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Turnover of the aggregates and cross-linked products of the D1 protein generated by acceptor-side photoinhibition of photosystem II

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Abstract

It is known that the reaction-center binding protein D1 in photosystem (PS) II is degraded significantly during photoinhibition. The D1 protein also cross-links covalently or aggregates non-covalently with the nearby polypeptides in PS II complexes by illumination. In the present study, we detected the adducts between the D1 protein and the other reaction-center binding protein D2 (D1/D2), the α-subunit of cyt b_{559} (D1/cyt b_{559}), and the antenna chlorophyll-binding protein CP43 (D1/CP43) by SDS/urea-polyacrylamide gel electrophoresis and Western blotting with specific antibodies. The adducts were observed by weak and strong illumination (light intensity: 50–5000 μE m^{-2} s^{-1}) of PS II membranes, thylakoids and intact chloroplasts from spinach, under aerobic conditions. These results indicate that the cross-linking or aggregation of the D1 protein is a general phenomenon which occurs in vivo as well as in vitro with photodamaged D1 proteins. We found that the formation of the D1/D2, D1/cyt b_{559} and D1/CP43 adducts is differently dependent on the light intensity; the D1/D2 heterodimers and D1/cyt b_{559} were formed even by illumination with weak light, whereas generation of the D1/CP43 aggregates required strong illumination. We also detected that these D1 adducts were efficiently removed by the addition of stromal components, which may contain proteases, molecular chaperones and the associated proteins. By two-dimensional SDS/urea-polyacrylamide gel electrophoresis, we found that several stromal proteins, including a 15-kDa protein are effective in removing the D1/CP43 aggregates, and that their activity is resistant to SDS.

Keywords: D1 protein; Photoinhibition; Photosystem II; Protein cross-linking and aggregation; Protein turnover; Photosynthesis

Abbreviations: PS II, photosystem II; D1, the reaction center-binding protein of PS II; Pheo, the primary electron acceptor pheophytin in PS II; P680, the primary electron donor chlorophyll in PS II; Tyr_Z, tyrosine secondary electron donor molecule in PS II; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; ECL, enhanced chemiluminescence; OEC33, the extrinsic 33-kDa protein subunit constituting the oxygen-evolving complex of PS II; Tris, tris(hydroxymethyl)aminomethane; MES, 2-(*N*-morpholino)-ethanesulfonic acid; CP43 and CP47, the antenna chlorophyll-binding proteins of PS II with molecular masses of 43 and 47 kDa, respectively

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1. Introduction

Visible light is essential for photosynthesis as the source of energy which drives the photochemical reactions in the primary process of photosynthesis. However, excessive exposure of chloroplasts to the light causes significant damage to the photosynthetic machinery and inhibits electron transport in chloroplasts. This phenomenon is referred to as photoinhibition of photosynthesis, and photosystem (PS) II has been shown to be the primary target of the photoinhibition. The photodamaged reaction center-binding protein D1 of PS II is degraded and finally replaced by newly synthesized D1 proteins by an efficient repair process of the D1 protein [1–4]. It was also shown that the D1 protein turns over fast even under weak illumination where light causes no significant stress [5–8]. Thus, the rapid turnover of the D1 protein is a process taking place in a wide range of light conditions, and it is reasoned that the rapid turnover of the D1 protein represents one of the strategies for protection of PS II from extensive photodamage, especially when the light is stressful for plants.

The details of photoinhibition by strong visible light have been studied in vitro for the most part. Thylakoid membranes, PS II membranes, PS II core complexes and PS II reaction center complexes have been used for analyses of the degradation of the D1 protein during strong illumination. Two possible mechanisms have been suggested to explain photoinhibition in PS II. In acceptor-side mechanism, strong illumination causes the over-reduction of PS II. It is likely that photodamage to the D1 protein is due to singlet oxygen molecules formed after charge recombination between the reduced primary acceptor $Pheo^-$ and the oxidized primary electron donor $P680^+$ [9] and/or to the superoxide anion radicals produced by donation of electrons to molecular oxygen on the reducing side of PS II [10], although experimental evidence supporting the latter case is not yet available. The site of damage in the D1 protein caused by the donor-side photoinhibition is suggested to be on the stroma-exposed DE loop of the protein, and a 23-kDa N-terminal and a 10-kDa C-terminal fragments are formed by the cleavage [11]. By contrast, in donor-side photoinhibition, damage to the D1 protein is caused by endogenous cationic radicals, such as $P680^+$ and Tyr_Z^+, that are generated by strong illumination [12]. Inactivation of the oxygen-evolving site of PS II is a prerequisite for the photodamage to the D1 protein. The cation radicals damage the D1 protein at the lumen-exposed AB loop, producing a 10-kDa N-terminal and a 24-kDa C-terminal fragments of the D1 protein [11]. Degradation products of 16–18 kDa region were also observed in vitro, which may be due to the cleavage in the lumenal loop connecting helices C and D [13–15].

Recently, we reported that significant aggregation of the damaged D1 protein to the surrounding polypeptides occurs as well during the illumination of PS II membranes [16,17]. A part of the aggregates may consist of covalently cross-linked products. In fact, cross-linking of the D1 protein and the α-subunit of cyt b_{559} was reported previously [18,19]. These aggregates and cross-linked products of the D1 protein can be detected by sodium dodecyl sulfate/urea-polyacrylamide gel electrophoresis (SDS/urea-PAGE), Western blotting with antibodies specific to the D1 protein, and the subsequent high-sensitivity fluorography with enhanced chemiluminescence (ECL) [16,17].

The molecular mechanism of cross-linking and aggregation is unknown. Oxidation of amino acids probably occurs first and covalent binding of the D1 protein via the oxidized amino acids to the nearby polypeptide follows. Both active oxygen molecules and endogenous cationic radicals are likely to be involved in the oxidation of amino acids. The relationship between the degradation and aggregation of the D1 protein is not clear. It is possible that the protein aggregation or cross-linking step represents a mechanism for circumventing protein degradation and that the adducts of the D1 protein, once formed, are not processed any further by a specific protease or proteases. If such is the case, the formation of the adducts of the D1 protein should compete with the degradation of the D1 protein and, therefore, the formation of the aggregates might limit the turnover of the damaged D1 proteins. There is also an argument that the aggregation and cross-linking of the D1 protein is not a process occurring in vivo. In green alga *Dunaliella salina*, photodamaged D1 pro-

teins were shown to form 160-kDa protein complexes composed of D1, D2 and other unknown proteins, and the formation of the high molecular weight aggregates was suggested to be an event involved in the damage and repair cycle of the D1 protein [20].

In the present study, we showed that the adducts of the D1 protein, namely D1/D2 heterodimers, cross-linked products between the D1 protein and the α-subunit of cyt b_{559} and aggregates of the D1 protein and CP43 are formed not only in the PS II membranes, but also in thylakoids and intact chloroplasts during illumination. We also demonstrated that the aggregates and cross-linked products of the D1 protein that are formed by illumination under aerobic conditions, probably by acceptor-side photoinhibition, are efficiently removed by the addition of the stromal fraction. Several stromal factors were identified, which are responsible for the removal of these adducts.

2. Materials and methods

2.1. Sample preparation and illumination conditions

Intact chloroplasts, thylakoids and PS II membranes were prepared from spinach according to the methods reported previously [21,22]. Intact chloroplasts and thylakoids were used fresh for the following photoinhibition experiments, while PS II membranes were frozen before the photoinhibition experiments because not much difference was detected in the pattern of proteins in SDS/urea-PAGE and Western blotting between the fresh and frozen PS II samples. To induce donor-side photoinhibition of PS II, the samples were treated with 0.8 M Tris (pH 9.0) [16]. For photoinhibition, we suspended the samples in a solution of 0.4 M sucrose, 10 mM NaCl and 40 mM MES-NaOH (pH 6.5), adjusted to 0.25 mg chlorophyll ml^{-1}, put them in microtubes kept in a water bath and then illuminated with white light (50–5000 μE m^{-2} s^{-1}) from a fluorescent lamp or the bulb of a slide projector at 25°C. The optical path of the suspension of the samples was 17 mm. Where indicated, samples were illuminated under the anaerobic conditions which were achieved by the addition of 0.1 mg ml^{-1} catalase, 0.1 mg ml^{-1} glucose oxidase and 10 mM glucose to the sample.

2.2. Isolation of stromal fraction

For isolation of the stromal fraction, the chloroplasts were suspended in a solution of 5 mM $MgCl_2$ and 10 mM MES-NaOH (pH 6.5) at 2.0 mg chlorophyll ml^{-1}. The suspension was incubated for 30 min on ice and then centrifuged at $10\,000 \times g$ for 10 min. The resultant supernatant was centrifuged again at $40\,000 \times g$ for 1 h and the final supernatant was used as the stromal fraction. The stromal fraction was used fresh in all the experiments.

2.3. SDS/urea-polyacrylamide gel electrophoresis and two-dimensional gel electrophoresis

SDS/urea-PAGE and Western blotting were carried out as described previously [16]. Before PAGE, the samples were dissolved in a lysis buffer containing 8 M urea, 5% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 5 mM EDTA, 5% (w/v) sucrose and 125 mM Tris-HCl (pH 6.8) at the concentration of 0.5 mg chlorophyll ml^{-1}, and incubated for 30 min at room temperature. The stacking and resolving gels (5% and 10–20% acrylamide, respectively) contained 6 M urea. Samples equivalent to 2.5 μg chlorophyll were loaded to each lane of the gels. Proteins after immunoblotting were detected with enhanced chemiluminescence reagents (ECL; Amersham, Japan). Two-dimensional gel electrophoresis was performed in two kinds of experiment as described by Barbato et al. [18]. In the first experiment, where the effects of the stromal fraction on the D1 protein and on the adducts of the D1 protein were examined, 13% polyacrylamide was used for both the first and second dimensions. In the first-dimensional electrophoresis, proteins in the illuminated PS II membranes were separated according to their molecular weights as usual. For the second-dimension electrophoresis, a given amount of the stromal fraction was included in the stacking gel. When the sample reached the bottom of the stacking gel, the current was turned off for 45 min to allow digestion or processing of the sample by the stromal fraction or by trypsin. After 45 min, electrophoresis was resumed and the products of processing or digestion were separated in the resolving gel. In the second experiment, where the stromal factors active in removing the adduct of the D1 protein were sought, 10–20% linear gradient

polyacrylamide was used in the resolving gels for both the first- and second-dimensional analyses. In the second-dimensional gel, illuminated PS II membranes were included in the stacking gel, and a strip of the gel, which was obtained by the first-dimensional gel electrophoresis and contained the separated stromal fraction, was layered on the top of the stacking gel. Then, the second-dimensional electrophoresis was started and when the stromal proteins reached the bottom of the stacking gel, current was turned off for 45 min to allow incubation of the illuminated PS II samples with the stromal fraction. After that, electrophoresis was resumed and the PS II membranes were separated in the resolving gel. By Western blotting with antibody against the D1 protein and fluorography with ECL, the stromal components responsible for the removal of the aggregates and cross-linked products of the D1 protein were detected as transparent bands in the dark background of the D1 adducts in the fluorogram of the second-dimensional gel. The amounts of the D1 adducts were quantified with a Personal Scanning Imager PD110 (Molecular Dynamics, USA). The antibody raised against the C-terminal part of the D1 protein from pea, which is a kind gift from Dr. P. Nixon of Imperial College of Science, Technology and Medicine, UK, was used in the Western analyses. Chlorophyll was quantified in 80% acetone with the absorption coefficient reported by Mackinney [23].

3. Results

3.1. Aggregation and cross-linking of the D1 protein with the nearby polypeptides in PS II during illumination

In addition to the degradation fragments, the aggregates and cross-linked products of the D1 protein were detected in the illuminated intact chloroplasts, thylakoids and PS II membranes, by SDS/urea-PAGE and Western analysis with specific antibody against D1 (Fig. 1). The comparison between the time courses for the appearance of the adducts during illumination of the samples shows that the band of D1/D2 heterodimer appeared first, followed by the band of a 41-kDa protein representing the cross-linked products between the D1 protein and the α-subunit of cyt b_{559} [18,19] by the illumination with intermediate light (intensity, 500 μE m^{-2} s^{-1}) (Fig. 1A–C). These adducts were detected also by the illumination with weak light (intensity, 50 μE m^{-2} s^{-1}) (Fig. 3). On the other hand, the band of larger aggregates of the D1 protein, having apparent molecular weights of 70–100 kDa, were detected only by strong illumination of the samples (light intensity, 1000–5000 μE m^{-2} s^{-1}) (Fig. 1C).

To identify the polypeptides that aggregate with the D1 protein to yield the larger adducts, we examined the cross-reactivities of the aggregates with various monospecific antibodies raised against the polypeptides of PS II other than the D1 protein. The antibody against CP43 cross-reacted with the aggregates, while antibodies against CP47, the D2 protein and cyt b_{559} did not (data not shown). Thus, the larger aggregates are probably formed by association of the D1 protein with CP43.

The exact ratio of the adducts and degradation products of the D1 protein detected here remains to be determined. Rough estimation shows that about 10% of the D1 protein may become the adducts (data not shown). The amounts of the adducts of the D1 protein seem to exceed those of the degradation products of the D1 protein. This is more clear when we illuminate PS II samples with weak light (Fig. 3). In intact chloroplasts and thylakoids, immunoreactive bands with lower molecular weights (less than 10 kDa) appeared during the illumination (Fig. 1). These bands were not detected in the PS II membranes and remain to be identified.

3.2. Differentiation between the adducts of the D1 protein formed by donor-side and by acceptor-side photoinhibition

The mechanism of aggregation and cross-linking of the D1 protein is not elucidated. Both donor-side and acceptor-side photoinhibition might be involved in the process. To differentiate between the adducts of the D1 protein formed by the two mechanisms, we illuminated native PS II membranes and the Tris-treated PS II membranes with strong light under aerobic and anaerobic conditions (Fig. 2). Under aerobic conditions where acceptor-side photoinhibition is expected, illumination induced the for-

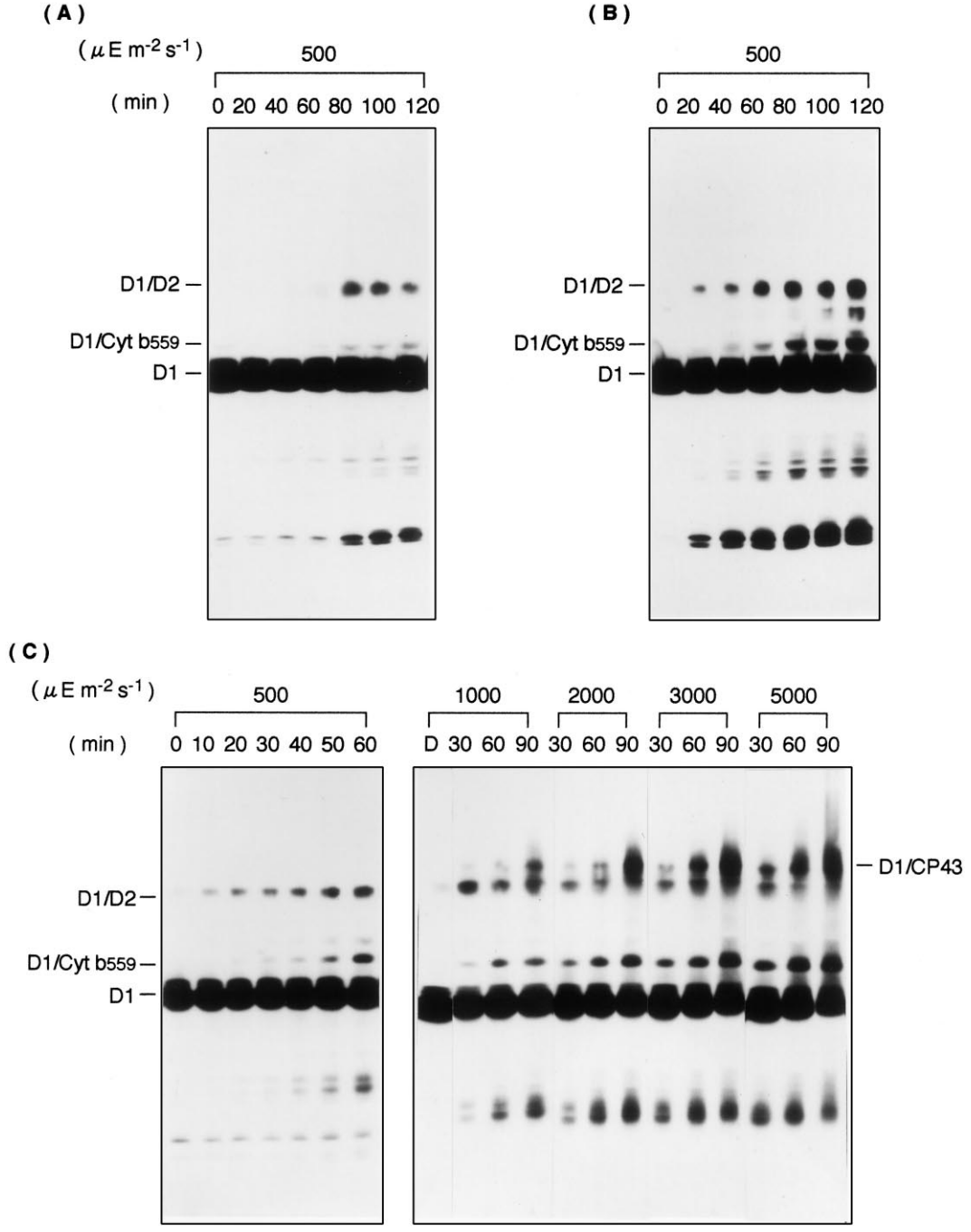

Fig. 1. Profile of the aggregates and the cross-linked products of the D1 protein generated by the illumination of intact chloroplasts (A), thylakoids (B) and PS II membranes (C) from spinach. The samples (0.25 mg chlorophyll ml^{-1}) were illuminated with white light of various intensities (500–5000 μE m^{-2} s^{-1}) for 0–120 min under the aerobic conditions at 25°C, and then subjected to SDS/urea-PAGE and Western blotting with specific antibodies against the C-terminus of the D1 protein. The immunoreactive bands were detected by high-sensitivity fluorography with ECL. The mobilities of the native D1 protein, the 41-kDa cross-linked product of the D1 protein and the α-subunit of cyt b_{559}, the D1/D2 heterodimer and the D1/CP43 aggregates are indicated at the left or both side of the gels.

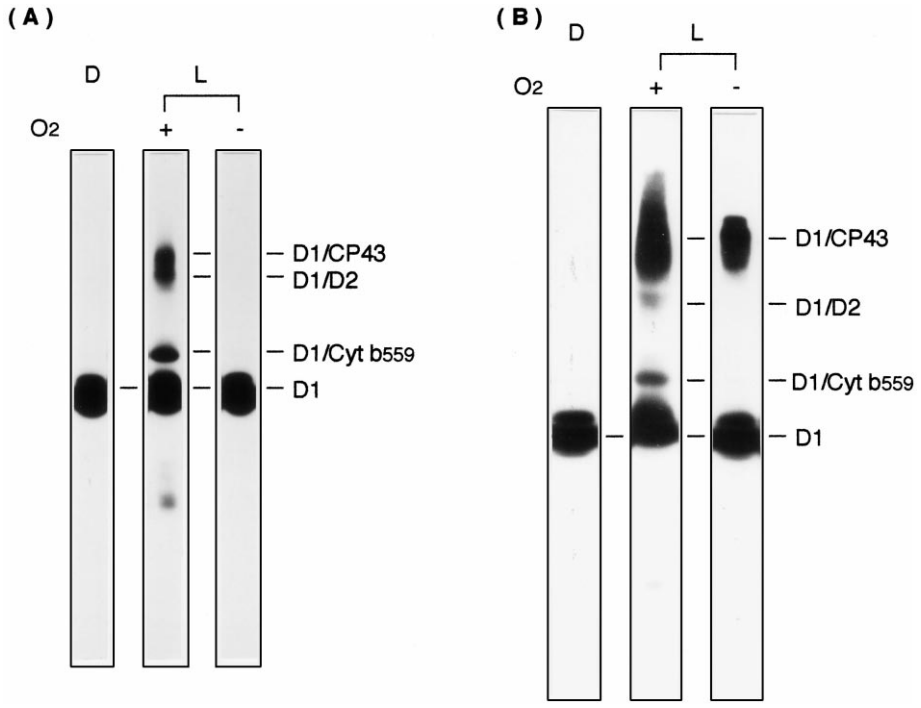

Fig. 2. The effects of oxygen on the formation of the adducts of the D1 protein. Native (A) and Tris-treated PS II membranes (B) were illuminated with strong light (5000 μE m^{-2} s^{-1}) for 20 min under either aerobic or anaerobic conditions (indicated by + and − O_2) at 25°C. After SDS/urea-PAGE and Western blotting with specific antibodies, the polypeptides in PS II were detected with ECL. D and L indicate the dark-control sample and the sample illuminated with strong light, respectively. The mobilities of the native D1 protein, the 41-kDa product of cross-linking between the D1 protein with the α-subunit of cyt b_{559}, the D1/D2 heterodimer, and the D1/CP43 aggregate are indicated at the right of the gels.

mation of the D1/D2 heterodimers, the D1/cyt b_{559} cross-linked products and the D1/CP43 aggregates. The yield of these adducts of the D1 protein was higher in the Tris-treated PS II membranes (Fig. 2B) than in the native PS II membranes (Fig. 2A). It is probably because acceptor-side, as well as donor-side, photoinhibition occurs during the illumination of Tris-treated samples in the absence of added electron acceptors. By the illumination of the Tris-treated PS II membranes, antenna chlorophylls instead of water molecules may be oxidized and electrons are possibly donated to oxygen molecules to produce superoxide radicals. In fact, significant photobleaching of chlorophylls were observed under these conditions (data not shown). Thus, under these conditions, the acceptor-side photoinhibition may occur in addition to the donor-side photoinhibition. It should be noted that under anaerobic conditions, only the D1/CP43 aggregates were formed by the illumination of the Tris-treated PS II membranes. Probably, in the absence of oxygen, illumination induces only the donor-side photoinhibition in the Tris-treated samples. Our results suggest that the D1/CP43 aggregates are formed both during the donor-side and acceptor-side photoinhibition.

3.3. *Removal of the aggregates and cross-linked products of the D1 protein by the addition of stromal fraction*

The aggregation and cross-linking of the D1 protein are more prominent in vitro than in vivo. For example, significant aggregation of the D1 protein was observed in PS II membranes illuminated with strong light, while the extent of the aggregation was less in intact chloroplasts (Fig. 1). A major difference between the isolated PS II membranes and intact chloroplasts is the presence of stroma in the latter samples. Therefore, it is possible that the photoinduced adducts of the D1 protein might be processed by stromal proteases, chaperones or other unknown stromal components in vivo. We examined the effects

of the stromal fraction on the aggregates and cross-linked products of the D1 protein. The PS II membranes were illuminated with either weak (50 μE m^{-2} s^{-1}) or strong light (5000 μE m^{-2} s^{-1}) under aerobic conditions to generate the D1 adducts, and then they were incubated with stromal fraction obtained from intact chloroplasts (Fig. 3). In the presence of the stromal fraction, the amounts of all the aggregates and cross-linked products of the D1 protein decreased significantly, while the level of the native D1 protein did not change apparently. No significant change in the level of the native D1 protein by the addition of the stromal fraction was confirmed by a series of experiments where different amounts of the PS II samples were subjected to SDS/urea-PAGE and Western analyses to avoid the overload or overexposure in ECL fluorography (data not shown).

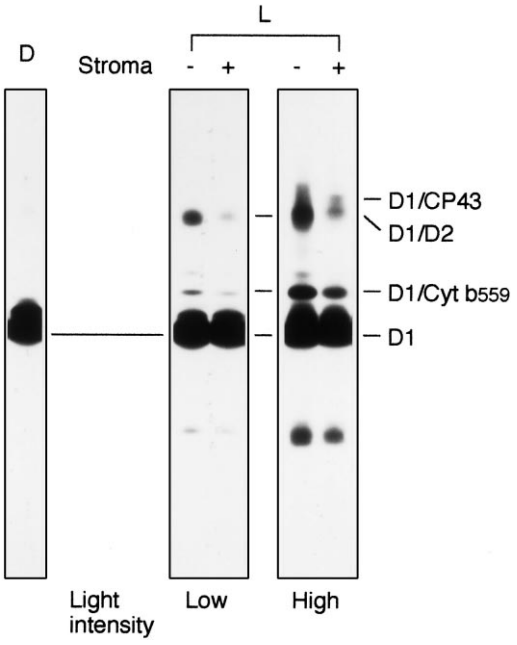

Fig. 3. The effects of a stromal fraction on the adducts of the D1 protein generated by illumination. The PS II membranes were illuminated with either weak (50 μE m^{-2} s^{-1}) or strong light (5000 μE m^{-2} s^{-1}) (indicated at the bottom of the gels) for 50 min under aerobic conditions and then incubated in darkness with (+) and without (−) the stromal fraction for 20 min at 25°C. D and L denote the dark control and the illuminated samples, respectively. Stromal fractions obtained from intact chloroplasts equivalent to 1.0 mg chlorophyll were added to 100 μl of the PS II membranes (concentration, 0.25 mg chlorophyll ml^{-1}) where indicated by +. In the control samples, which are indicated by −, a solution containing 5 mM $MgCl_2$ and 10 mM MES-NaOH (pH 6.5) was added instead of the stromal fraction. The location of the bands of the native D1 protein and the adducts of the D1 protein is shown at the right of the gels.

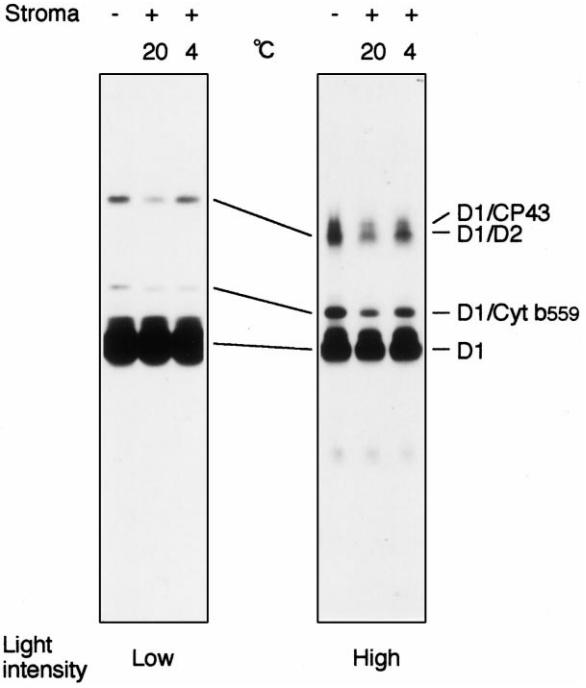

Fig. 4. Effects of temperature on the removal of the adducts of the D1 protein by the stromal fraction. PS II membranes were illuminated with either weak (50 μE m^{-2} s^{-1}) or strong light (5000 μE m^{-2} s^{-1}) under aerobic conditions and incubated with (+) and without (−) the stromal fraction in darkness at 4 or 20°C for 20 min. The amount of the stromal fraction added was the same as that shown in the legend to Fig. 3.

The effects of the stromal fraction depended on the temperature of the reaction medium. When the temperature was lowered from 25°C to 4°C, the rate of loss of the D1 adducts was significantly reduced (Fig. 4). In this figure, we tried to show the discrete bands of D1/D2 and D1/CP43, and to demonstrate the effects of the stromal fraction on these adducts clearly. For that, we had to expose the X-ray film shorter in time in fluorography, which inevitably reduced the level of the degradation products of the D1 protein. The results indicate that some proteinaceous factors in the stromal fraction are involved in the removal of the aggregates and cross-linked products of the D1 protein. Our stromal fraction failed to process the D1/CP43 aggregates formed upon illumination of Tris-treated PS II under anaerobic conditions (data not shown). The stromal components might be able

to process only the D1/CP43 adducts generated at the stromal side of PS II by the action of oxygen radicals produced by the acceptor-side photoinhibition.

3.4. Characterization of the activity that participates in the processing of the adducts of the D1 protein

To see if the action of the stromal fraction is specific to the adducts of the D1 protein and not to the native D1 protein, we carried out two-dimensional gel electrophoresis of photodamaged PS II membranes (Fig. 5). In the Western analysis, the antibody against the C-terminal of the D1 protein was used. The results show that the stromal component(s) can efficiently remove the adducts of the D1 protein but not the native D1 protein. It should be noted here that no off-diagonal spots of the D1 protein were detected below the major D1/CP43 adducts and the native D1 protein. The present data indicate that the stromal factors responsible for the processing of the D1 adducts are stable in the presence of SDS, and can process the D1 adducts efficiently. For comparison, the photodamaged PS II membranes were incubated with trypsin and analyzed by the two-dimensional gel electrophoresis. The native D1 protein was digested by trypsin and several off-diagonal fragments of the D1 protein were produced, while the adducts of the D1 protein were insensitive to trypsin (data not shown). The difference in sensitivity between the D1 adducts and the intact D1 protein to trypsin suggest that the conformation of the D1 protein in the adducts is different from that of the native D1 protein. We also carried out the same experiment using the antibody against the whole D1 protein. The result was the same as that obtained with the antibody against the C-terminal of the D1 protein (data not shown).

As the stromal factors were shown to be SDS-stable, we next carried out another type of two-dimensional SDS/urea-PAGE to identify the stromal component(s) active in processing of the aggregated or cross-linked forms of the D1 protein (Fig. 6). In the first SDS/urea-PAGE, the proteins in the stromal fraction obtained from intact chloroplasts was separated according to their molecular weights. In the second SDS/urea-PAGE, the strip of the first gel was loaded and the proteins in the gel strip were incubated for a certain period in the stacking gel which contained the PS II membranes that had been illuminated with strong light. Removal of the D1 adducts in the second gel by the action of a specific stromal component derived from the first gel should result in a transparent area(s) in the bands of the D1 adducts in the fluorogram of the second

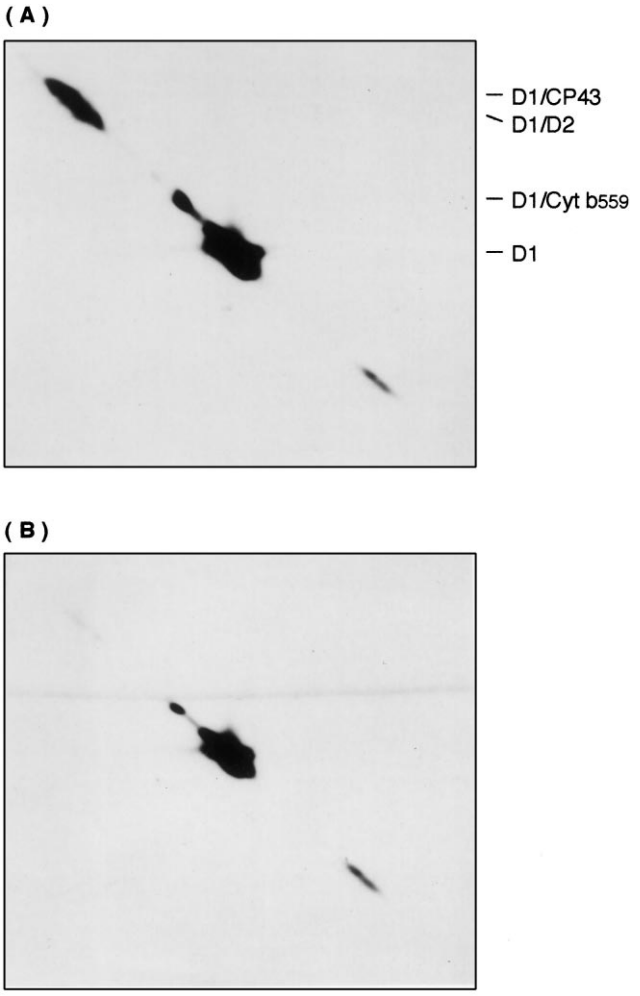

Fig. 5. Fluorograms of the two-dimensional gel electrophoresis of the illuminated PS II membranes incubated with the stromal fraction. The PS II samples were illuminated (intensity, 500 μE m^{-2} s^{-1}) for 30 min and subjected to the first-dimensional SDS/urea-PAGE gel. A strip of the gel containing the illuminated PS II sample, where the proteins were separated from left to right in the order of decreasing their apparent molecular weights, was then applied to the second dimensional gel without (A) and with (B) the incubation with the stromal fraction in the stacking gel. The stromal fraction from intact chloroplasts equivalent to 800 μg chlorophyll was used. The location of the native D1 protein and the adducts of the D1 protein is shown at the right of the second gel.

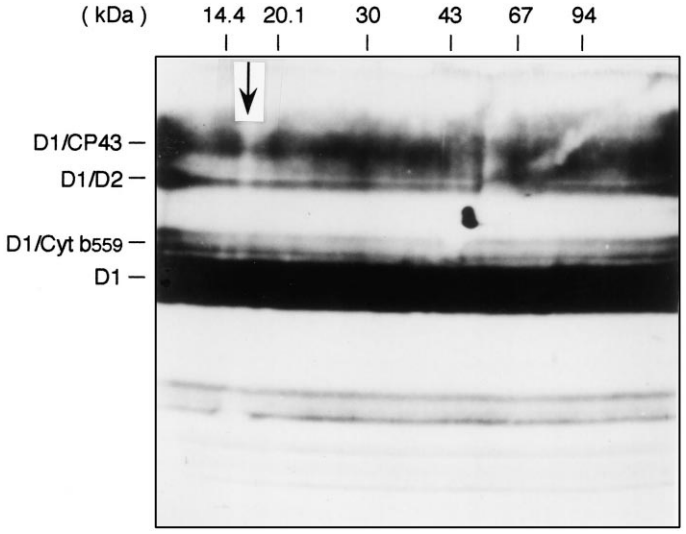

Fig. 6. Identification of the stromal factor that removes the aggregates and cross-linked products of the D1 protein. Two-dimensional SDS/urea-polyacrylamide gel electrophoresis and Western blotting analysis with the antibody against the C-terminal region of D1 protein were performed as described in Section 2. The stromal proteins in the first gel were separated from right to left in the order of decreasing their apparent molecular weights. The mobility of marker proteins in the first dimensional gel is shown at the top of the fluorogram. In the second gel, stromal proteins were incubated with the photodamaged samples for 45 min in the stacking gel, and the proteins in the samples were separated in the resolving gel according to their molecular weights. After Western blotting, with the antibody against the D1 protein and fluorography, the bands of the D1 protein and the adducts of the D1 protein were identified. The transparent bands in the dark backgrounds of the adducts of the D1 protein represent the stromal factor active in removing the adducts. The arrow shows the 15-kDa component in the stromal fraction. The bands of the native D1 protein and the adducts of the D1 protein are shown at the left of the second gel.

gel. By this method, we actually found such several transparent areas in the first-dimensional gel. The most clear spot was seen around 15 kDa.

4. Discussion

In the present study, we detected the photoinduced aggregation and cross-linking of the D1 protein not only in the PS II membranes, but also in thylakoids and intact chloroplasts by SDS/urea-PAGE and Western blotting. Although there is no evidence for the existence of the adducts of the D1 protein in vivo so far, our present observation strongly suggest that the aggregation and cross-linking of the D1 protein with its neighbor polypeptides is a phenomenon occurring in vivo as well as in vitro under the illumination. The mechanism of generation of these D1 adducts in PS II remains to be elucidated. In the acceptor-side photoinhibition of PS II, oxidation of amino acids in the D1 protein, which is induced by oxygen radicals generated in the illuminated PS II, is probably the primary cause for the event. Oxidation of amino acids in the D1 protein may occur at the stroma-exposed portion of the D1 protein, and the damaged D1 protein should then cross-react with the surrounding polypeptides. In fact, covalent binding of the D1 protein to the α-subunit of cyt b_{559} was demonstrated by Barbato et al. [18,19]. Local change in the conformation of the D1 protein by the photodamage may also induce aggregation of the D1 protein and the neighbor polypeptides. The association of polypeptides in the latter case is probably noncovalent. In the donor-side photoinhibition, endogenous cationic radicals, such as $P680^+$ and Tyr_Z^+ that are produced during illumination on the donor side of PS II, are also responsible for photodamage to the D1 protein. These endogenous cationic radicals are strong oxidants that should also oxidize the D1 protein to induce adducts of the D1 protein. Sharma et al. recently demonstrated the oxidation of amino acids in the D1 and D2 proteins by mass spectroscopy [24]. The oxidized amino acids are distributed around the photoreactive components of PS II. This

oxidation might serve as a trigger for the subsequent covalent and non-covalent association of the proteins.

In the present study, the aggregates and cross-linked products of the D1 protein were resolved by SDS/urea-PAGE, where weakly associated proteins should be dissociated under the rather strong denaturing conditions employed here. A part of the aggregates can be generated by the addition of high concentration of salts to PS II membranes or incubation of PS II membranes with alkaline pH solution in darkness. As these aggregates are still detectable by SDS/urea-PAGE and Western analysis, it indicates that the interaction of the related polypeptides resolved under the present electrophoretic conditions is not necessarily covalent (data not shown).

The acceptor-side and donor-side photoinhibition can be distinguished from each other since the former should be dependent on oxygen, while the latter may occur irrespective of the presence of oxygen. The D1/D2 heterodimers and the D1/cyt b_{559} cross-linked products were formed by the acceptor-side photoinhibition, while the D1/CP43 adducts were generated by both the acceptor-side and donor-side photoinhibition (Fig. 2). In an earlier work, Barbato et al.[19] also showed that the light-induced cross-linking between the D1 protein and the α-subunit of cyt b_{559} adducts is dependent on oxygen, and we confirmed their results. As to the D1/CP43 adducts, participation of the donor-side photoinhibition in their production is rather striking. In our previous study, we showed that an extrinsic protein of PS II, OEC33, effectively suppresses the photoinduced aggregation of the D1 protein with CP43 under aerobic conditions, and thereby facilitates degradation [17]. The same and more extensive effects of OEC33 were observed in the experiments where the PS II samples were illuminated under anaerobic conditions and therefore the donor-side photoinhibition was prevailing (data not shown). From these results, it is suggested that the D1/CP43 adducts formed by the illumination under anaerobic conditions are due to the reaction between the two polypeptides at the portions exposed to thylakoid lumen, and that the presence of OEC33 prevents the polypeptide interaction.

In the present study, we also showed that the aggregates and cross-linked products of the D1 protein disappear after the addition of the stromal fraction to the photodamaged samples (Figs. 3–6). Probably the stroma contains proteases, molecular chaperones and a set of associated proteins which can process damaged, incompletely folded or misfolded proteins including the D1 adducts resulted from photodamage. Specific conformation of the D1 protein induced by either photodamage itself or the following aggregation and cross-linking with the other polypeptides might be recognized by the specific components in the stroma. The intact D1 proteins were insensitive to the stromal fraction (Fig. 5). It is noteworthy that only the D1 adducts formed on the stromal side of PS II by the acceptor-side photoinhibition were processed by the stromal fraction; the D1/CP43 aggregates formed on the lumenal side by the donor-side photoinhibition were not removed by the stromal fraction (data not shown). The PS II membranes that we used were not vesicles but, rather, particles with exposed surfaces and, thus, proteases and other responsible factors in the stromal fraction had access to both the stromal and the lumen sides of PS II. The lack of sensitivity of the aggregates produced by the donor-side photoinhibition to the stromal fraction suggests that the interacted portion of the lumen-exposed D1 protein and CP43 is not recognized by such stromal components.

It is interesting to know if the cross-linked or aggregated neighbors of the D1 protein are degraded, or return to the initial level by the addition of the stromal fraction. We examined if degradation of D2, Cyt b_{559} and CP43 takes place after the D1 adducts are processed by stromal fraction, by Western blotting with specific antibodies. In our preliminary experiments, the degradation fragments of the D2 protein and CP43 were detected, but cyt b_{559} was not affected (data not shown). The degradation of the aggregated neighbors of the D1 protein suggests the presence of a protease(s) which recognizes the adducts of the D1 protein in the stromal fraction.

Taking account of the fact that the stromal components responsible for removal of the aggregates and cross-linked products of the D1 protein are resistant to SDS (Fig. 5), we carried out two-dimensional SDS/urea-PAGE to identify stromal proteins which remove the D1 adducts (Fig. 6). Several protein components were apparently effective in removing the adducts and a component having an apparent molecular weight of 15 kDa was shown to have the

most significant effect. Recently, several SDS-stable proteases were reported with spinach chloroplasts [25]. Three of them, having apparent molecular weights of 14, 30, and 54 kDa, were found in stromal extracts. At the present stage, it is not known whether the 14-kDa stromal protease is identical to our 15-kDa stromal protein. By analogy with bacterial systems, it is suggested that chloroplasts contain ATP-dependent soluble endoproteases that correspond to Lon [26] as well as Clp [27]. FtsH protease is another ATP-dependent protease, which is nuclear-encoded and bound to the thylakoid membranes [28]. We examined the effects of ATP (+Mg) or GTP (+Mg), but they were not so significant, probably because we assayed their effects using unfractionated stroma (data not shown). To determine if a proteolytic enzyme(s) is involved in the degradation of the cross-linked products of the D1 protein, we examined the effects of inhibitors of several types of proteases including serine proteases, sulfhydryl proteases and metalloproteases. Among the inhibitors that we tested, leupeptin, phenylmethanesulfonyl fluoride (PMSF) and aprotinin, which are all inhibitors of serine-type proteases, were partially effective in preventing the processing of D1 aggregates (20–50% inhibition of the activity in the stromal fraction) (data not shown). Thus, there is a possibility that a serine-type protease(s) is involved in the processing of the D1 adducts. It should be noted, however, that degradation fragments of the cross-linked products of the D1 protein by the stromal fraction were not detected in the present study. This is probably due to efficient degradation of the damaged D1 proteins to smaller fragments by proteases, or due to participation of other components, such as molecular chaperones, in this processing steps, alternatively. Resistance of the active stromal components to SDS may be useful for further purification of the components, which is now being undertaken.

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