(s) bound to the thylakoid membrane selectively digests the heterodimer (data not shown).

It is also likely that the digestion of fragments of the D1 protein is catalyzed by various proteases inside the chloroplast. Although the FtsH protease digests the 22 kDa fragment (*25*, *44*), the 16−18 kDa fragments do not seem to be digested by this protease: we observed in thylakoids that the presence of zinc ion, which is an activator of the FtsH protease, reduced the level of the 22 kDa fragment but not at all those of the 16−18 kDa fragments (data not shown). Taken together, it is suggested that, once damaged, the D1 protein is degraded completely by the action of a variety of proteases that digest abnormal proteins.

In general, oxidatively modified proteins have an increased surface hydrophobicity, a common feature of non-native proteins, which is recognized by proteases for abnormal proteins (*2*, *4*). Cleavage in the DE loop likely exposes hydrophobic domains of the D1 protein to the outer surface of the PSII reaction center complex, or it generates a nonpolar C-terminus for protease recognition as discussed previously for digestion of the 22 kDa fragment by the FtsH protease (*44*). Similarly, intermolecular cross-linking made the D1 protein susceptible to digestion by the stromal protease(s) (Figure 4). It is thus likely that both chemical and enzymatic cleavages and also cross-linking reactions promote complete degradation of the D1 protein *in vivo*. It still remains to be determined if the 32 kDa D1 protein can be directly digested by proteases to small peptides and/or amino acids. Such digestion, if any, would proceed much more slowly than that of fragments and adducts, as judged from the time courses of the loss of the D1 protein of 32 kDa in intact chloroplasts and leaf disks (Figure 1B).

The proteases involved in degradation of the D1 protein that have been identified so far are all located in either the stroma or the stroma-exposed surface of the nonappressed region of the thylakoid membrane, which is separated from the appressed granal region where the functional PSII is located. This is consistent with the photoinhibition and repair cycle model (*8*, *12*, *14*), in which PSII damaged under illumination migrates from the granal region to the non-appressed region, which is the site of *de novo* synthesis of the D1 protein (*47*). The DE loop of the D1 protein, the site of initial damage, is located on the stromal side of the membrane, and this enables the damaged regions to be recognized by the proteases.

The chloroplast is a major site of generation of active oxygen species under stress conditions (*7*). In particular, PSII can generate active oxygen species even under nonstress conditions because of the high redox potential of its photochemistry [E_m of P680 = 1.2 V (*15*)]. Efficient mechanisms for the removal of the damaged D1 protein would be essential in maintaining the activity of PSII. The D1 protein is the most susceptible of all PSII proteins to attack by exogenous active oxygen species, even when solubilized with SDS (*24*). This feature, which can be ascribed to the primary structure of its DE loop, and also the site-specific generation of active oxygen species within the PSII reaction center complex would accomplish the selective turnover of the D1 protein. The turnover of the D1 protein itself would serve as the quality control system of the chloroplast, ensuring that the cost in repair of the entire PSII is kept to a minimum.

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