Role of an Extrinsic 33 Kilodalton Protein of Photosystem II in the Turnover of the Reaction Center-Binding Protein D1 during Photoinhibition†

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ABSTRACT: The reaction center-binding protein D1 of photosystem II (PS II) undergoes rapid turnover under light stress conditions. In the present study, we investigated the role of the extrinsic 33 kDa protein (OEC33) in the early stages of D1 turnover. D1 degradation was measured after strong illumination (1000−5000 μE m^{-2} s^{-1}) of spinach manganese-depleted, PSII-enriched membrane and core samples in the presence and absence of the OEC33 under aerobic conditions at room temperature. PSII samples lacking the OEC33 were prepared by standard biochemical treatments with Tris or $CaCl_2/NH_2OH$ while samples retaining the OEC33 were prepared with NH_2OH or $NaCl/NH_2OH$. The degradation of D1, monitored by SDS/urea−polyacrylamide gel electrophoresis and Western blotting using specific antibodies against D1, proceeds to a greater extent in NH_2OH-treated samples than in Tris-treated samples over a 60 min illumination period. Under the same conditions, significantly more aggregation of D1 occurs in the Tris-treated samples than in the NH_2OH-treated samples. The lower level of D1 degradation in Tris-treated samples is not due to secondary proteolysis, as judged from the time course for degradation at 25 °C or the degradation pattern at 4 °C. Similarly, for $NaCl/NH_2OH$-treated samples, D1 degradation is greater and D1 aggregation less than in $CaCl_2/NH_2OH$-treated samples. The effect of the presence of the OEC33 on D1 degradation and aggregation is confirmed by reconstitution experiments in which the isolated OEC33 is restored back to Tris-treated samples. During very strong illumination, significant loss of CP43 also occurs in Tris-treated but not in NH_2OH-treated samples. Structural analysis of PS II core complexes by Fourier transform infrared (FT-IR) spectroscopy revealed very little change in the protein secondary structure after 10 min illumination of NH_2OH-treated samples while a large 10% decrease of α-helix content occurs in Tris-treated samples. On the basis of these results, we suggest that either (1) the OEC33 stabilizes the structural integrity of PS II such that it prevents the photodamaged D1 protein from aggregating with nearby polypeptides and thereby facilitating degradation or (2) the OEC33 specifically stabilizes CP43, a putative D1-specific protease, which normally promotes the efficient degradation of D1.

Oxygenic photosynthetic organisms are susceptible to photoinhibition under strong illumination, where the polypeptides, photosynthetic pigments, and electron transport activity in thylakoid membranes are damaged through oxidative stress. The D1 protein[1] is one of the reaction center-binding proteins in PS II and is the primary target for photodamage (*1*−*4*). Under illumination, the D1 protein turns over rapidly, being replaced by a newly synthesized protein in a unique repair process (*4*).

In the damage/repair cycle for the D1 protein, the degradation process has been studied mostly in vitro, because it can be induced under variously controlled conditions and a larger amount of degradation products can be detected than in vivo. Two mechanisms for D1 degradation have been proposed. In the so-called acceptor-side mechanism, which occurs under very strong light, the degradation of D1 is thought to be mediated by the "over-reduction" of the plastoquinone components. In particular, upon double reduction of the plastoquinone electron acceptor Q_A and dissociation from its binding site, the probability for charge recombination between the oxidized form of the chlorophyll primary electron donor P680 and the reduced form of the pheophytin primary electron acceptor Phe increases. This in turn leads to an enhancement in the formation of P680 triplet states which can react with molecular oxygen to produce singlet oxygen, a potentially very damaging species to proteins. It is thought that the localization of singlet oxygen formation to the immediate vicinity of the D1 protein is the initiating event that causes D1 cleavage (*5*).

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[1] Abbreviations: D1 and D2 proteins, reaction center-binding proteins of photosystem II; PS II, photosystem II; Q_A, primary plastoquinone electron acceptor; Q_B, secondary plastoquinone electron acceptor; P680, primary electron donor of PS II; Y_Z, tyrosine secondary electron donor molecule; Chl, chlorophyll; CP43 and CP47, antenna−chlorophyll-binding proteins of PS II; Tris, tris(hydroxymethyl)aminomethane; OEC33, -24, and -18, extrinsic proteins of the oxygen-evolving complex; Mes, 2-(*N*-morpholino)ethanesulfonic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; ECL, enhanced chemiluminescence; FT-IR, Fourier transform infrared.

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An alternative interpretation of acceptor-side photoinhibition involves the interaction between the plastoquinone electron acceptor Q_B and molecular oxygen. In this case, reduced Q_B is oxidized by O_2 to form superoxide anion radicals, which is another type of active oxygen species that can be harmful to proteins. Recent results have indicated that reversible conformational changes around the Q_B binding site occur during D1 degradation in the light. It is suggested that the reversible conformational changes lead to an irreversible structural deformation which inactivates the D1 protein (*6*). The evidence that herbicides which interact with the Q_B-binding pocket suppress the light-induced degradation of D1 supports this idea (*7, 8*) as does the finding that the binding of the Q_B inhibitor *N*-octyl-3-nitro-2,4,6-trihydroxybenzamide (PNO8) triggers the specific cleavage of D1 into 23-kDa N-terminal and 9-kDa C-terminal fragments in the dark which is enhanced upon preillumination with weak light (*9*). Thus, it appears that certain protein structural changes are required for the efficient degradation of D1.

In the so-called donor-side mechanism of photoinhibition, it is proposed that D1 degradation is mediated through long-lived, highly oxidative species, such as $P680^+$ and the oxidized tyrosine electron donor Y_Z^+. These species become long-lived when PS II complexes lose the catalytic Mn cluster (*10, 11*) or when the final O_2-evolving step is inhibited such as under low chloride conditions (*12, 13*). Donor-side photoinhibition is easily demonstrated with in vitro system, because it has a much larger quantum yield than acceptor-side photoinhibition and can occur even under weak illumination (*14, 15*). In light-stressed pumpkin leaves, the D1 degradation pattern is similar to that observed in the donor-side mechanism indicating that donor-side photoinhibition occurs in vivo (*16*).

Even though molecular oxygen has been shown to be required in acceptor-side photoinhibition but not in donor-side photoinhibition (*13, 17*), both mechanisms involve some kind of active radical species. Regardless of the exact radical species involved, however, D1 degradation may be further exacerbated by the action of specific proteases (*4*), although such proteases have not yet been identified.

While D1 seems to be the most photosensitive protein in PS II, other proteins have also been shown to undergo degradation during strong illumination. In particular, the D2 protein, which is the D1 counterpart in the reaction center heterodimer, breaks down in parallel with D1 but to a much lesser extent (*18, 19*). More recently, we have shown that the antenna−chlorophyll-binding protein CP43 can also be degraded, at least during donor-side photoinhibition of PS II-enriched membranes treated with 0.8 M Tris (pH 9.0) (*20*). Since CP43 is not directly involved in electron transport of PS II, the observed degradation may be due to its location in the vicinity of the long-lived strong oxidants created by the D1 protein in the light. It should be noted that relatively strong light is required for the degradation of CP43 and that the extrinsic 33 kDa protein of PS II (OEC33) appears to protect CP43 from photodegradation (*20*).

Although the cleavage of polypeptides in PS II during strong illumination is relatively well documented, not much information is available on the repair process. It has been proposed that when PS II complexes that are mainly located in the appressed granal regions of the thylakoid membranes are photoinhibited, they migrate to the nonappressed stroma regions where the damaged D1 protein is replaced by the insertion of newly synthesized D1 precursor (*21*). Nevertheless, it is known that located near the D1 protein are the three extrinsic proteins (OEC33, -24, and -18) which bind to the lumenal surface of the PSII complex and regulate the O_2-evolving activity. Interestingly, the three OEC subunits were shown to be released from PS II during strong illumination in vivo (*22*) and in vitro (*23*) during acceptor-side photoinhibition. The kinetics of release correlated well with the kinetics for D1 degradation. It could be that acceptor-side photoinhibition is followed by donor-side photoinhibition which then induces the release of the OEC subunits.

Of the OEC subunits, OEC33 is most closely associated with D1, probably at the C-terminal end which is exposed on the lumenal side of the PS II complex (*24*). To address the question whether the OEC33 has a specific function in the early stages of D1 turnover, we investigated the degradation and aggregation of D1 and any associated protein secondary structural changes after high light treatment of PS II-enriched membrane and core samples in the presence and absence of the OEC33. Our results show that the presence of the OEC33 strongly influences the light-induced degradation and aggregation of D1 and that these effects are possibly related to the function of the OEC33 in maintaining the structural integrity of PS II needed for the efficient turnover of the D1 protein.

MATERIALS AND METHODS

Preparation of PS II Samples. PS II-enriched membranes were prepared from spinach chloroplasts by the method of Kuwabara and Murata (*25*). PS II core complexes were isolated from the PS II membranes using heptyl thioglucoside according to the procedure of Enami et al. (*26*). Both samples were frozen with liquid nitrogen and stored at −80 °C until used. Treatment of the PS II-enriched membrane and core samples with 0.8 M Tris (pH 9.0) or with 3 mM NH_2OH (pH 6.5) was carried out as described previously (*20*). Both Tris and NH_2OH solutions included 3 mM EDTA to trap the Mn ions released from PS II. Alternatively, PS II membranes were treated with NaCl (1 M)/NH_2OH (3 mM, pH 6.5) or with $CaCl_2$ (1 M)/NH_2OH (3 mM, pH 6.5) to remove OEC24, OEC18, and Mn, or to remove OEC33, OEC24, OEC18, and Mn, respectively. After the treatments, the PS II samples were washed twice and suspended with a buffer solution containing 40 mM Mes−NaOH, 0.4 M sucrose, and 10 mM NaCl, pH 6.5 (buffer A). All preparation procedures and subsequent treatments were carried out in darkness or under a safe light.

For isolation of the OEC33 protein, PS II-enriched membranes were washed first with 1 M NaCl and then with 1 M $CaCl_2$, where the OEC33 is released in the second wash step. For the crude Tris supernatant, PS II-enriched membranes were washed with 0.8 M Tris (pH 9.0). All wash steps were carried out by centrifugation at 35000*g* for 10 min. The final supernatants in both extractions were dialyzed against 10 mM Mes−NaOH (pH 6.5) and concentrated by an Amicon ultrafiltration cell. For reconstitution, Tris-treated, PS II-enriched membrane samples were washed with buffer A twice and then incubated with either the concentrated OEC33 or Tris supernatant for 30 min on ice. The

reconstituted samples were washed again twice and resuspended with buffer A. The reconstitution was done in darkness. Chlorophyll was determined in 80% acetone using the extinction coefficient reported by Mackinney (*27*).

Illumination of PS II Samples with Strong Light. For the photoinhibitory treatment under aerobic conditions, microtubes (volume 0.5 mL) containing the PS II samples suspended in buffer A were put into a circulating water bath in which the temperature was kept at 25 °C. In the experiment where the effect of a lower temperature on the photodamage of the D1 protein was examined, samples were incubated at 4 °C. The concentrations of chlorophyll for the PS II-enriched membranes and for the PS II core complexes were adjusted to 0.5 mg mL^{-1} and 0.2 mg mL^{-1}, respectively. The microtubes were then illuminated from the side through a water layer for the indicated periods. Strong white light (5000 μE m^{-2} s^{-1}) and intermediate light (1000 μE m^{-2} s^{-1}) were obtained from slide projectors. The microtubes were then centrifuged and the PS II samples recovered as pellets.

Gel Electrophoresis and Immunoblotting. SDS/urea−polyacrylamide gel electrophoresis (PAGE), Western blotting, and fluorography by enhanced chemiluminescence (ECL) were performed according to the procedures described earlier (*20*). The amounts of the proteins detected by ECL in X-ray films were quantified with the use of a Personal Scanning Imager PD110 (Molecular Dynamics, USA). The antibodies against D1, D2, CP43, and CP47 were prepared as described previously (*20, 28*). The antibodies against the cyt b_{559} α-subunit, the N-terminal segment (N2−N238) of the D1 protein from wheat, and a synthesized C-terminal region (C1−C12) which is conserved in the D1 proteins from various plant species were the gifts from Prof. B. Andersson of the University of Stockholm, Prof. R. Barbato of the University of Padova, and Dr. T. Ono of the Institute of Chemical Physics, Riken, respectively.

FT-IR Measurements. Fourier transform infrared (FT-IR) absorption spectra were recorded at 2 cm^{-1} resolution using a Bruker IFS66 spectrometer equipped with a DTGS detector and a KBr beam splitter. Aliquots of 10 μL of the concentrated PS II preparations (10−15 mg of chlorophyll mL^{-1}), with and without illumination, were loaded between two CaF_2 windows using a 6 μm aluminum spacer. The sample compartment was continuously purged with nitrogen gas to eliminate absorption by water vapor in the light path. Background absorptions were measured under identical conditions and digitally subtracted so as to generate a flat base line from 1900 to 1740 cm^{-1} and to remove the water band around 2130 cm^{-1}. Each spectrum was an average of 100−200 scans. Protein secondary structural contents were calculated by pattern recognition analysis of the amide I band (1600−1700 cm^{-1}) using the Bruker OPUS/Quantitative Analysis 2 software package and a calibration set of 15 proteins whose secondary structure has been determined by X-ray crystallography (*29*).

RESULTS

Comparison of D1 Degradation between Tris- and NH_2OH-Treated PS II Samples after Photoinhibition. To evaluate the possible role of the OEC33 in degradation of D1 during donor-side photoinhibition, spinach PS II-enriched membranes were treated with either Tris (0.8 M, pH 9.0) or NH_2OH (3 mM, pH 6.5) and illuminated with strong white light (5000 μE m^{-2} s^{-1}) for 60 min at 25 °C. Treatment of the PS II-enriched membranes with Tris removes all OEC subunits (*30*), while treatment with NH_2OH does not remove the OEC33 (*31*). Both treatments do, however, cause the release of the catalytic Mn ions (*31, 32*). The effects of Tris and NH_2OH treatments on the PS II protein composition of PS II-enriched membrane samples are shown by the Coomassie-stained, SDS/urea−PAGE patterns of Figure 1. With Coomassie staining, all of the OEC subunits appear to be released by Tris treatment, consistent with previous observations, while after NH_2OH treatment a large part of the OEC24 and -18 is extracted but not the OEC33. Upon using the more sensitive immunodetection method by Western analysis with specific antibodies against the individual OEC subunits, we were able to quantify the amounts of the OEC subunits which remain in the PS II-enriched membrane samples after treatment (data not shown). For Tris-treated samples, 15% and 11% of the original OEC24 and OEC18, respectively, remain bound while all of the OEC33 is completely removed. For NH_2OH-treated samples, 75% of the OEC33, 70% of the OEC24, and 38% of the OEC18 are retained. Even after strong illumination over 60 min less than 10% of the total OEC33 is lost in the NH_2OH-treated samples (Figure 1). Thus, the significant retention of the OEC33 in NH_2OH-treated samples allows one to study the specific role of the

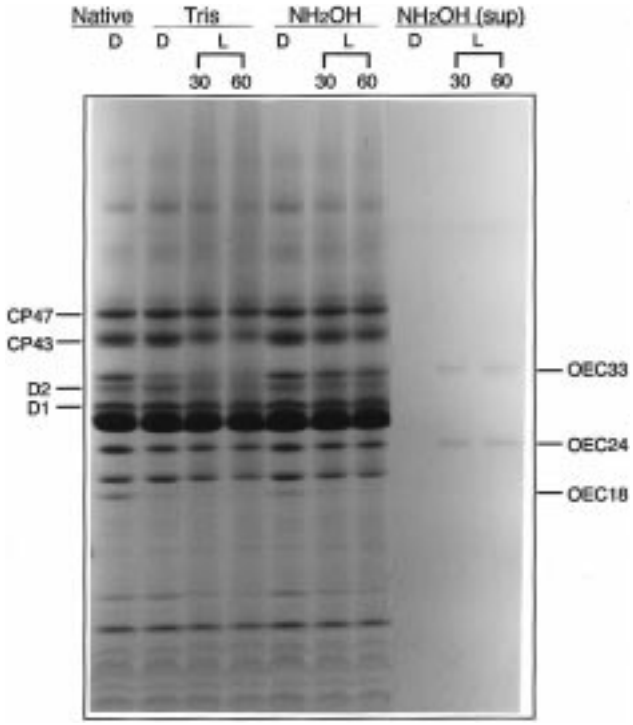

FIGURE 1: SDS/urea−PAGE profile of the polypeptide composition in Tris-treated and NH_2OH-treated spinach PS II-enriched membranes samples before and after high light treatment. Samples were illuminated with white light (5000 μE m^{-2} s^{-1}) for 30 or 60 min. The proteins were stained by Coomassie brilliant blue. Native indicates the untreated control sample. D and L denote in the dark and after illumination, respectively. Locations of PS II proteins are identified by the lables at the right and left sides of the gel. The three lanes on the right side of the gel are the supernatants of the NH_2OH-treated samples before and after illumination. See the text for other details.

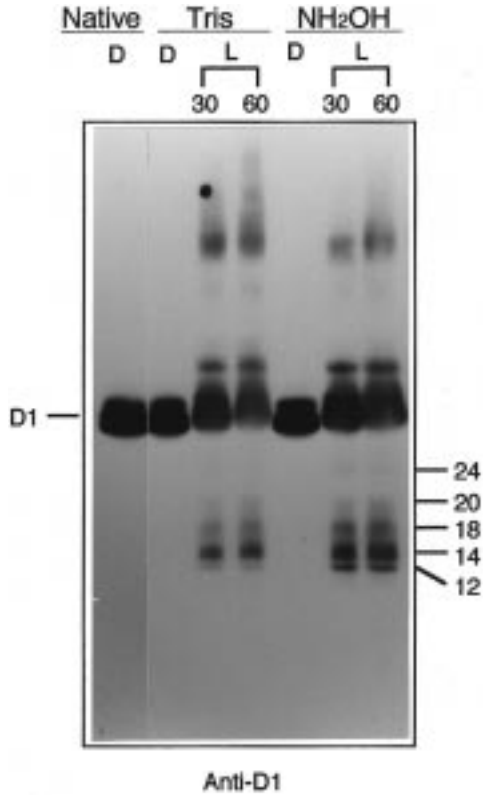

FIGURE 2: Light-induced degradation and aggregation of the D1 protein in PS II-enriched membrane and PS II core samples after treatment with Tris or NH_2OH. Samples in the dark (D) and after illumination (L) with white light (5000 μE m^{-2} s^{-1}) for 30 or 60 min at 25 °C under aerobic conditions are shown. The degradation and cross-linked products were detected by SDS/urea−PAGE, Western blotting with a specific antibody against the D1 protein, and fluorography by enhanced chemiluminescence (ECL). The sizes of the protein fragments are indicated on the right side of the gel patterns. See text for other details.

OEC33 in the light-induced degradation of D1 by comparison with Tris-treated samples.

Figure 2 shows the results from SDS/urea−PAGE and Western blots realized by ECL fluorography for both Tris-treated and NH_2OH-treated PS II-enriched membranes after strong illumination. D1 degradation yields the same pattern for both types of samples, which include fragments with relative molecular masses of 24−12 kDa, but is clearly more extensive in the NH_2OH-treated samples than in the Tris-treated samples under identical illumination conditions. Ten to twenty percent of D1 is degraded in the NH_2OH-treated samples by the illumination for 60 min (data not shown). Upon immunoblotting of the NH_2OH-treated PS II-enriched membranes with antibodies against an N-terminal (N2−N238) and a C-terminal (C1−C12) segment of the D1 protein, the 24 kDa fragment was found to contain an N-terminal segment while the 18 kDa fragment contained a C-terminal segment (data not shown). Thus, NH_2OH treatment of PS II apparently allows more significant photodegradation of D1 than does Tris treatment. In these experiments, artificial electron acceptors were not included in the suspension medium during illumination because even a very small residual electron transport activity that may occur in Tris- and NH_2OH-treated PS II could retard D1 degradation which would make comparison of the degradation patterns more difficult. Illumination of the PS II samples in the absence of electron acceptors may induce acceptor-side photoinhibiton as well as donor-side photoinhibition. Detection of a small amount of the N-terminal 24 kDa fragment, which is typical of acceptor-side photoinhibition, shows the possibility.

Comparison of D1 Aggregation between Tris- and NH_2OH-Treated PS II Samples after Photoinhibition. Previously we reported significant cross-linking of the D1, CP43, and CP47 proteins after strong illumination of Tris-treated PS II-enriched membranes (*20*). The cross-linked products (hereafter referred to as aggregation products or aggregates) were detected in the region of relatively high molecular mass as revealed by Western blotting using specific polyclonal antibodies against D1, CP43, and CP47. The smeared bands of the aggregates showed lower mobility than the band of the D1/D2 heterodimer. Upon illumination of NH_2OH-treated samples, the aggregates were also detected, but the amounts were much reduced compared with those in Tris-treated samples. This behavior is shown in Figure 2, where the aggregates have apparent molecular masses of 100, 84, 78, and 70 kDa. The aggregates are probably byproducts of the photodegradation of the D1 protein. With PS II core complexes, a similar D1 aggregation pattern was observed, but variations depended upon the pretreatment of the samples (data not shown). The less significant aggregation of the D1 protein in NH_2OH-treated samples compared with Tris-treated samples suggests that the OEC33 effectively suppresses the aggregation process during the illumination period.

Figure 3 shows the difference in the levels of D1 degradation and aggregation between Tris- and NH_2OH-treated PS II membranes over a 60 min time course of strong illumination. It should be noted that the D1 degradation patterns detected during the early stages of illumination are not much different from the pattern after prolonged exposure to strong light.

Comparison of D1 Degradation and Aggregation Induced by Strong Illumination at Room Temperature and a Lower Temperature. We have used the relative amount of D1 fragments as a quantitative maker of D1 degradation. However, this approach may have certain limitations since the fragments are prone to further proteolysis. To examine possible secondary degradation of D1, we illuminated Tris- and NH_2OH-treated samples at room temperature (25 °C) and at a lower temperature (4 °C). The results are shown in Figure 4. After Tris treatment, significant D1 degradation is observed, even at 4 °C where proteolytic enzymes may not work efficiently. Aggregation of D1 is also induced under these conditions. The degradation and aggregation of D1 are probably caused by the endogenous radicals formed during donor-side photoinhibition as well as by oxygen radicals that are produced by the acceptor side. When Tris-treated samples are illuminated at 25 °C, the amount of D1 degradation products decreases compared with the amount observed at 4 °C. However, smaller D1 fragments corresponding to possible secondary degradation were not detected by fluorography. By contrast, the D1 aggregates increase significantly by raising the ambient temperature. Accumulation of a 41 kDa cross-linked product between D1 and the α-subunit of cyt b_{559} (*33*) was also enhanced at 25 °C compared with 4 °C. From these results, we assume that the decrease in D1 degradation in Tris-treated samples upon

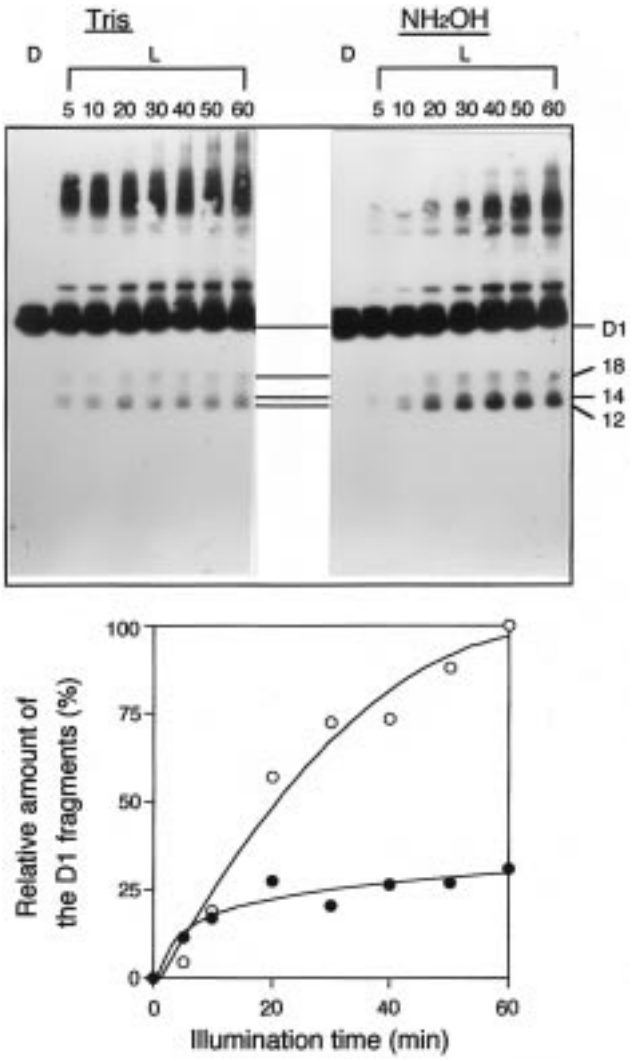

FIGURE 3: Time courses for the degradation of the D1 protein during strong illumination of PS II-enriched membrane samples treated with either Tris or NH_2OH. D and L denote dark control and illuminated samples, respectively. The pretreated PS II membranes were illuminated and aliquots withdrawn at given periods of illumination (5, 10, 20, 30, 40, 50, and 60 min) prior to Western blot analysis. The conditions of illumination and protein assay are the same as those described in Figure 2. The antibodies against the C-terminal parts of the D1 protein were used. The bands of D1 and the degradation fragments (18, 14, and 12 kDa) are shown at the right side of the gel. At the bottom, the relative amounts of the 12 and 14 kDa fragments are plotted as the function of the illumination time. (●) Tris-treated samples; (○) NH_2OH-treated samples.

raising the temperature is not due to stimulation of the secondary degradation but rather to the increased D1 aggregation. In NH_2OH-treated samples, D1 degradation was stimulated upon raising the temperature, while the level of D1 aggregation stayed low, in contrast to the Tris-treated samples.

Light-Induced Degradation and Aggregation of the D1 Protein in NaCl/NH_2OH- or $CaCl_2$/NH_2OH-Treated PS II-Enriched Membranes. Although the Tris and NH_2OH treatments employed here are well-known procedures to inactivate the O_2-evolving system of PS II, they are different from each other, especially with respect to the pH during the treatment. Therefore, a possibility remains that other changes occur in PSII that are not attributed to presence or

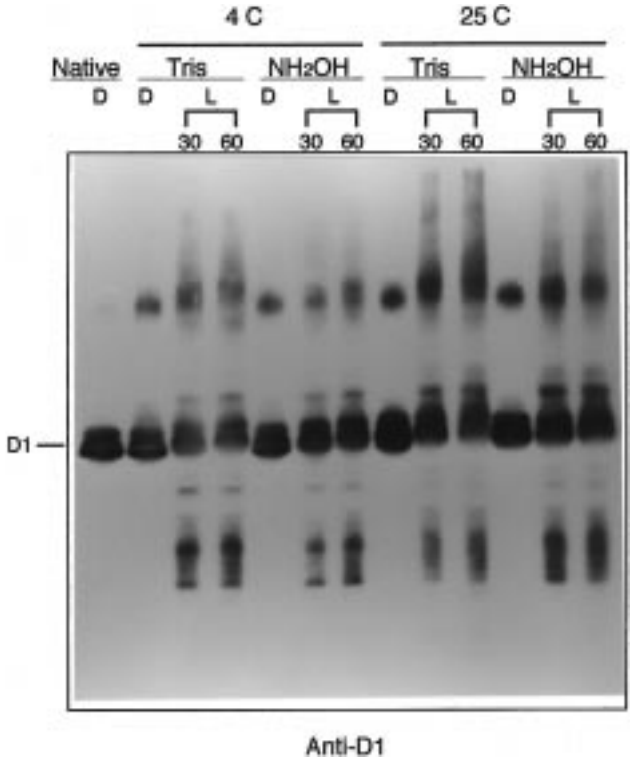

FIGURE 4: Comparison of the behavior of D1 degradation in the PS II-enriched membrane samples during strong illumination at 4 and 25 °C. All the experimental conditions were the same as those described in the legend to Figure 2.

absence of the OEC33. To confirm that the OEC33 is the primary factor in promoting D1 degradation, we used alternative methods to release the OEC subunits. Treatment of PS II-enriched membranes with 1 M NaCl removes only the OEC24 and -18, while washing with 1 M $CaCl_2$ takes off all the OEC subunits from the membranes. During these treatments, the catalytic Mn is not released. However, the Mn can be removed by subsequent treatment with NH_2OH.

Figure 5 shows that in NaCl/NH_2OH-treated PS II-enriched membranes, degradation of D1 by strong illumination is more extensive than in $CaCl_2$/NH_2OH-treated samples. By contrast, D1 aggregation is more prominent in the $CaCl_2$/NH_2-OH-treated samples. These results suggest again that OEC33 has an important role in the processes related to D1 turnover under photoinhibitory conditions.

Reconstitution of the OEC33 in Tris-Treated PS II Samples. The possible role of the OEC33 in D1 degradation was further tested by reconstitution experiments. Upon incubation of Tris-treated PS II-enriched membranes with the purified OEC33, the protein rebinds efficiently as shown by the SDS/urea−PAGE patterns in Figure 6A. When the reconstituted samples are illuminated, the level of D1 degradation increases and that of D1 aggregation decreases compared with the nonreconstituted Tris-treated samples. These results are shown in Figure 6B,C. The effect of reconstitution on D1 degradation was more prominent upon illumination with intermediate light than with strong light, probably because the bound OEC33 is partially released from PS II during the strong illumination (data not shown). Almost the same results were obtained upon reconstitution of the Tris-treated PS II core complexes with the OEC33 (data not shown).

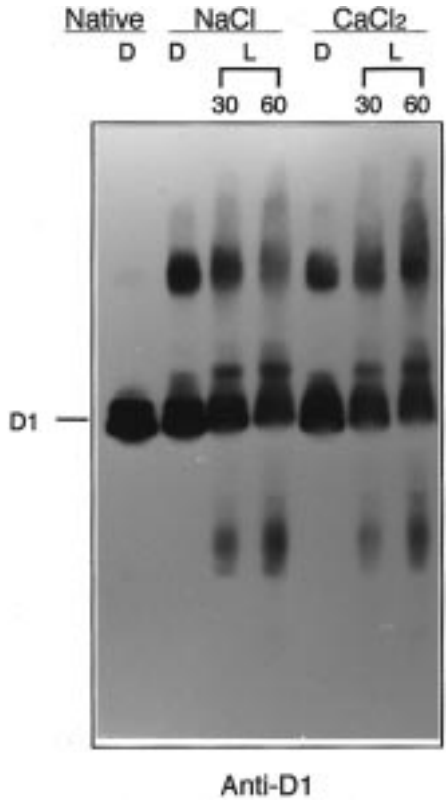

FIGURE 5: Light-induced degradation and aggregation of D1 in the PS II-enriched membrane samples treated with NaCl/NH_2OH or $CaCl_2$/NH_2OH. The PS II membranes were treated either with 1 M NaCl/3 mM NH_2OH (pH 6.5) or with 1 M $CaCl_2$/3 mM NH_2-OH (pH 6.5) (denoted as NaCl or $CaCl_2$ at the top of the gel) to remove OEC24, OEC18, and Mn, or to remove all the OEC subunits and Mn, respectively. All the other experimental conditions were the same as those described in the legend to Figure 2.

There is, however, a possibility that proteins other than the OEC33 that are removed from PSII by Tris washing may be involved in the regulation of the light-induced D1 degradation and aggregation. Thus, we also carried out reconstitution experiments of Tris-treated PS II-enriched membranes with the supernatant fraction after Tris washing. In this case, there was not much difference in the observed behavior of D1 compared to after reconstitution with the purified OEC33 (data not shown).

Changes in the Protein Secondary Structural Content after Photoinhibition As Measured by FT-IR Spectroscopy. To examine if the effect of the OEC33 in enhancing the degradation and suppressing the aggregation of D1 is detectable as a protein structural change in PS II, we analyzed Tris- and NH_2OH-treated PS II core complexes by FT-IR measurements. The infrared absorption in the amide I band contains inherent information on the protein secondary structure. Relative contents of various structural domains such as α-helix, β-sheet, and turn can be obtained by pattern recognition analysis of the amide I band compared with a set of known protein standards (*29*, *34*). Figure 7A shows the FT-IR absorption spectra over the range 1600−1700 cm^{-1} for Tris-treated PS II core complexes during a 20 min illumination period. Subtle changes are revealed in the second-derivative spectra as shown in Figure 7B, where the major feature at 1657 cm^{-1} attributed to α-helix decreases with increasing illumination time. Analysis of the spectra in terms of relative protein secondary structure content reveals a gradual decrease in the α-helix domain and a parallel increase in the β-sheet domain with increasing illumination time. As given in Table 1, the relative change in the protein secondary structure after 10 min illumination is about 10% in the Tris-treated PS II core and only a few percent in the native and NH_2OH-treated PS II core samples. Longer illumination periods of the latter samples will lead to greater changes in the protein secondary structures, and all samples will reveal major structural changes after very long illumination times (data not shown). These observations are consistent with the notion that the presence of the OEC33 is effective in stabilizing the secondary structure of PS II, at least in the early stages of photoinhibition.

Light-Induced Structural Change of CP43 in Tris-Treated PS II Samples. The FT-IR measurements indicate that a large conformational change initially takes place during the illumination of Tris-treated samples. The SDS/urea−PAGE protein pattern under these conditions reveals a significant decrease in the amount of CP43 as shown in Figure 1. This change is probably due to CP43 degradation itself and cross-linking with D1 and CP47 (*20*). More intriguing, however, is that the loss of CP43 is not as extensive after NH_2OH treatment, as also shown in Figure 1. These results suggest that the OEC33 may also prevent the degradation of CP43 during illumination. There is apparently no loss of CP47 under the same conditions. Thus, it is possible that the absence of OEC33 induces a significant structural change in CP43, which then affects PS II in a way that decreases the efficiency of D1 degradation during strong illumination.

DISCUSSION

The OEC33 is one of the extrinsic proteins of PS II located on the lumenal surface of the thylakoid membranes. Its function has been assigned to stabilizing the catalytic Mn in the O_2-evolving mechanism (*35*−*38*) since upon its removal there is a rapid loss of the functional Mn and inactivation of O_2 evolution (*30*). In cyanobacteria, the OEC33 is also required for optimizing O_2 evolution but appears not to be essential for the mechanism because OEC33-deletion mutants can grow photoautotrophically (*39*−*41*). It has also been suggested that this protein is important in stabilizing PS II structure (*42*), particularly the CP43 during early stages of chloroplast development and during photoinhibition (*20*, *43*).

During strong illumination of inside-out thylakoids from spinach and *Chlamydomonas* cells, the OEC33 is partially released from PS II in parallel with the degradation of the D1 protein (*22*, *23*). These results were obtained under the conditions of the so-called acceptor-side photoinhibition where samples are illuminated with strong light in the absence of artificial electron acceptors. Interestingly, the released OEC33 appears to be stored in the lumen of the thylakoid until needed after the reinsertion of functionally competent D1 (*22*). The presence of a pool of free OEC proteins in the thylakoid lumen and their competence in the assembly of PS II complexes have been demonstrated recently using *in organello* protein transport assays (*44*).

The site of cleavage of the D1 protein during acceptor-side photoinhibition is thought to be in the DE loop that is exposed toward the stromal side of the membrane (*3*, *4*, *45*, *46*). Since the OEC33 is known to be associated with the lumenal-exposed C-terminal end of the E-helix of D1 (*24*),

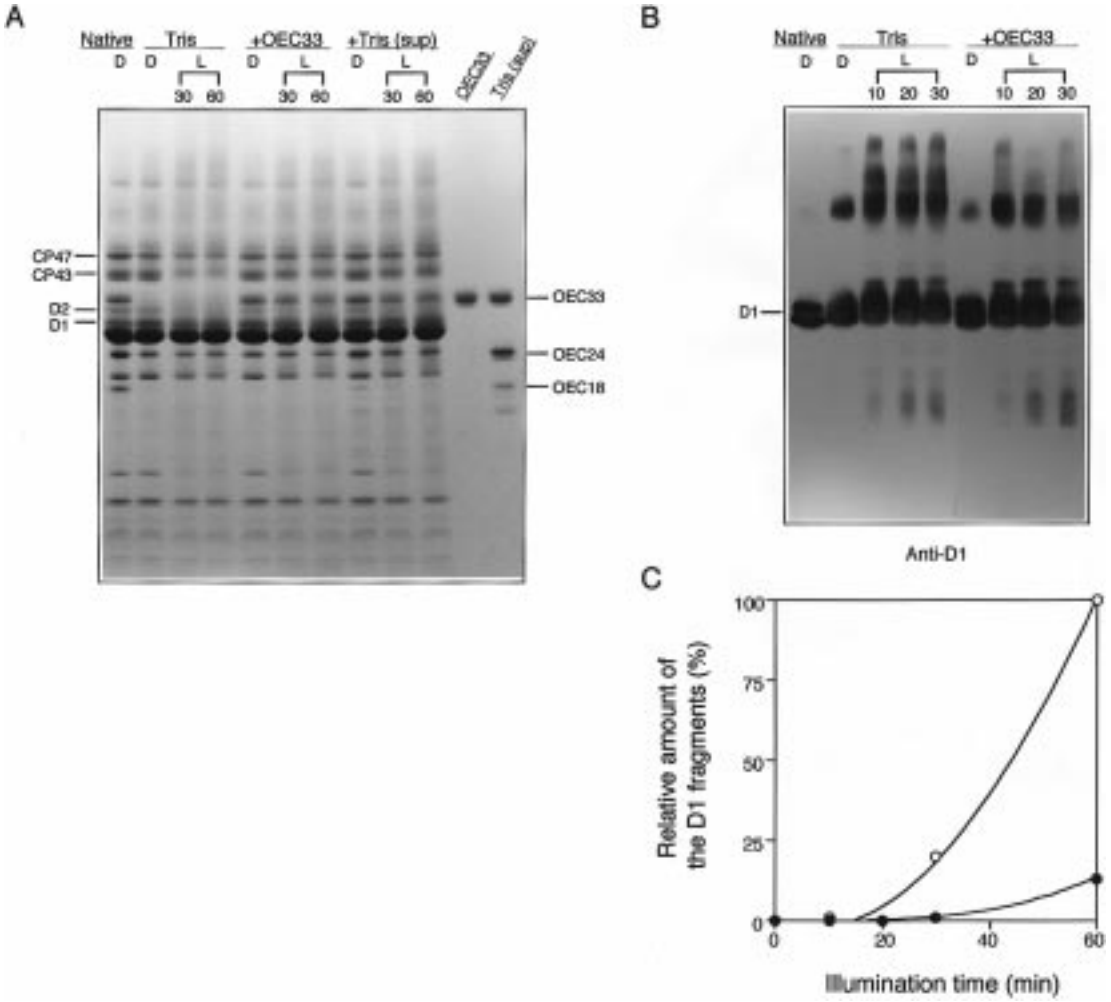

FIGURE 6: Light-induced degradation and aggregation of D1 in Tris-treated PS II-enriched membrane samples before and after reconstitution with the OEC33. (A) A SDS/urea−PAGE gel showing that OEC33 is rebound to the Tris-treated PS II membranes by the reconstitution. Tris, +OEC33, and +Tris (sup) indicate the Tris-treated sample, the OEC33-reconstituted sample, and the sample reconstituted with the supernatant after Tris washing, respectively. Saturating amounts of the purified OEC33 and supernatant fraction were used for the reconstitution. The isolated OEC33 and the supernatant of Tris washing are shown in the two lanes at the right side of the gel. (B) Typical fluorograms showing the degradation and aggregation of D1 by the illumination of nonreconstituted (Tris) and reconstituted (+OEC33) PS II samples. D and L denote the dark control and illuminated sample, respectively. Samples were illuminated with an intermediate light (1000 μE m^{-2} s^{-1}) for 10, 20, and 30 min. (C) Relative amount of D1 degradation fragments plotted as a function of illumination time. The total amounts of all the major fragments of D1 were estimated by a Personal Scanning Imager PD110 (Molecular Dynamics, USA). The data are the average of five independent experiments, and the deviations are within 10%.

cleavage in the DE loop would have to transmit a configurational change through the transmembrane E helix in order to cause the release of OEC33 from the PS II complex.

In contrast, during donor-side photoinhibition, long-lived, strong oxidants are generated upon perturbation of the catalytic Mn. It is likely that donor-side photoinhibition is initiated by the release of the OEC33. This has been readily demonstrated through in vitro studies where the OEC33 and the catalytic Mn can be selectively removed biochemically. However, the complete removal of the OEC33 and catalytic Mn is probably not the usual sequence of events during photoinhibition in vivo even though release of the OEC33 can apparently be caused by the degradation of the D1 protein itself (*22*). In the present study, we suggest that the retention of the OEC33 by PS II is actually required for the efficient degradation of the D1 protein in situ and that its observed release from PS II occurs at a later stage during in vivo photoinhibition. This conclusion comes from the following results.

(1) After strong illumination of NH_2OH-treated PS II samples, no significant release of the OEC33 is observed (Figure 1) even though D1 is extensively degraded by the high light conditions (Figure 2). In addition, the release of the OEC33 did not occur to a significant extent during the acceptor-side photoinhibition of untreated PS II membranes and PS II core complexes after strong illumination for up to 2 h in the absence of electron acceptors (data not shown).

(2) After strong illumination of Tris-treated or $CaCl_2/NH_2$-OH-treated PS II samples where the OEC33 is released as well as the catalytic Mn, the extent of D1 degradation is less compared with NH_2OH-treated or $NaCl/NH_2OH$-treated samples where the OEC33 is retained (Figures 2, 3, and 5). On the other hand, OEC33-depleted samples show much more aggregation of the D1 protein than OEC33-retaining samples.

(3) Reconstitution of Tris-treated PS II samples with purified OEC33 protein reveals that the presence of the

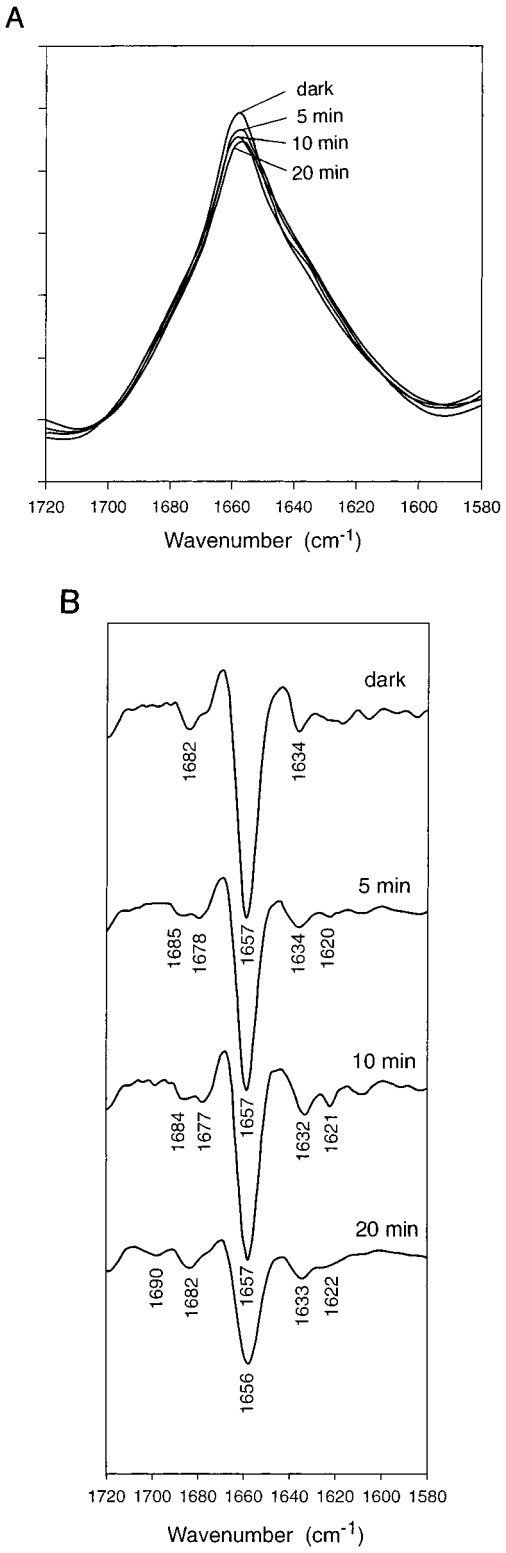

FIGURE 7: FT-IR spectra measured between 1580 and 1720 cm^{-1} for PS II core samples during strong illumination. The absorption (A) and second-derivative (B) spectra are shown for the dark control and after 5, 10, and 20 min illumination with strong white light. The data were averaged over 100 scans. The illumination conditions are the same as those described in Figure 1. Other details are given in the text.

OEC33 does indeed stimulate the degradation of D1 and suppress the formation of aggregates (Figure 6).

If the OEC33 regulates D1 turnover as the above evidence indicates, then the question becomes what is the role of the OEC33 in this process. One possible explanation is that the OEC33 helps to maintain the structural integrity of PS II during photoinhibition. For the efficient removal and replacement of the damaged D1, the other subunits in PS II probably need to maintain their original conformation to avoid inter-polypeptide reactions that could retard the integration of newly synthesized D1 protein. Thus, the absence of OEC33 allows more extensive aggregation of D1 during illumination and thereby decreases the extent of degradation (Figures 2−5). In this interpretation, the OEC33 functions as a 'molecular chaperone' which prevents damaged polypeptides from interacting or folding incorrectly.

Table 1: Percent Change in Protein Secondary Structures for Variously Treated PS II Core Samples after 10 min Strong Illumination[a]

sample	% α-helix	% β-sheet
native PS II core	−3	+2
Tris-treated PS II core	−9	+8
NH_2OH-treated PS II core	−1	+1

[a] Relative changes in the content of α-helix and β-sheet structure were derived from FT-IR spectra as shown in Figure 7 by pattern recognition analysis of the amide I band using a set of 15 protein standards of known secondary structure (see Zhang et al. for details). + and − indicate relative increase and decrease, respectively.

In the comparison of D1 degradation between Tris-treated and NH_2OH-treated samples, the level of secondary D1 degradation may be of importance. It is possible that in the Tris-treated samples more efficient secondary proteolysis takes place, which would cause an apparent reduction in the amount of the primary D1 degradation products observed. However, when D1 degradation is analyzed at 4 °C, the pattern of degradation products (including the relative ratios) is almost the same at 25 °C (Figure 4). Although the total amount of degradation products at room temperature was smaller than that at lower temperature, it can be mostly attributed to the increased aggregation of D1. Considering the results of D1 degradation at the lower temperature, as well as the time course of D1 degradation (Figure 3), we conclude that the lower level of D1 degradation products in Tris-treated samples is not due to additional proteolysis under these conditions.

The active role of the OEC33 in D1 degradation is also demonstrated by reconstitution experiments (Figure 6). However, it is known that Tris washing releases not only the OEC subunits and the catalytic Mn, but also a 10 kDa protein or the *psb* R gene product (*47*). This protein appears to be involved in the binding of the OEC24 to the PS II complex. When the concentrated supernatant after Tris washing is reconstituted with the Tris-treated sample, the level of D1 degradation and aggregation was not much different from the purified OEC33-reconstituted samples (data not shown). From these results, we conclude that OEC33 is the principal component involved in the regulation of D1 degradation.

An alternate explanation for the role of the OEC33 in stimulating D1 degradation during photoinhibition is that it stabilizes the possible PS II protease that is responsible for D1 cleavage. A serine-specific protease is thought to be involved since D1 degradation is considerably slowed at low temperature and is reduced by serine-protease inhibitors (*4*, *48*−*50*). Recently, CP43 was suggested to function as the

protease, at least during acceptor-side photoinhibition (*51*). It is possible that the OEC33 stabilizes the putative enzymatic activity and thereby, by its presence, enhances D1 degradation. Certainly, a large difference in the amount of CP43 is observed between Tris- and NH_2OH-treated samples after strong illumination (Figure 1) where there is significant less CP43 in the absence of the OEC33. It was shown earlier that the OEC33 protects CP43 from photodegradation (*20*). We are not able to judge from the present data whether the OEC33 stimulates the primary degradation of D1 or its subsequent degradation. The finding that not only the degradation products of D1 itself but also those of D1−cytochrome b_{559} cross-linked products are detectable at the lower molecular mass range by immunoblotting with the antibodies against D1 makes it difficult to identify the exact origin of the degradation fragments (data not shown).

Active oxygen species have been proposed to cause the damage and aggregation of D1 (*20*, *52*, *53*). In donor-side photoinhibition, electrons are not derived from water, but active oxygen species are probably still produced at the reducing side of PS II. Since the integrity of the PS II complex is required for electron transport, the OEC33 possibly assists in the production of active oxygen species by maintaining the functional structure of PS II. A study on the role of the OEC33 in the production of toxic oxygen molecules during donor-side photoinhibition is currently being undertaken.

Protein secondary structural changes of PS II can be measured by FT-IR (*29*, *54*). The first FT-IR measurements of D1 conformational changes during strong illumination were carried out using PS II reaction center samples (*55*). In this study, the α-helix and β-sheet contents changed by as much as 30%. It was suggested that the photoinduced degradation of D1 involves an autocatalytic mechanism that depends on the composition of the suspending medium (*50*). However, for the PS II core samples which we used here, the FT-IR detectable conformational changes were triggered only by illumination. Conformational changes in PS II may be dependent not only upon the cleavage of polypeptides but also upon light-induced structural rearrangements. The conformational changes monitored in the PS II core samples by FT-IR spectroscopy are probably not due to secondary structural changes in the D1 protein only, because we could not detect any major changes in the amide I band during the illumination of NH_2OH-treated samples where significant D1 degradation still takes place. Thus, we assume that the light effects on the FT-IR spectra of Tris-treated samples represent more extensive conformationalal changes in PS II triggered by the photodamage to the D1 protein. Degradation to the CP43 in the absence of OEC33 may be central to these conformational changes.

In conclusion, we suggest that the structural integrity of the PS II complex that is maintained by the presence of the OEC33 is required for the efficient turnover of the D1 protein after it has been damaged by strong illumination. Degradation of the D1 protein and the removal of its breakdown products from the PS II complex are crucial steps in the repair cycle, and the OEC33 probably plays an essential role in regulating these steps.

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REFERENCES

1. Ohad, I., Kyle, D. J., and Arntzen, C. J. (1984) *J. Cell Biol. 99*, 481−485.
2. Kyle, D. J. (1987) in *Photoinhibition* (Kyle, D. J., Osmond, C. B., and Arntzen, C. J., Eds.) pp 197−226, Elsevier, New York.
3. Barber, J., and Andersson, B. (1992) *Trends Biochem. Sci. 17*, 61−66.
4. Aro, E.-M., Virgin, I., and Andersson, B. (1993) *Biochim. Biophys. Acta 1143*, 113−134.
5. Vass, I., Styring, S., Hundal, T., Koivuniemi, A., Aro, E.-M., and Andersson, B. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 1408−1412.
6. Ohad, I., Adir, N., Koike, H., Kyle, D. J., and Inoue, Y. (1990) *J. Biol. Chem. 265*, 1972−1979.
7. Kyle, D. J., Ohad, I., and Arntzen, C. J. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4070−4074.
8. Zer, H., and Ohad, I. (1995) *Eur. J. Biochem. 231*, 448−453.
9. Nakajima, Y., Yoshida, S., Inoue, Y., and Ono, T. (1996) *J. Biol. Chem. 271*, 17383−17389.
10. Blubaugh, D. J., and Cheniae, G. M. (1990) *Biochemistry 29*, 5109−5118.
11. Klimov, V. V., Shafiev, M. A., and Allakhverdiev, S. I. (1990) *Photosynth. Res. 23*, 59−65.
12. Theg, S. M., Filar, L. J., and Dilley, R. A. (1986) *Biochim. Biophys. Acta 849*, 104−111.
13. Jegerschold, C., and Styring, S. (1991) *FEBS Lett. 280*, 87−90.
14. Callahan, F. E., Becker, D. W., and Cheniae, G. M. (1986) *Plant Physiol. 82*, 61−269.
15. Eckert, H.-J., Geiken, B., Bernarding, J., Napiwotzki, A., Eichler, H.-J., and Renger, G. (1991) *Photosynth. Res. 27*, 97−108.
16. Kettunen, R., Tyystjaervi, E., and Aro, R.-M. (1996) *Plant Physiol. 111*, 1183−1190.
17. Jegerschold, C., Virgin, I., and Styring, S. (1990) *Biochemistry 29*, 6179−6186.
18. Schuster, G., Timberg, R., and Ohad, I. (1988) *Eur. J. Biochem. 177*, 403−410.
19. Barbato, R., Friso, G., Rigoni, F., Frizzo, A., and Giacometti, G. M. (1992b) *FEBS Lett. 311*, 33−36.
20. Yamamoto, Y., and Akasaka, T. (1995) *Biochemistry 43*, 9038−9045.
21. Adir, N., Shochat, S., and Ohad, I. (1990) *J. Biol. Chem. 21*, 12563−12568.
22. Eisenberg-Domovich, Y., Oelmüller, R., Herrmann, R. G., and Ohad, I. (1995) *J. Biol. Chem. 270*, 30181−30186.
23. Hundal, T., Virgin, I., Styring S., and Andersson, B. (1990) *Biochim. Biophys. Acta 1017*, 235−241.
24. Diner, B. A., Ries, D. F., Cohen, B. N., and Metz, J. G. (1988) *J. Biol. Chem. 263*, 8972−8980.
25. Kuwabara, T., and Murata, N. (1982) *Plant Cell Physiol. 23*, 533−539.
26. Enami, I., Kamino, K., Shen, J.-R., Satoh, K., and Katoh, S. (1989) *Biochim. Biophys. Acta 977*, 33−39.
27. Mackinney, G. (1941) *J. Biol. Chem. 140*, 315−322.
28. Yamamoto, Y. (1988) *Biochim. Biophys. Acta 993*, 165−171.
29. Zhang, H., Yamamoto, Y., Ishikawa, Y., Zhang, W., Fisher, G., and Wydrzynski, T. (1997) *Photosynth. Res. 52*, 215−223.
30. Yamamoto, Y., Doi, M., Tamura, N., and Nishimura, M. (1981) *FEBS Lett. 133*, 265−268.
31. Yamamoto, Y., and Nishimura, M. (1983) in *The Oxygen Evolving System of Photosynthesis* (Inoue, Y., et al., Eds.) pp 229−238, Academic Press, Tokyo.
32. Becker, D. W., Callahan, F. E., and Cheniae, G. M. (1985) *FEBS Lett. 192*, 209−214.

33. Barbato, R., Friso, G., de Laureto, P. P., Frizzo, A., Rigoni, F., and Giacometti, G. M. (1992) *FEBS Lett. 309*, 165−169.
34. Lee, D. C., Haris, P. I., Chapman, D., and Mitchell, R. C. (1990) *Biochemistry 29*, 9185−9193.
35. Murata, N., and Miyao, M. (1985) *Trends Biochem. Sci. 10*, 122−124.
36. Yamamoto, Y. (1988) *J. Biol. Chem. 263*, 497−500.
37. Ghanotakis, D. F., and Yocum, C. F. (1990) *Annu. Rev. Plant Physiol. Plant Mol. Biol. 41*, 255−276.
38. Hansson, O., and Wydrzynski, T. (1990) *Photosynth. Res. 23*, 131−162.
39. Burnap, R. L., and Sherman, L. A. (1991) *Biochemistry 30*, 440−446.
40. Philbrick, J. B., Diner, B. A., and Zilinskas, B. A. (1991) *J. Biol. Chem. 266*, 13370−13377.
41. Vass, I., Cook, K. M., Deak, Z., Mayes, S. R., and Barber, J. (1992) *Biochim. Biophys. Acta 1102*, 195−201.
42. Mayfield, S. P., Bennoun, P., and Rochaix, J.-D. (1987) *EMBO J. 6*, 313−318.
43. Hiramatsu, H., Mizobuchi, A., Mori, H., and Yamamoto, Y. (1991) *Plant Cell Physiol. 32*, 881−889.
44. Hashimoto, A., Yamamoto, Y., and Theg, S. M. (1996) *FEBS Lett. 391*, 29−34.
45. Mattoo, A. K., Marder, J. B., and Edelman, M. (1989) *Cell 56*, 241−246.
46. Greenberg, B. M., Gaba, V., Mattoo, A.K., and Edelman, M.-(1987) *EMBO J. 6*, 2865−2869.
47. Lautner, A., Klein, R., Ljungberg, U., Reilaender, H., Bartling, D., Andersson, B., Reinke, H., Beyreuther, K., and Herrmann, R. G. (1988) *J. Biol. Chem. 263*, 10077−10081.
48. Aro, E.-M., Hundal, T., Carlberg, I., and Andersson, B. (1990) *Biochim. Biophys. Acta 1019*, 269−275.
49. Virgin, I., Ghanotakis, D. F., and Andersson, B. (1990) *FEBS Lett. 269*, 45−48.
50. Shipton, C. A., and Barber, J. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 6691−6695.
51. Salter, A. H., Virgin, I., Hagman, A., and Andersson, B. (1992) *Biochemistry 31*, 3990−3998.
52. Miyao, M., Ikeuchi, M., Yamamoto, N., & Ono, T. (1995) *Biochemistry 34*, 10019−10026.
53. Chen, G.-X., Kazimir, J., and Cheniae, G. M. (1992) *Biochemistry 31*, 11072−11083.
54. Haris, P. I., and Chapman, D. (1992) *Trends Biochem. Sci. 17*, 328−333.
55. He, W.-Z., Newell, W. R., Haris, P. I., Chapman, D., and Barber, J. (1991) *Biochemistry 30*, 4552−4559.

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