Studies on the photoinhibition of photosystem II in chloroplasts

- Degradation and cross-linking of polypeptides in photosystem II -

Hiroki Mori
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The following abbreviations are used: Caps, 3-cyclohexylamino-1-propanesulfonic acid; CF₄CF₃, chloroplast coupling factor; Ches, 2-(cyclohexylamino)ethanesulfonic acid; CP43, CP47 and CP29, antenna chlorophyll-binding proteins of PS II with molecular masses of 43 kDa, 47 kDa and 29 kDa, respectively; D1 and D2, reaction center-binding proteins of PS II; DABCO, 1,4-diazabicyclo[2.2.2]octane; DCIP, 1,5-dichloronaphthalene; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DPC, 1,4-diphenylcarbazide; Hepes, N-2-hydroxyethylpiperazine-N’-2-ethane-sulfonic acid; E, Einstein; LDS, lithium dodecyl sulfate; LHC II, light-harvesting chlorophyll-protein complex associated with PS II; Mes, 2-(N-morpholino)ethanesulfonic acid; P680, primary electron donor chlorophyll of PS II; PAGE, polyacrylamide gel electrophoresis; PC, plastocyanin; PVDF, poly (vinyliden difluoride); PQ and PQH₂, oxidized and reduced form of plastocyanine, respectively; PS I and II, photosystem I and II, respectively; QA and QB, primary and secondary plastoquinone electron acceptors of PS II, respectively; SDS, sodium dodecyl-sulfate; Taps, N-tris (hydroxymethyl) methyl-3-amino-propanesulfonic acid; Tricine, N-[tris(hydroxymethyl)] methyl-glycine; Tris, tris (hydroxy-methyl) aminomethane; Y₂ and Y₂, redox-active tyrosine residues of the D1 protein and the D2 protein, respectively

General Introduction

In photosynthesis, electron donors (H₂A) are oxidized by light and carbon dioxide is reduced to produce carbohydrates (CH₂O), as shown in the following equation:

\[ 2 \text{H}_2\text{A} + \text{CO}_2 \rightarrow (\text{CH}_2\text{O}) + 2 \text{A} + \text{H}_2\text{O} \]

Photosynthetic bacteria use various organic and inorganic compounds such as H₂, S₂ and S₂O₇²⁻ as electron donors (H₂A). On the other hand, higher plants, algae and cyanobacteria use water molecules, which are inexhaustibly present on the earth, as the electron donor. Oxygen is evolved as the by-product of oxidation of water molecules. Photosynthesis that carried out by higher plants, algae and cyanobacteria is called oxygenic photosynthesis. Oxygen, which is thought to be absent at the primitive atmosphere, has accumulated as the result of oxygenic photosynthesis in billion years and accounts for 20% of the present atmosphere.

Oxygenic photosynthesis is an important reaction to support our ecosystem on the earth. Almost all organisms including our human beings take carbohydrates produced by photosynthesis. Oxygen that was generated in oxygenic photosynthesis and accumulated in the atmosphere, led the biological evolution: (i) The ozone layer, which was formed from oxygen of the atmosphere by the illumination with ultraviolet light and electric discharge, absorbs the ultraviolet light harmful to organisms. The formation of ozone layer allowed organisms that had lived under water, to live on the land. (ii) After accumulation of oxygen, aerobic respiration, which uses oxygen as the terminal electron acceptor, emerged. The efficiency of energy production of aerobic respiration is higher than that of fermentation.

Photosynthesis consists mainly of two reactions, a photochemical reaction (Equation 1) and a reaction related to fixation of carbon dioxide (Equation 2).

\[ \text{H}_2\text{O} + \text{NADP}^+ + \text{ADP} \rightarrow \frac{1}{2} \text{O}_2 + \text{NADPH} + \text{ATP} + \text{H}^+ \quad (\text{Eq. 1}) \]
\[ \text{CO}_2 + \text{NADPH} + \text{ATP} \rightarrow (\text{CH}_2\text{O}) + \text{NADP}^+ + \text{ADP} \quad (\text{Eq. 2}) \]

The former reaction (Eq. 1) occurs in the thylakoid membranes of chloroplasts and light energy is converted to chemical energy, i.e., NADPH and ATP. By the latter reaction (Eq. 2), carbon dioxide is assimilated in the stroma of chloroplasts with the aid of NADPH and ATP, which are supplied by the photochemical reaction (Eq. 2).

The photochemical reaction in thylakoid membranes of chloroplasts are divided into four separate reactions, i.e.,

\[ \text{H}_2\text{O} + \text{PQ} \rightarrow \frac{1}{2} \text{O}_2 + \text{PQH}_2 \quad (\text{Eq. 3}) \]
\[ \text{PQH}_2 + \text{oxidized PC} \rightarrow \text{PQ} + \text{reduced PC} \quad (\text{Eq. 4}) \]
\[ \text{Reduced PC} + \text{NADP}^+ \rightarrow \text{Oxidized PC} + \text{NADPH} \quad (\text{Eq. 5}) \]
\[ \text{ADP} + \text{Pi} \rightarrow \text{ATP} \quad (\text{Eq. 6}) \]

These reactions (Eq. 3 - 6) are catalyzed by multi-subunit complexes, i.e., PS II complex, cytochrome b₆/f complex, PS I complex and CF₄CF₃ complex, in the thylakoid membranes.
Photosystem II is a multi-subunit complex (Fig. 2) in the thylakoid membranes of chloroplasts, consisting of the reaction center-binding proteins D1 and D2 (Namba and Satoh, 1987), antenna chlorophyll a-binding proteins CP43 and CP47 (Delepeilaire and Chua, 1979), the extrinsic 33, 24, 18 kDa proteins (Yamamoto et al., 1981), alpha- and beta-subunits of cytochrome b559 (Widger et al., 1985), psb I gene products (Ikeuchi and Inoue, 1988), and the other components of low molecular weight (Ikeuchi, 1992). The electron transfer components from Z to Qb of PS II bind to the D1 and D2 proteins of PS II. Mn clusters are stabilized by the extrinsic 33 kDa protein (Ono and Inoue, 1983), which may bind to CP43, CP47 and the D1 protein (Isogai et al., 1985, Enami et al., 1987, Mei et al., 1989). Extrinsics 24 and 18 kDa proteins function as the concentrator of Ca2+ and Cl- essential for water oxidation (Murata and Miyao, 1985).

Strong illumination of oxygenic photosynthetic organisms results in the loss of their photosynthetic activity (Powles, 1984). The primary event in this process is impairment of PS II (Kyle, 1987). Since PS II is the site of the primary photoreaction of oxygenic photosynthesis, the impairment affects the whole reaction of photosynthesis consequently. The impairment of PS II during illumination (photoinhibition) involves photoinactivation of PS II activity and specific degradation of the D1 protein (Prähl et al., 1992, Aro et al., 1993). After the degradation of the D1 protein, PS II complex migrates from grana thylakoid to stroma thylakoid. In the stroma thylakoid, the D1 protein is synthesized and incorporated to PS II complex lacking the D1 protein (Mattoo et al., 1987, Adir et al., 1990). The PS II complex migrates back to grana thylakoid, where finally the PS II complex is activated by light to have a capacity of evolving oxygen.

Two distinct types of photoinhibition of PS II have been proposed, so-called acceptor-side and donor-side photoinhibition (Barber and Andersson, 1992, Aro et al., 1993). In the

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**Fig. 1** Photosynthetic electron transfer in the thylakoid membrane. Electron transfer components are depicted with their redox-potentials.

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**Fig. 2** Schematic illustration of the polypeptide components of PS II complex. Solid arrows show the direction of electron transfer.
acceptor-side photoinhibition, photodamage to PS II may be initiated by the overreduction of Qa. The resulting irreversible lesions are thought to be caused by the highly oxidizing and reactive singlet oxygen (\(O_2^*\)) that is formed in a reaction between oxygen and the triplet state of P680, the latter being generated by charge recombination between reduced phytochrome and P680 after dissociation of doubly reduced QA from PS II (van Mieghem et al., 1989, Vass et al., 1992). In the donor-side photoinhibition, photodamaging events are initiated by one or some of the highly oxidizing PS II radicals \(Y_2^*\), \(Y_1^*\), or \(P_680^*\) (Blubaugh et al., 1991, Eckert et al., 1991), which accumulate in the light following perturbation or inhibition of the oxygen-evolving complex by depletion of its Mn, Cl, or Ca\(^{2+}\). Photodamages in the acceptor-side photoinhibition require much greater light intensities and occur at a much slower rate than those in the donor-side photoinhibition (Eckert et al., 1991). Singlet oxygen (\(O_2^*\)) and the oxidizing species generated in this way cause irreversible damage to the reaction center and induce the subsequent degradation of the D1 protein.

The photo-induced degradation of the D1 protein has been examined \textit{in vitro} with a various preparations of PS II in detail. The primary sites of cleavage of the D1 protein differ depending on the illumination conditions (De Las Rivas et al., 1992). When PS II complexes with functional oxygen-evolving complex were illuminated under conditions that induce the acceptor-side photoinhibition, a 23 kDa (N-terminal), a 16 kDa (C-terminal) and a 9 kDa (C-terminal) fragments were detected (Salter et al., 1992). The 23 kDa and 9 kDa fragments are the degradation products that are derived by cleavage of the loop connecting helices D and E of the D1 protein. The cleavage site is close to that of the degradation product detected \textit{in vivo} (Greenberg et al., 1987). In the donor-side photoinhibition, when PS II reaction center complexes, which consist of the D1 and D2 protein and \textit{psb} I gene products and carry out charge separation exclusively, were illuminated, the D1 protein was cleaved in the loop connecting helices A and B on the luminal side of the thylakoid membranes, giving rise to a 24 kDa fragment (C-terminal) (Barbato et al., 1991). A 16 kDa fragment (C-terminal) was detected in Tris-treated thylakoid membranes and in various preparations of PS II other than the PS II reaction center complex (Barbato et al., 1992a), which are devoid of the oxygen-evolving complex.

With respect to the degradation of the D1 protein, two mechanisms have been mainly suggested: (i) involvement of an endogenous protease of PS II complex, (ii) involvement of active oxygen species generated in PS II under illumination. In the former mechanism, it is suggested that a serine-type protease intrinsically present in PS II complex, possibly as a component of PS II itself, catalyzes the degradation of the D1 protein. This possibility is inferred from the observation that inhibitors of serine-type protease such as dithiopropyl fluorophosphate and phenylmethylsulfonyl fluoride suppressed the degradation of the D1 protein in isolated PS II complexes (Virgin et al., 1991, Shipton and Barber, 1992). In the latter mechanism, it is suggested that active oxygen such as hydrogen peroxide is generated at the specific sites of PS II under illumination and that the D1 protein is cleaved at specific sites only by the action of active oxygen (Bradley et al., 1991, Chen et al., 1992, Miyao, 1994).

Recently, in addition to the degradation of the D1 protein, the degradation of other components of PS II and the cross-linking of components of PS II induced by illumination were reported \textit{in vivo} (Kim et al., 1993, Mishra et al., 1994a, Christopher and Mullet, 1994). These phenomena seem to occur when repair cycle of PS II does not catch up with the damage of the D1 protein. Without repair cycle of PS II, the degradation of other components of PS II or the cross-linking of components of PS II induced by illumination may occur easily \textit{in vitro}.

I found that the PS II components other than the D1 protein are also affected when PS II membranes are illuminated under conditions where the three extrinsic proteins of PS II are removed and the donor-side photoinhibition of PS II occurs. I describe deletion of PS II components in the donor-side photoinhibition in \textsc{CHAPTER I}, further characterization of the deletion of PS II components in \textsc{CHAPTER II}, and degradation of the D1 protein in the donor-side photoinhibition in \textsc{CHAPTER III}. 


CHAPTER I

Deletion of PS II components during Tris-treatment of PS II membranes

Introduction

One of the remarkable features of PS II is its high susceptibility to photoinhibition (Powles, 1984). When chloroplasts are illuminated with strong light, electron transport is inhibited and degradation of the D1 protein is accelerated (Kyle et al., 1984; Kyle, 1985; Ohad et al., 1985). It was shown that the turnover of the D1 and D2 proteins increases under illumination with weak light in the hydroxyamine-treated chloroplasts (Callahan et al., 1986). Acceleration of the degradation of D1 and D2 proteins is also observed in Cl-depleted thylakoid membranes where transition from S2 to S3 in the S-cycle of oxygen evolution is reversibly inhibited (Jegerschöd et al., 1990). Thus, it is probable that the removal of Mn from the catalytic site of water oxidation, or the blocking of oxidation of Mn, accelerates inevitable degradation of the D1 and D2 proteins under the illumination.

There are some treatments which inactivate the oxygen-evolving complex of PS II membranes by extracting cofactors responsible for water oxidation. They are low pH (Ono and Inoue, 1988), NaCl- (Miyao and Murata, 1984), CaCl2- (Ono and Inoue, 1983), NH3OH- (Cammarata et al., 1984) and Tris-treatments (Yamamoto et al., 1981), and all of them enhance susceptibility of PS II to photoinhibition. Tris-harshest among them, and it removes Mn atoms and the extrinsic proteins from the PS II membranes (Yamamoto et al., 1981).

In this chapter, I describe the results when I treated the spinach PS II membranes with 0.8 M Tris under illumination with weak light. By this treatment, CP43 was shown to be significantly deleted from the PS II membranes, depending on the pH of Tris and also on illumination. Deletion of the D1 protein and CP47 also occurred, but the extent was less, compared with the deletion of CP43 under the same conditions. This is the first evidence showing that the PS II components, other than the reaction center-binding proteins, are significantly affected under photoinhibitory conditions.

Materials and Methods

Preparation of PS II membranes

PS II membranes were prepared from spinach by the method of Kuwabara and Murata (1982), suspended in a solution containing 30% ethylene glycol, 0.4 M sucrose, 40 mM Mes-NaOH, 10 mM NaCl and 5 mM MgCl2 (pH 6.0), and stored at -80°C until use. Chlorophyll was determined in 80% acetone using the absorption coefficients reported by Mackinney (1941).

Tris- and CaCl2-treatment of PS II membranes

The PS II membranes were washed once with a solution containing 0.4 M sucrose, 40 mM Mes-NaOH and 10 mM NaCl (pH 6.5) (SMN), suspended in 0.8 M Tris-HCl to a concentration of 0.5 mg chlorophyll ml⁻¹ and incubated at 4°C for 30 min, with slow stirring either in the dark or light. For illumination, white fluorescent lamps were used and the incident intensity was 20 μE m⁻² s⁻¹ above the suspension of the PS II membranes, unless otherwise stated. The pH of Tris-HCl, which was varied from 7.0 to 10.0, is specified in each figure legend. The Tris-treated PS II membranes were recovered by centrifugation at 16,000 x g for 15 min and washed twice with SMN in the dark. Treatment of the PS II membranes with 1 M CaCl2 to remove the extrinsic 33 kDa protein was carried out in the light (20 μE m⁻² s⁻¹) by the method of Ono and Inoue (1983). The CaCl2-treated PS II membranes were recovered by the same centrifugation step used for Tris-treatment, but without further washing, to avoid removal of Cl⁻ from the membranes.

Protein analysis

SDS-PAGE was done according to Laemmli (1970) expect that the concentration of Tris-HCl (pH 8.8) in the resolving gel (10-20% acrylamide) was raised from 0.375 to 0.6 M, and 6 M urea was included in the gel to increase resolution of polypeptides (Ikeuchi and Inoue, 1988). The proteins in the gel were stained by Coomassie brilliant blue R-250 and quantified by measuring absorption at 565 nm with a dual-wavelength TLC scanner CS-930 (Shimadzu, Japan). The non-denaturating lithium dodecyl sulfate (LDS)-PAGE was done according to the method of Cammi and Green (1989). The PS II membranes were solubilized with a solution containing 0.9% n-octyl-β-D-glucopyranoside, 10% (v/v) glycerol and 2 mM Tris-maleate (pH 7.0) to the concentration of chlorophyll of 0.6 mg ml⁻¹. After incubation for 10 min, the solution was centrifugated at 16,000 x g for 10 min and the supernatant was subjected to electrophoresis at 4°C in the dark. Western blotting was carried out as previously described (Yamamoto, 1988). Proteins of the PS II membranes after SDS/urea-PAGE were blotted onto PVDF membranes (0.45 μm, Millipore, Japan) and cross-reacted with specific antibodies and alkaline phosphatase-conjugated secondary antibodies. The amount of the protein was estimated from the densitogram, where the wavelength of the measuring beam of the TLC scanner was set at 585 nm.
Results

The PS II membranes were treated with 0.8 M Tris-HCl in the light (20 μE m⁻² s⁻¹), and the proteins in the membranes were analyzed by SDS/urea-PAGE (Fig. 3). With increasing the pH from 7.0 to 10.0 in the Tris-treatment, the three extrinsic proteins were gradually released from the membranes, and above pH 8.0 all of them were completely removed, as reported previously (Yamamoto et al., 1981). Mn was also released from the membranes under these conditions. I found here that the amounts of the apoproteins of CP43 and CP47 in the PS II membranes, determined from the densitogram of the Coomassie-stained bands, decreased with increasing the pH from 7.5 to 8.5. The amounts of the apoproteins at pH 8.5 were about 75% of those in the control PS II membranes incubated with SMN (pH 6.5) (Fig. 4). Above pH 8.5, the apoproteins of CP43 still decreased with pH, and apparently 40% of the protein originally present in the membranes disappeared at pH 10.0. In contrast with that, no further decrease was observed in the amount of the apoprotein of CP47 above pH 8.5. To see if there is a change in the amount of the D1 protein in parallel with the decrease in the apoproteins CP43 and CP47, I determined the amount of the D1 protein by Western blotting with a specific antibody (Fig. 4). The pattern of decrease in the amount of the D1 protein was almost the same as that of the apoprotein of CP47. The amount continued to decrease from pH 7.5 to 8.5, but there was no further change beyond pH 8.5. The D2 protein was not assayed here. Thus, among the polypeptide components of PS II examined, CP43 was particularly sensitive to the Tris-treatment in the light.

The decrease in CP43 and CP47, which depends on the pH of Tris-treatment, was also detected by non-denaturing LDS-PAGE of the PS II membranes (Fig. 5). Here, the PS II membranes were solubilized with n-octyl-β-D-glucopyranoside, and the green bands were detected by electrophoresis. The green bands corresponding to the holocomplexes of CP43 and CP47, were reduced significantly after the Tris-treatment in the light. Other major proteins were also partially removed.
Fig. 6 Chlorophyll a/b ratio of PS II membranes after Tris-treatment at various pH under weak illumination. Chlorophyll a/b ratio was determined with the PS II membranes used in Fig. 3, and depicted as a function of pH of Tris-treatment. Square shows the chlorophyll a/b ratio of the PS II membranes incubated with SMN (Fig. 3, lane 1).

of the PS II membranes, such as LHC II and cytochrome b559, were not affected at all under the same conditions, judging from both the SDS/urea-PAGE and non-denaturing LDS-PAGE. Figure 6 shows the decrease in chlorophyll a/b ratio of PS II membranes depending on the pH of Tris-treatment. All chlorophyll b in the thylakoid membrane are bound to the light-harvesting chlorophyll complexes of PS I and PS II. The core complexes of PS I and PS II contain only chlorophyll a (Green, 1988). The decrease of chlorophyll a/b ratio of PS II membranes indicates either release of chlorophyll a from CP43 and CP47, or bleaching of chlorophyll a in CP43 and CP47 during Tris-treatment under illumination.

In the Tris-treatment of the PS II membranes, no change has so far been reported in the amount of intrinsic polypeptide, because more attention has been paid to the removal of the extrinsic proteins and Mn from PS II by the treatment (Yamamoto et al., 1981). Usually Tris-treatment was carried out either in complete darkness or under dim light, and there is no comparative study on the effects of light and dark conditions on the intrinsic membranes proteins of PS II. I studied the effect of light during the Tris-treatment on CP43. Tris-treatment of the PS II membranes induced only slight deletion of PS II component in the dark (Fig. 7, lane 2). By contrast, decrease in the amounts of CP43 and CP47 was significant with the same treatment in the light (Fig. 7, lane 3). In the range of light intensity from 20 to 84 μE m⁻² s⁻¹, no difference was observed in the effect of Tris (pH 9.0) on CP43 (Fig. 7, lane 3, 6 and 7), suggesting that the incident intensity used here (20 μE m⁻² s⁻¹) is saturating for the effect. The

Fig. 7 Comparison of the effects of Tris-treatment on the PS II membranes in the light and in the dark.
Lane 1, control PS II membranes incubated with SMN in the light. Lane 2, PS II membranes treated with Tris-HCl (0.8 M, pH 9.0) in the dark. Lane 3, PS II membranes treated with Tris-HCl in the light. Lane 4, the same as lane 3 but with 0.5 mM DPC. Lane 5, PS II membranes treated with 1 M CaCl₂ in the light. Other conditions are the same as those described in the legend to Fig. 3.

deletion of CP43 observed here is in contrast with the degradation of the D1 protein that occurs under illumination with much stronger light (Kyle et al., 1984, Kyle, 1985, Obad et al., 1985). Removal of the three extrinsic proteins was also attained by CaCl₂-treatment under the illumination (20 μE m⁻² s⁻¹), but in this case, the deletion of CP43 was not so significant (Fig. 7, lane 5). In the CaCl₂-treated PS II membranes, which are depleted of the 33 kDa protein, the activity of oxygen evolution is retained as long as Cl⁻ is supplied to the depleted membranes and the Mn in the catalytic site is stabilized (Miyao and Murata, 1984). These results suggest that the destructive effect of light on CP43 appears when the function of Mn atoms is hindered. Treatment of the PS II membranes with 1 M NaCl, which removes extrinsic proteins other than the 33 kDa protein, but no Mn, from the membranes, had no significant effect on CP43 (data not shown).

Considering the synergistic effect on the deletion of CP43 of alkaline-Tris and illumination with weak light, I assume that excitation of PS II induces oxidizing radicals at the donor side of PS II during the Tris-treatment of PS II membranes in the light. It is expected that the electron donors to PS II (reductants) block the formation of oxidizing radicals and, therefore, that the deletion of CP43 does not occur. When 0.5 mM DPC was added to the PS II membranes in the light (20 μE m⁻² s⁻¹), apparently no significant decrease was detected in the
Table 1 The effects of reductants and of DCMU on CP43 in the Tris-treatment (0.8 M, pH 9.0) of the PS II membranes in weak light.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Amount of CP43 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubated with SMN in the dark</td>
<td>100</td>
</tr>
<tr>
<td>Treated with Tris in the dark</td>
<td>87.0</td>
</tr>
<tr>
<td>Treated with Tris under light irradiation</td>
<td>63.6</td>
</tr>
<tr>
<td>+DPC (0.5 mM)</td>
<td>98.3</td>
</tr>
<tr>
<td>+sodium ascorbate (0.5 mM)</td>
<td>91.7</td>
</tr>
<tr>
<td>+NH₂OH (0.5 mM)</td>
<td>91.8</td>
</tr>
<tr>
<td>+potassium ferrocyanide (0.5 mM)</td>
<td>95.1</td>
</tr>
<tr>
<td>+hydroquinone (0.5 mM)</td>
<td>92.0</td>
</tr>
<tr>
<td>+DCMU (20 µM)</td>
<td>100</td>
</tr>
</tbody>
</table>

Amount of CP43 during the Tris-treatment (Fig. 7, lane 4). The other reductants, including hydroquinone, sodium ascorbate, hydroxylamine and potassium ferrocyanide, had the same effect as DPC (Table 1). The addition of DCMU in the light eliminated the effect of Tris-treatment on CP43 (Table 1), which suggests the involvement of electron transport of PS II in the formation of the radicals.

Discussion

Tris-treatment of PS II membranes removes three extrinsic proteins and Mn atoms from PS II membranes (Yamamoto et al., 1981). When PS II membranes are treated with Tris in the weak light, some components of PS II, i.e., CP43, CP47 and the D1 protein, were deleted from the membranes. CP43 was deleted during Tris-treatment in the weak light most prominently. Weak-light photoinhibition of PS II has been reported (Callahan et al., 1986, Callahan and Cheniae, 1985, Blubaugh and Cheniae, 1990), but no evidence was presented for the deletion or degradation of the PS II-related proteins during the photoinhibition process. The data presented here are the first to show the significant deletion of CP43, CP47 and the D1 protein, as a result of the weak-light photoinhibition of PS II.

Deletion of PS II components during Tris-treatment is observed at low intensity of irradiation (20 µE m⁻² s⁻¹). This is in contrast with the degradation of the D1 protein that occurs under illumination with much stronger light in vitro (Kyle et al., 1984, Kyle, 1985, Ohad et al., 1985). The difference of efficiency between degradation of the D1 protein, which required strong illumination in vitro, and deletion of PS II protein components observed here are probably related to the absence of oxygen-evolving complex and the pH.

Light-induced decrease of holocomplexes of CP47 and CP43 detected by non-denaturing LDS-PAGE and that of chlorophyll a/b ratio may reflect release of chlorophyll a from CP47 and CP43, which may result from degradation or conformational change of CP47 and CP43, or bleaching of chlorophyll a in CP47 and CP43, which may result from oxidation of chlorophyll a in CP47 and CP43 by cation radicals such as chlorophyll•⁺, P680⁺.

The oxidized form of the reaction-center chlorophyll P680, and of the secondary electron donor YZ, are candidates for the oxidizing radicals produced in the Tris-treated PS II membranes. It was reported that P680⁺ and YZ⁺ are oxidants strong enough to oxidize the nearby amino acid residues or redux components (Thompson and Brudvig, 1988, Metz et al., 1989). When P680 and YZ are oxidized in the Tris-treated PS II membranes in the light, they may have a destructive effect on the surrounding polypeptides. In the experiment examining the effect of the reductants (Table 1), the hydrophobic reductants (hydroquinone and DPC) were more effective than the hydrophilic reductants (ascorbate, ferrocyanide and hydroxylamine) in the inhibition of deletion of CP43. These results may be interpreted by assuming that the oxidizing radicals are produced at the hydrophobic region in the oxidizing side of PS II, probably on the D1 protein. As low irradiation of PS II was enough to induce deletion of CP43, the efficiency of the light-induced effect should be quite high. Judging from the most prominent deletion of CP43 among the proteins of PS II observed here, it is suggested that a portion of CP43 polypeptide is located closely to P680 or YZ, or both and in that portion there are amino acid residues quite susceptible to the effects of cation radicals. However, an alternative possibility is that long-lived diffusible radicals are formed, following the oxidation of P680 and YZ, which affect the surrounding polypeptides non-specifically. Illumination of the PS II membranes in the Tris-treatment induced deletion of CP43 as well as the D1 protein, and CP47 (Figs. 3 - 5). The deletion of CP43 was dependent on the duration of illumination (data not shown), suggesting the accumulation of deteriorative molecular species under these conditions. All these results favor the view that long-lived oxidizing radicals are produced on the donor side of PS II.

The deletion of CP43 may be due to the degradation of the protein. However, a definite degradation product of CP43 has not been found so far, in the PS II membranes after Tris-treatment, with the SDS/urea-PAGE and Western blotting analysis with a specific antibody against CP43 (data not shown). I also tried to find a degradation product in the soluble fraction after Tris-treatment of the PS II membranes by protein sequencing of the minor proteins detectable in SDS/urea-PAGE, but the fragment corresponding to the segment of CP43 was not found (data not shown). Such difficulty in detecting the degradation product of CP43 is probably due to high efficiency of the degradation process. It is known that CP43 can be removed from the PS II complex by an appropriate treatment with detergents (Imaoka et al.,
1986), which suggest relative weakness of the binding of CP43 to the PS II reaction center complex. Once the degradation of CP43 is triggered by light, the polypeptide may be removed from the PS II complex easily. Alkaline pH is probably required for the efficient degradation of the polypeptide, but details of the effect of pH in this process remain to be explored.

Weak-light photoinhibition of PS II has been reported (Callahan et al., 1985, Blubaugh and Cheniae, 1990), but no evidence was presented for the deletion or degradation of the PS II-related proteins during the photoinhibition process. The data presented here are the first to show the significant deletion of CP43, CP47 and the D1 protein, as a result of the weak-light photoinhibition of PS II. Further analysis of the photoinhibition on the polypeptides of PS II membranes should provide more accurate information, not only on the mechanism of photoinhibition, but also on the molecular organization of the oxygen evolution system of PS II.

Summary

Treatment of spinach PS II membranes by Tris (0.8 M, pH 7.5-10) in weak light (20 μE m⁻² s⁻¹) induced partial deletion of intrinsic proteins of PS II as well as release of three extrinsic proteins and Mn from the membranes. Most prominently deleted was CP43, which was detected by SDS/urea-PAGE. The D1 protein and CP47 were also affected by the same treatment but to a lesser extent. Deletion of CP43 did not occur when the PS II membranes treated with Tris in the dark, or treated in the light in the presence of reductants for PS II. The results suggest that CP43 is located close to the water-oxidation site and is damaged by the oxidizing radicals produced at the donor side of PS II during the Tris-treatment of the PS II membranes in the weak light.

CHAPTER II

Further characterization of deletion of PS II components in the donor-side photoinhibition

Introduction

I described in chapter I that the D1 protein, CP43 and CP47 are lost from the PS II membranes during treatment with Tris in weak light (20 μE m⁻² s⁻¹). The loss of the proteins appeared to be caused by the donor-side inhibition of PS II since it was prevented in the presence of artificial donors of electron to PS II. Illumination of Mn-extracted PS II membranes with light of low intensity induced inactivation of the donor side of PS II. The inactivation occurred with a high quantum yield (Blubaugh and Cheniae, 1991), while strong light is required for the photoinactivation of the acceptor side of PS II (Eckert et al., 1991). However, it is not known how inactivation of the donor side of PS II affects the D1 protein and other protein components of PS II.

In the donor-side photoinhibition, strong illumination induces the degradation of the D1 protein. As the degradation products, a 24-kDa (C-terminal) and a 9-kDa (N-terminal) fragments were identified with the PS II reaction center complexes (Shipton and Barber, 1991), and a 16-kDa (C-terminal) fragment was found in Tris-treated thylakoid membranes and in various preparations of PS II other than the reaction center complexes (Barbato et al., 1992a). A proteolytic activity within PS II, but not active oxygen, has been shown to be responsible for the cleavage (Shipton and Barber, 1992).

In chapter II, I characterize in greater detail the deletion of CP43 during illumination of PS II membranes and of oxygen-evolving complexes of PS II that had been treated with Tris at alkaline pH in the dark, and present data showing that significant cross-linking among CP43, CP47 and the D1 protein is induced under these conditions. When cations were added to these PS II membranes, a 16 kDa fragment of the D1 protein appeared by illumination. Photo-induced cross-linking was differently affected by the addition of monovalent and divalent cations.

Materials and Methods

Preparation of PS II membranes and PS II complexes

Membranes of PS II were prepared from spinach thylakoids (Kuwahara and Murata, 1982), PS II complexes that retained the extrinsic 33 kDa protein and manganese were obtained by solubilization of PS II membranes with n-octyl β-D-glucopyranoside (Ghanotakis et al., 1987), except for the presence of 0.6 M NaCl during solubilization. For treatment with Tris, PS II membranes and PS II complexes were separately suspended in 0.8 M Tris-HCl (pH 9.0), at 0.5 mg chlorophyll ml⁻¹, incubated on ice for 20 min in darkness, pelleted by
centrifugation and washed with either a solution of 0.4 M sucrose, 40 mM Mes-NaOH and 10 mM NaCl (pH 6.5) (SMN), or a solution of 0.4 M sucrose, 40 mM Chex-NaOH and 10 mM NaCl (pH 9.0) (SCN). Each solution used in washing step contained 2 mM EDTA. 

**Photoinhibition**

Tris-treated PS II membranes and the PS II complexes were resuspended in SMN or SCN and the both suspensions were adjusted to 0.5 mg chlorophyll ml⁻¹ and 0.1 mg chlorophyll ml⁻¹, respectively. These preparations were illuminated with weak light (20 µE m⁻² s⁻¹) as described in chapter I. All procedures prior to and following the illumination was done in dim green light. Anaerobic conditions were achieved by the addition of 10 mM glucose, 0.2 mg ml⁻¹ glucose oxidase and 0.2 mg ml⁻¹ catalase to the cuvette that contained the suspension of PS II membranes. Silicone oil was layered on the surface of the solution. Lowering the tension of oxygen to the zero level was monitored by an oxygen electrode (Rank Brothers, UK). After incubation at 25°C for 5 min in darkness, the cuvette was illuminated as described above.

**Assay for electron transfer activity of PS II membranes**

Photoreduction of DCIP was measured at 580 nm with a Hitachi 356 dual-wavelength double-beam spectrophotometer. Actinic light was provided through a heat-absorbing filter HA-50 (Hoya, Japan) and a red cut-off filter VR-67 (Toshiba, Japan). The photomultiplier was guarded from strong actinic light with a band pass Corning 4-96 filter (Corning, USA) and an interference KL-58 filter (Toshiba, Japan). The reaction mixture (1 ml) contained 0.4 M sucrose, 40 mM Mes-NaOH, 10 mM NaCl, 0.5 mM DPC, 40 µM DCIP (pH 6.5) and PS II membranes equivalent to 5 µg of chlorophyll.

**Protein analysis**

An aliquot withdrawn from each irradiated suspension was added to an equal volume of a solution of 125 mM Tris-Cl, 6% SDS, 8% β-mercaptoethanol, 6 mM EDTA and 8 M urea (pH 6.8). Proteins in the preparations of PS II were assayed by SDS/urea-PAGE (10-20% polyacrylamide gradient gel with 6 M urea) as described in chapter I.

 Antibodies were raised against the D1 protein, CP47, CP43 and the D2 protein with the proteins obtained by preparative SDS-PAGE of reaction center complexes or core complexes of PS II from spinach as the antigens. The antibody against the α-subunit of cytochrome b559 was a gift from Dr. Bertil Andersson, University of Stockholm. Further purification of the antibodies was performed as described Sambrook et al. (1989). Immunological detection of the proteins by western blotting was performed with an enhanced chemiluminescence system (Amersham, Japan) according to the manufacturer’s protocol.

**Source of enzymes**

Catalase (bovine liver) and glucose oxidase were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and Biozyme Laboratories Ltd. (USA), respectively.

**Results**

Deletion of some intrinsic components of PS II (CP43, CP47 and the D1 protein) was observed during Tris-treatment of PS II membranes in weak light (chapter I). In this case, the following two processes occur simultaneously: (1) release of the extrinsic 33, 24 and 18 kDa proteins and Mn cluster from PS II, (2) photodamage to the donor side of PS II and deletion of the protein components of PS II. I separated these two processes from each other to further characterize the deletion of the PS II components. First, PS II membranes were treated with alkaline Tris in the dark to remove the three extrinsic proteins and Mn atoms from PS II complexes and resuspended in appropriate buffer solutions with different pH. The Tris-treated PS II membranes were then illuminated with weak light (intensity, 20 µE m⁻² s⁻¹) at 4°C (Fig. 8). In this case, CP43, CP47 and the D1 protein were deleted only at alkaline pH as described in chapter I, which indicates that removal of the extrinsic proteins and Mn from PS II itself has no significant effect on the deletion of the protein components of PS II and that illumination under alkaline pH conditions is required for the deletion.

When Tris-treated PS II complexes were exposed to weak light (20 µE m⁻² s⁻¹) at pH 9.0 for 30 min at 25°C, reductions in levels of CP43, CP47, the D1 protein and 10 kDa polypeptide were demonstrated by SDS/urea-PAGE, as was observed with the PS II.

**Fig. 8** Photo-induced loss of components from Tris-treated PS II membranes under alkaline pH conditions. Tris-treated PS II membranes were suspended in a medium containing 0.4 M sucrose, 10 mM NaCl and 40 mM of the following buffer: Mes for pH 6.5, Chex for pH 9.0 or phosphate for pH 11.0, and illuminated (at the light intensity of 20 µE m⁻² s⁻¹) at 4°C for 30 min. Polypeptides were separated by SDS/urea-PAGE, and stained with Coomassie brilliant blue R-250.
membranes (Fig. 9). The amounts of these proteins did not change significantly in the control PS II complexes that had been treated by alkaline Tris and then kept at pH 6.5. The PS II complexes are devoid of light-harvesting chlorophyll-protein complexes and, therefore, have more simple protein composition than the PS II membranes. In the PS II complexes, there was a protein band just below the D1 protein in the SDS/urea-PAGE gel. The protein, which did not cross-react either with the antibody against the D1 protein or with that against the D2 protein, may correspond to CP29.

I was not able to detect the degradation fragments of CP43 and the D1 protein by western blotting in chapter I, probably because of the low titer of the specific antibodies used and the low sensitivity of the immunodetection system. In this chapter, I used newly prepared antibodies and a sensitive immunodetection system to identify the products of the photoinhibition of PS II membranes (Fig. 10, panel A) and of PS II complexes (Fig. 10, panel B) that had been treated with Tris. SDS-PAGE and western blotting of the illuminated preparations of PS II revealed that the antibody against the D1 protein recognized, in addition to the normal D1 band at about 34 kDa, two polypeptides with molecular masses of 41 and 38 kDa, respectively, as well as several polypeptides with molecular masses greater than that of the D1/D2 heterodimer (Fig. 10). No new bands of protein were produced as a result of the illumination that cross-reacted with the antibody against the D2 protein. The polypeptide with higher molecular masses that cross-reacted with the antibody against the D1 protein were also recognized by the antibodies against CP43 and CP47. At least part of the photo-induced loss of CP43 and CP47 from PS II observed under alkaline conditions may be attributable to cross-linking among the D1 protein, CP43 and CP47. In particular, the products of cross-linking between the D1 protein and CP43 seemed to be especially prominent. These results indicate

Fig. 9 Photo-induced loss of proteins from Tris-treated PS II complexes.
Tris-treated PS II complexes were suspended in SMN (pH 6.5) or SCN (pH 9.0; see text for details) at 100 μg chlorophyll ml⁻¹ and illuminated at 20 μE m⁻² s⁻¹ at 25°C for 30 min. Polypeptides were separated by SDS/urea-PAGE (10-20% gradient gel with 6 M urea) and stained with Coomassie brilliant blue R-250. L, PS II complexes that were illuminated; D, PS II complexes that were kept in darkness (dark control).

Fig. 10 Detection of photo-induced products of cross-linking in Tris-treated PS II complexes and PS II membranes.
Tris-treated PS II membranes (panel A) and PS II complexes (panel B), suspended in SCN (pH 9.0), were illuminated as described in the legend to Fig. 9. After illumination, polypeptides were separated by SDS/urea-PAGE; transferred to PVDF membranes and analyzed immunologically by the use of antibodies against D1, D2, CP47, CP43 and the α-subunit of cytochrome b559. D, preparations of PS II that were kept in the dark; L, preparations of PS II that were illuminated.
that CP43 is located close to the D1 protein and that these proteins form a heterodimer during photoinhibition.

The 41 kDa polypeptide detected with the antibody against the D1 protein was also recognized by the antibody against the α-subunit of cytochrome b559 (Fig. 10). This 41 kDa polypeptide has been observed regardless of the conditions of photoactivation (Shipton and Barber, 1991, Virgin et al., 1990, Aro et al., 1990), and it has been identified as a product of cross-linking between the uncleaved D1 protein and the α-subunit of cytochrome b559 (Barbato et al., 1992b). The antibody against the D1 protein also recognized a polypeptide of 38 kDa. Since none of the antibodies against the D2 protein, CP43, CP47 and the α-subunit of cytochrome b559 cross-reacted with the 38 kDa protein (Fig. 10), it is possible that an unidentified polypeptide with a molecular mass of 5 kDa in PS II cross-reacts with the D1 protein to produce the 38 kDa polypeptide. The yield of the 41 kDa cross-linking products between the D1 protein and the α-subunit of cytochrome b559, and the yield of the 38 kDa polypeptide were dependent on the preparations of PS II. The 41 kDa band was observed more clearly in the PS II complexes, whereas it was easier to see the 38 kDa band in the PS II membranes than in the PS II complexes.

Fig. 11 Effects of pH and temperature on the photo-induced loss of CP43 from Tris-treated PS II membranes.

Tris-treated PS II membranes were resuspended in buffers at various pH values (0.4 M sucrose, 10 mM NaCl plus one of the following buffers: Hepes (pH 7.0), Tris (pH 8.0), Ches (pH 9.0), or Cap (pH 10.0)) at 0.5 mg chlorophyll ml⁻¹ and exposed to weak light (at the light intensity of 20 µE m⁻² s⁻¹) at the temperature shown in the figure. Polypeptides were separated by SDS/urea-PAGE, stained with Coomassie brilliant blue R-250 and quantified with a densitometer.

The photo-induced loss of CP43 from Tris-treated PS II membranes depended on both pH and temperature. The amount of CP43 decreased with increase in pH or temperature of the suspension of PS II membranes (Fig. 11). It should be noted, however, that the loss of CP43 was still observed near 0°C at alkaline pH. This result contrasts with the degradation of the D1 protein by the acceptor-side inactivation of PS II during illumination with strong light. In this latter case, the degradation does not occur at low temperature (Aro et al., 1990). The optimum pH for the degradation of the D1 protein by the acceptor-side mechanism is 7.5 (Salter et al., 1992), and the profile with respect to pH is different from that of the loss of CP43 observed here.

Next, I studied the relationship between the photo-induced loss of CP43 (Fig. 12) and photo-inactivation of electron transfer activity of Tris-treated PS II membranes (Fig. 13), which was measured by photoreduction of DCP with DPC as an electron donor. When Tris-treated PS II membranes were illuminated in the presence of DPC at pH 6.5 (open triangles) or at pH 9.0 (closed triangles) at 4°C, or kept in the dark at pH 6.5 (open squares) or pH 9.0 (closed squares) at 4°C, the level of CP43 (Fig. 12) and the electron transfer activity of PS II (Fig. 13) did not change before and after the illumination. However, when Tris-treated PS II membranes were illuminated in the absence of DPC (circles) at 4°C, the level of CP43
Fig. 13 Time courses of photoinactivation of Tris-treated PS II membranes. Tris-treated PS II membranes were illuminated as described in legend to Fig. 12. Samples were withdrawn at the times indicated. Photoreduction of DCIP supported by DPC was immediately measured. Symbols are the same as those of Fig. 12.

significantly decreased at pH 9.0 (closed circle), or did not change at pH 6.5 (Fig. 12, open circle). Under these conditions, electron transfer activity of Tris-treated PS II membranes was inactivated with similar kinetics, irrespective of pH of the suspension (Fig. 13, circles). These results indicate that the inactivation of electron transfer activity of PS II is not enough to induce the deletion of PS II components, i.e., cross-linking among the PS II components, and that the cation radicals produced in PS II by illumination and elevated pH and temperature are necessary for the cross-linking reaction.

Active oxygen is involved in the degradation of the D1 protein in the acceptor-side photoinactivation of PS II (Hundal et al., 1990). By contrast, the degradation of the D1 protein by donor-side inactivation with PS II reaction center complex is independent of oxygen (Shipton and Barber, 1992). In the present study, loss of CP43 during photoinhibition was assayed in the presence or absence of oxygen. The loss of CP43 occurred even in the absence of oxygen, although the rate of loss was lower than that under aerobic conditions (Fig. 14). This result indicates that active oxygen is not involved in the deletion of CP43 during photoinhibition.

The photo-induced cross-linking of CP43 and the D1 protein observed at alkaline pH in Tris-treated PS II membranes was affected by cations. When MgCl₂ was added to the PS II membranes, levels of cross-linked products between CP43 and the D1 protein decreased significantly (Fig. 15). At the same time, a degradation product of the D1 protein appeared.

Fig. 14 Time-courses of the photo-induced loss of CP43 under aerobic and anaerobic conditions. Tris-treated PS II membranes were suspended in SCN and illuminated as described in the legend to Fig. 8 at 25°C under aerobic (circles) and anaerobic conditions (triangles). Samples were withdrawn at the times indicated and immediately solubilized with a solubilization medium for SDS/urea-PAGE. After separation of proteins by SDS/urea-PAGE, the amounts of CP43 were quantified with a densitometer.

with a molecular mass of 16 kDa, it may correspond to the polypeptide reported by Barbato et al. as the degradation product of the D1 protein in the donor-side photoinhibition (Barbato et al., 1992a). As the level of MgCl₂ rose, the 38 kDa band became clearly visible in the SDS/urea-PAGE gel. The same effects were also observed by the addition of CaCl₂ (data not shown).

Figure 16 shows dependence of the photo-induced depletion of CP43 and generation of the 16 kDa fragment of the D1 protein and the 38 kDa polypeptide on the concentrations of divalent and monovalent cations in Tris-treated PS II membranes. The 38 kDa polypeptide increased with increase in the concentration of Mg²⁺ and it was at the maximum level at about 2 mM Mg²⁺. Addition of a monovalent cation, Na⁺, had a little effect on the generation of 38 kDa polypeptide (panel A). The depletion of CP43 was inhibited by Mg²⁺ and the inhibition was saturated at about 2 mM Mg²⁺. By contrast, the level of CP43 decreased by the addition of low concentration of Na⁺ and increased again by the addition of higher concentration of Na⁺ (panel B). The cross-linking reaction seems to be regulated specially by divalent cation in PS II complex. Level of the 16 kDa fragment of the D1 protein increased with increase in the concentration of Mg²⁺, and reached a saturation level at about 20 mM with an apparent half-
Fig. 15 Effects of cations on the degradation of the D1 protein and on the cross-linking between the D1 protein and CP43 in Tris-treated PS II membranes. Tris-treated PS II membranes were suspended in SCN (pH 9.0) that contained MgCl₂ (concentrations shown in the figure) and exposed to weak light (20 μE m⁻² s⁻¹) at 25°C for 30 min. Polypeptides were separated by SDS/urea-PAGE, electroblotted onto PVDF membranes and analyzed immunologically with antibodies against the D1 protein and CP43. 'D' represents PS II membranes that were kept in darkness.

Saturating concentration of 2.5 mM. When NaCl was added, the level of the 16 kDa fragment saturated at 500 mM, with a half-saturating concentration of 100 mM (panel C). The degradation of the D1 protein to generate the 16 kDa fragment seems to be regulated electrostatically in the PS II complex. Similar effects of cations were also observed with thylakoid membranes (data not shown).

Fig. 16 Dependency on the concentration of divalent and monovalent cations of the degradation of the D1 protein, deletion of CP43 and generation of a 38 kDa polypeptide in Tris-treated PS II membranes. MgCl₂ (circles) and NaCl (squares) were added to the suspension of Tris-treated PS II membranes. Experimental conditions were the same as those described in Fig. 15. Panel A, the 38 kDa polypeptides; B, CP43; C, the 16 kDa fragment of the D1 protein.
Discussion

I described the loss of CP43, CP47 and the D1 protein from PS II membranes during treatment with Tris in weak light (20 μE m⁻² s⁻¹) by SDS/urea-PAGE (chapter I). Although the loss of CP43 was the most prominent among these processes, no products of degradation of CP43 were detected. In this chapter, I again failed to detect degradation products, even with a preparation of PS II with a simpler protein composition, antibodies with improved specificity and titer, and a sensitive detection system. Instead, a smeared band was detected over the range of molecular masses from 70 to 150 kDa in the gel after SDS/urea-PAGE (Fig. 10). Since the band cross-reacted with antibodies both against the D1 protein and CP43, I concluded that at least a part of the band represents a product of the cross-linking reaction between the two proteins. When PS II membranes that had been subjected to photoinhibition were treated with lysylendopeptidase, the smeared band disappeared and new bands with higher mobility appeared in the gel (data not shown). The monomeric D1 protein, which contains no lysine, was unaffected under these conditions. I assume that a cross-linking reaction also occurs between the D1 protein and CP47, or between CP43 and CP47, judging from the cross-reactivity with the specific antibodies, but the D2 protein and the other protein components of PS II seem to be no involved. The relative reactivity of the antibody against the D2 protein may be critical to show that there is no cross-linking products between the D2 protein and other proteins. As both the antibodies against the D1 and D2 proteins cross-reacted with the D1-D2 heterodimer with similar efficiency (Fig. 10), I suggest that the titer of the antibody against the D2 protein is high enough to detect the cross-linking products between the D2 protein and other protein components, if they are really present. Prät et al. discussed the aggregation of the D1 protein and other proteins of PS II under photoinhibitory conditions (1992), and their observation may correspond to the cross-linking observed here.

The details of the putative cross-reactions remain to be elucidated. Cross-linking of the proteins in PS II with chemical cross-linker that generates heterodimers results in a well-defined band after SDS-PAGE. The corresponding molecular mass is exactly equal to the sum of the molecular masses of the respective proteins involved (Hashimoto et al., 1993). Since the band observed in our study was diffuse, the products of the cross-linking probably consist of several components that are formed by reactions between the photo-damaged and decomposed fragments of the proteins and/or the photo-damaged but undegraded proteins. The stoichiometry of the proteins involved in the cross-linking is not known.

Both the photo-induced cross-linking of the proteins in PS II and the degradation of the D1 protein caused by the donor-side photoinactivation of PS II were affected by the ambient temperature. The reactions were retarded when the temperature was low (Fig. 11), suggesting the participation of enzyme-dependent processes. It should be noted, however, that, in contrast to the degradation of the D1 protein, the cross-linking proceeds even at temperature close to 0°C. A possible explanation is that the temperature-independent cross-linking of the proteins occurs even in the absence of the degradation of the proteins. Cross-linking was observed under both aerobic and anaerobic conditions. Therefore, active oxygen molecules, which may be produced by the irradiation of PS II in the presence of oxygen under certain conditions and damage the proteins in PS II, are not directly related to the cross-linking process. The optimum pH differs between the degradation of the D1 protein and the cross-linking reaction. The degradation of the D1 protein was most prominent at pH 7.5-8.0 (Saher et al., 1992), while cross-linking was enhanced with increasing pH (Fig. 11). These results suggest that the degradation of the D1 protein is driven by an enzyme-dependent process, while the cross-linking of the proteins in PS II includes a process that is independent of enzymatic reactions.

Irreversible damage to PS II, which leads to the degradation of the D1 protein, is caused by either the acceptor-side or donor-side photoinhibition of PS II (Aro et al., 1993). Irreversible photodamage to PS II at low light intensity was also observed in Tris-treated PS II membranes (Fig. 13). The photodamage and the loss of CP43 (cross-linking of CP43 with the D1 protein or CP47, or both) were prevented in the presence of DPC, a detergent of PS II (Fig. 12 and 13), indicating that the photo-induced loss of CP43 is caused by the donor-side photoinhibition of PS II. The mechanism of the donor-side photoinhibition has been studied in detail with PS II membranes that were treated with hydroxylamine (Blubaugh and Cheniae, 1990, Blubaugh et al., 1991, Ono and Inoue, 1991). Blubaugh et al. indicated that the components on the donor side of PS II are destroyed by weak light and the order of susceptibility of the components to such damage was proposed to be chlorophyll/carotenoid > Y₃ > Y₁ > P₆80, phoephptin, QA (1991). Under strong light, emission of A₁ band of thermoluminescence from PS II, which reflects the charge recombination of QA and a putative oxidized histidine residue (Ono and Inoue, 1991a), as well as the photoreactivation of PS II, was rapidly impaired, as compared with the loss of the EPR signals that correspond to Yₚ and Y₃⁺ (Ono and Inoue, 1991b). Thus, illumination of PS II that has been deprived of Mn results in destruction or modification of amino acid residue in the D1 protein and/or in the other protein components of PS II. Preparations of PS II that have been treated with Tris and exposed to weak light are probably inactivated by the same mechanism as the mechanism that is operative upon treatment with hydroxylamine because, in both cases, the membranes are devoid of the catalytic Mn that is required for the evolution of oxygen and are susceptible to donor-side photoinhibition. I assume that the cation radicals, namely, P₆80⁺, Yₚ⁺ and chloroprophyl⁺, which are produced on the donor side of PS II, are responsible for the cross-linking reactions of the proteins observed here. The cross-linking reaction seems to occur in a limited domain of PS II, probably near the site at which cation radicals are produced, at alkaline pH.

Cations affect the cross-linking among the components of PS II and induces the degradation of the D1 protein under the present conditions (Fig. 15 and 16). The addition of divalent cations induced a decrease in the level of the product of cross-linking between CP43 and the D1 protein and an increase in the level of the 38 kDa cross-linking product of the D1
protein (Fig. 16). Cross-linking reaction between CP43 and the D1 protein, and that between the D1 protein and a 5 kDa polypeptide in PS II to generate the 38 kDa polypeptide was not affected so much by the addition of NaCl. I suggest that divalent cation regulates cross-linking of the D1 protein with CP43 or 5 kDa polypeptide in PS II, near the site at which cation radicals are produced. Yamamoto and Akasaka reported that cross-linking reaction among components of PS II was not induced, when NH$_2$OH-treated PS II membranes, which mostly retains the extrinsic 33 kDa proteins, and Tris-treated PS II membranes reconstituted with the extrinsic 33 kDa proteins of PS II were illuminated with strong light (1995). The effect of divalent cation on the suppression of cross-linking reaction may be related to stabilization of conformation of the PS II complex lacking the extrinsic 33 kDa protein.

No specific cation was linked to the photo-induced degradation of the D1 protein under the present conditions (Fig. 16). Thus, the effect of cations in the degradation of the D1 protein is apparently not chemical but electrostatic. It seems to be controlled by electrostatic interactions between related proteins in PS II. But, I can not exclude the possibility that chemical interaction specific for divalent cation may be involved in the degradation of the D1 protein, because Mg$^{2+}$
induced two times as much the 16 kDa fragment of the D1 protein as Na$^{+}$ in their saturating concentrations. The actual concentration of free Mg$^{2+}$ in the stroma is about 1 mM in the dark and this value increases to about 2-4 mM upon illumination (Pfützer, 1973; Gimmler et al., 1975). It is possible that Mg$^{2+}$ regulates degradation of the D1 protein under the conditions where the concentration of Mg$^{2+}$ in the thylakoid lumen also varies during illumination and Mn and the extrinsic proteins are not tightly associated with PS II core proteins. This may be the case in the early step of greening of chloroplasts and in the stroma thylakoids of mature chloroplasts.

Kim et al. detected a 160 kDa cross-linking product that was recognized with the antibody against the D1 protein and appeared in vivo both under irradiance-stress conditions and upon inhibition of chloroplast protein biosynthesis by chloramphenicol (1993). The 160 kDa cross-linking product may be one of the cross-linking products observed here. In the present study, I detected a cross-linking product using PS II membranes, which do not have a capability for de novo biosynthesis of protein. Cross-linking among the components of PS II observed here may be associated with repair cycles of PS II, and serves as a stopgap measure under the conditions that the photo-damage to PS II and disassembly of PS II are accelerated, when the repair cycle of PS II does not work. Alternatively, cross-linking among the components of PS II may be an inevitable reaction in the photodamaged and partially disassembled PS II complex such as Tris-treated PS II membranes, when the repair cycle of PS II does not work.

Summary

The photo-induced reduction in the level of the antenna chlorophyll-binding proteins CP43 and CP47, as well as the reaction-center forming protein D1, in PS II was studied with PS II membranes and PS II complexes that had been treated with alkaline Tris. The loss of CP43, as detected by SDS/urea-PAGE, was attributed mostly to specific cross-linking reactions among CP43, CP47 and the D1 protein. The cross-linking of the D1 protein and the α-subunit of cytochrome b559 was also observed. The relationship between the photo-induced loss of CP43, i.e., cross-linking of CP43 with the D1 protein and CP47, and the photo-inactivation of electron transfer of Tris-treated PS II membranes was also studied. Electron transfer activity of PS II was inactivated by illumination irrespective of pH. But, the photo-induced loss of CP43 was observed with dependence of pH. The cross-linking reaction was stimulated by elevated pH and temperature. These results suggest that photo-inactivation of electron transfer activity of PS II itself is not enough to induce the cross-linking reaction and that elevated pH and temperature are required additionally for the cross-linking reaction in Tris-treated PS II membranes. The cross-linking reaction was independent of oxygen. Addition of divalent or monovalent cations induced a 16 kDa degradation product of the D1 protein. A simultaneous decrease in the amount of the product of cross-linking between the D1 protein and CP43, and increase in the amount of a 38 kDa cross-linking form of the D1 protein were observed only when divalent cations were added. The cross-linking reactions seem to be regulated specifically by divalent cations in PS II complex.
CHAPTER III
Degradation of the D1 protein in the donor-side photoinhibition

Introduction
Photoinhibition causes impairment of electron transfer activity of PS II, and results in degradation of the D1 protein. (Aro et al., 1993). Two major mechanisms for photoactivation and degradation of the D1 protein have been proposed, i.e. acceptor-side and donor-side photoinhibition (Barber and Andersson, 1992).

In the acceptor-side photoinactivation, singlet oxygen damages the D1 protein (Vass et al., 1992). In the donor-side photoinactivation, strong oxidizing species, such as P680*, Chl*, and Yz*, produced at the donor side of PS II, damage the D1 protein and induce the subsequent degradation of the D1 protein. This process is independent of active oxygen (Eckert et al., 1991), but the generation of superoxide radicals, which damage the D1 protein, has been reported in the donor-side photoinactivation (Chen et al., 1992).

In the donor-side photoinactivation, a 24-kDa (C-terminal) and a 9-kDa (N-terminal) fragments were identified as the degradation products with strongly illuminated PS II reaction center complexes (Shipton and Barber, 1991). A 16-kDa (C-terminal) fragment was found in Tris-treated thylakoid membranes and in various preparations of PS II other than the reaction center complexes (Barbato et al., 1992a). A proteolytic activity within PS II, but not active oxygen species, has been shown to be responsible for the cleavage (Shipton and Barber, 1992). Recently, it is also reported that the damaged D1 protein is cleaved by the action of active oxygen (Miyao, 1994; Yamamoto and Akasaka, 1995).

In chapter II, I described detection of a 16 kDa degradation fragment of the D1 protein with illumination of the Tris-treated PS II membranes under alkaline pH in the presence of cations. The degradation of the D1 protein seems to be regulated by electrostatic interaction in PS II. In chapter III, I studied the degradation of the D1 protein at weak light under alkaline pH in the presence of cations, using Tris-treated thylakoids and PS II membranes. I found that the 16 kDa fragment of the D1 protein is the C-terminal part of the D1 protein, and that the photo-induced degradation of the D1 protein was suppressed completely under anaerobic conditions and partially in the presence of histidine. These results suggest involvement of singlet oxygen in the degradation of the D1 protein in the donor-side photoinhibition.

Materials and Methods
Preparation of thylakoids and PS II membranes
Spinach was obtained from a local market. The leaves were ground in a blender with an isolation medium containing 0.33 M sorbitol, 50 mM Tricine-KOH, 15 mM NaCl and 5 mM MgCl2 (pH 7.6). The homogenates were filtered through 4 layers of cheese cloth and centrifuged at 3,000 × g for 5 min. The precipitates were suspended in the isolation medium without 0.33 M sorbitol. After removing unbroken cells by centrifugation at 500 × g for 30 sec, thylakoid membranes were recovered, washed twice with a solution containing 0.1 M sorbitol, 50 mM Tricine-KOH, 15 mM NaCl and 5 mM MgCl2 (pH 7.6) (buffer A), and finally resuspended in buffer A supplemented with 30% ethyleneglycol and stored at -80°C.

Wheat seeds were planted in vermiculite and watered with half-strength Hoagland's nutrient solution. Seedlings were grown for 9-11 days at 25°C in the light (6 W m⁻²) as described previously (Hashimoto et al., 1993). Thylakoid membranes of wheat were prepared as described above.

PS II membranes were prepared from spinach thylakoid membranes with Triton X-100 (Kuwabara and Murata, 1982), suspended in 0.4 M sucrose, 40 mM Mes-NaOH, 10 mM NaCl, 5 mM MgCl2 and 30% ethyleneglycol (pH 6.0) and stored at -80°C until use.

For Tris treatment, PS II membranes were suspended in 0.8 M Tris-HCl and 3 mM EDTA (pH 8.5), at about 0.5 mg chlorophyll ml⁻¹, incubated on ice for 30 min in the dark, recovered by centrifugation at 15,000 × g for 15 min and washed twice with 0.4 M sucrose and 40 mM Mes-NaOH (pH 9.0) (buffer B). Tris-treatment of thylakoid membranes was carried out in the same procedure as that used for PS II membranes except that a medium containing 0.1 M sorbitol and 50 mM Mes-NaOH (pH 9.0) (buffer C) was used in the washing step.

Photoinhibition
Tris-treated PS II membranes or thylakoid membranes were resuspended in buffer B or C supplemented with the additions indicated in the legends to figures. These membranes were illuminated as described in chapter II. The incident intensity was 20 μE m⁻² s⁻¹. All handling of membranes prior to and following the illumination was done in dim green light. Anaerobic conditions were achieved as described in chapter II.

Protein analysis
Proteins in the illuminated samples were analyzed as described in chapter II. When samples were treated with lysylendopeptidase, a solubilization medium containing 0.4 M Mes-NaOH, 5% SDS, 5% β-mercaptoethanol, 10% glycerol and 0.005% bromophenol blue (pH 6.0) was used. The polypeptides were quantified by scanning the bands developed on X-ray films with a Personal Densitometer PD110 (Molecular Dynamics, U.S.A.).

Source of Enzyme
The sources of enzymes used in this work were as follows: lysyl endopeptidase and catalase (bovine liver) from Wako Pure Chemical Industries Ltd. (Osaka, Japan); Cu, Zn-
superoxide dismutase (bovine erythrocytes) from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and glucose oxidase from Biozyme Laboratories Ltd. (U.S.A.).

Results

As described in chapter II, when Tris-treated PS II membranes were illuminated with weak light (20 μE m⁻² s⁻¹) under alkaline pH conditions in the presence of cations, a 16 kDa degradation fragment of the D1 protein was produced. This fragment was also detected with Tris-treated thylakoid membranes under the same conditions (Fig. 17, lane 2). I used thylakoid membranes of wheat in order to examine the cleavage site of the D1 protein (Fig. 17). The D1 protein of wheat contains one lysine residue at position 238 (Marder et al., 1988), while the D1 of spinach contains no lysine residue (Zurawski et al., 1982). Illumination of thylakoid membranes of wheat in the presence of MgCl₂ under alkaline pH generated a band with a molecular mass of 16 kDa (lane 4). The size of the band is apparently the same as the 16 kDa.

Fig. 17 Generation of a 16 kDa fragment of D1 protein in thylakoid membranes from spinach and wheat. Tris-treated thylakoid membranes from spinach (lanes 1-2) and wheat (lanes 3-6) were suspended in buffer B supplemented with 10 mM MgCl₂ at 0.1 mg chlorophyll ml⁻¹, incubated without (lanes 1, 3 & 5) and with illumination (lanes 2, 4 & 6) with weak light (20 μE m⁻² s⁻¹) for 30 min at 25°C. The reaction was stopped by the addition of an equal volume of solubilization medium in the absence (lanes 1-4) or presence (lanes 5 & 6) of 0.5 mg/ml lysolecithin. After incubation for 2 hr at 25°C, the polypeptides were analyzed by SDS/urea-PAGE and immunodetected with the antibody against the D1 protein.

Fig. 18 Effects of oxygen on generation of the 16 kDa fragment of the D1 protein. Tris-treated PS II membranes were suspended in buffer C supplemented with 10 mM MgCl₂ at 0.5 mg chlorophyll ml⁻¹, and illuminated (light intensity of 20 μE m⁻² s⁻¹) at 25°C for 30 min under the aerobic (lane 2) or anaerobic (lane 3) conditions. The polypeptides were analyzed by SDS/urea-PAGE and immunodetected with the antibody against the D1 protein. D denotes dark control.

![Image of a gel with bands](image)

![Image of a bar graph](image)

Fig. 19 Effects of scavengers of oxygen radicals on the production of the 16 kDa fragment of the D1 protein. Experimental conditions were the same as those described in the legend to Fig. 18. In each reaction mixture, one of the following scavengers was added: 20 mM histidine, 2 mM DABCO, 20 mM mannitol, 0.1 mg/ml catalase and 5 U/ml SOD. After immunodetection, the 16 kDa fragment of the D1 protein was quantified.
fragment observed in the thylakoid membranes of spinach, indicating that the cleavage site of the D1 protein of wheat is the same as that of spinach (lanes 2 and 4). Treatment of thylakoid membranes of wheat with lysylendopeptidase before electrophoresis eliminated the 34 kDa band of the D1 protein and made two bands appear, which correspond to the N-terminal fragment from N-acetyl-O-phospho-Thr-2 to Lys-238 and a C-terminal fragment from Phe-239 to Ala-344 of the wheat D1 protein (lanes 5 and 6). The 16 kDa fragment of the wheat D1 protein was also digested with lysylendopeptidase (lane 6), indicating that the 16 kDa fragment of the D1 protein contains a lysine residue at position 238.

Involvement of various active oxygen species in photoinactivation of PS II and in degradation of the D1 protein has been proposed (Bradley et al., 1991, Chen et al., 1992, Miyao, 1994). Figure 18 shows involvement of active oxygen in the degradation of the D1 protein under alkaline pH at weak light. In anaerobic conditions, no 16 kDa fragment of the D1 protein was detected (lane 3). Dependence of the degradation of the D1 protein on active oxygen observed here is different from the results obtained by Shipton and Barber (1992), which showed that the photo-induced degradation of the D1 protein is dependent of active oxygen in the donor-side photoinhibition of PS II.

Effects of active oxygen scavengers on the oxygen-dependent degradation of the D1 protein under alkaline pH conditions in weak light were investigated (Fig. 19). The scavengers used were histidine and DABCO (for singlet oxygen), mannitol (for hydroxyl radical), catalase (for hydrogen peroxide), SOD (for superoxide), or a combination of catalase and SOD. Among the scavengers investigated, only histidine, a radical scavenger for singlet oxygen, suppressed the generation of the 16 kDa fragment significantly. These results suggest that singlet oxygen is involved in the degradation of the D1 protein under alkaline pH in weak light.

In Tris-treated PS II membranes, histidine is accessible to both the luminal andstromal surfaces of PS II complexes because PS II membranes are non-vesicular fragments consisting of the appressed grana membranes (Dumahay et al., 1984). I studied the site of action of histidine, namely, the site where singlet oxygen involved in the degradation of the D1 protein is produced (Fig. 20). When Tris-treated thylakoid membranes were illuminated at alkaline pH, histidine did not inhibit the degradation of the D1 protein (lane 6). If singlet oxygen is produced in the stromal surface of PS II complex, histidine should be effective in suppression of photo-induced degradation of the D1 protein with both Tris-treated thylakoid membranes and PS II membranes. These results indicate that the singlet oxygen is produced at the luminal surface of PS II complexes.

Figure 21 shows dependence of the generation of the 16 kDa degradation fragment of the D1 protein on pH. A 16 kDa degradation fragment of the D1 protein was generated more efficiently at alkaline pH (Fig. 21). The dependence on pH of the 16 kDa fragment observed here is different from that of the loss of CP43, where CP43 was deleted gradually with increase of pH (Fig. 11). The optimum pH for the degradation of the D1 protein by the

![Fig. 20](image) Effects of histidine on the production of the 16 kDa fragment in thylakoids and PSII membranes. Tris-treated PS II membranes (lanes 1, 2) and thylakoid membranes (lanes 3, 4) were resuspended in buffer B supplemented with 10 mM MgCl₂, and 20 mM histidine. Other experimental conditions were the same as those in Fig. 18.

![Fig. 21](image) Dependency on pH of the production of the 16 kDa fragment of the D1 protein. Tris-treated PS II membranes, resuspended in buffer B supplemented with 10 mM MgCl₂, were illuminated and analyzed as described in the legend to Fig. 18. pH of the suspension was adjusted with buffers containing Mes (pH 6.0), Mops (pH 7.0), Tricine (pH 8.0), Caps (pH 10.0), which were added to buffer B in place of Ches.
acceptor-side photoinactivation (Salter et al., 1992) and by the donor-side photoinactivation (Shipton and Barber, 1992, Yamamoto and Akasaka, 1995) is 7.5 and 6.5, respectively.

Discussion

Illumination of Tris-treated PS II membranes with weak light in the presence of cations at alkaline pH induced a 16 kDa degradation fragment of the D1 protein. I tried to determine the cleavage site of the degradation of the D1 protein by direct sequencing of the 16 kDa fragment, but it was not successful because of the limited amount of the fragment available. I tried to estimate the cleavage site of the D1 protein by another way. The amino acid sequence was deduced from cDNA (Marler et al., 1988). Considering acetylation and phosphorylation at the N-terminal threonine residue (Michel et al., 1988), palmitoylation at cysteine residue (Mattoo and Edelman, 1987) and processing on the COOH side of Ala at position 344 (Takahashi et al., 1988), molecular weight of the wheat D1 protein is calculated to be 38324. Treatment of the wheat D1 protein with lysyl endopeptidase results in two fragments, the N-terminal and C-terminal fragments, having a calculated molecular weight of 26311 and 12031, respectively, because of the presence of a lysine residue at position 238 in wheat D1 protein. From relationship between electrophoretic mobility on SDS/urea-PAGE and calculated molecular weight of the wheat D1 protein and the two fragment of the wheat D1 protein obtained by digestion with lysylendopeptidase, I estimated molecular weight of the photo-induced 16 kDa fragment to be 19120 7 936. Considering that the 16 kDa fragment of wheat D1 protein contains a lysine residue at position 238 and assuming that the 16 kDa fragment contains a native C-terminus of the D1 protein, the above estimation suggests that the cleavage site is located somewhere at the region from Gly-164 to Phe-182 of the D1 protein, which is located in the loop connecting the C and D helices (the so-called CD loop) exposed to the luminal surface (Fig. 22). Barbato et al. detected a 16 kDa fragment of the D1 protein when Tris-treated thylakoid membranes and various PS II preparations were illuminated with strong light, and suggested that the 16 kDa fragment is produced by cleavage at the CD-loop of the D1 protein (1992a). It is possible that the 16 kDa fragment observed here is identical with the 16 kDa fragment observed by Barbato et al. A 16 kDa fragment observed in the acceptor-side photoinhibition (Virgin et al., 1990) may also be cleaved at the same site, i.e., the CD-loop of the D1 protein. The fragment is thought to be cleaved at the DE-loop of the D1 protein (Salter et al., 1992). However, I think that a molecular mass of the fragment is too small if the D1 protein is cleaved at the DE-loop and the fragment contains the C-terminus of the D1 protein.

I fail to detect N-terminal fragment of the D1 protein except for the 16 kDa fragment. N-terminal fragment of the D1 protein may not have been detected because of the poor recognition by the antibodies used. In fact, after treatment of wheat D1 protein with lysylendopeptidase, the antibodies cross-reacted more effectively with the C-terminal fragment than with the N-terminal fragment of the D1 protein (Fig. 17).

Histidine, a radical scavenger for singlet oxygen, greatly suppressed the photo-induced degradation of the D1 protein under the present conditions. But, DABCO did not affect the degradation of the D1 protein. This results may reflect the low accessibility of their scavengers to the site of singlet oxygen formation because singlet oxygen react mainly at the site where it is formed. In respect to the efficiency of scavenging singlet oxygen, histidine has already been demonstrated to be a nice scavengers (Pfliiger, 1973). Mishra and Ghosnatsik reported that the degradation of the D1 protein in PS II complexes exposed to chemically generated singlet oxygen is inhibited by the addition of histidine (1994b). It is probable that singlet oxygen is responsible for the degradation of the D1 protein observed here.

Strong illumination of oxygen-evolving PS II complexes and thylakoid membranes produces singlet oxygen that is formed by the reaction of triplet state of P680 (P680) with the ground state of oxygen. The formation of singlet oxygen by the reaction between triplet state of P680 and oxygen requires light strong enough to overreduce QA and to dissociate QA from PS
II complex (Vass et al., 1992). But, light intensity at 20 μE m⁻² s⁻¹ used here is not enough to overdrive QA. Thus, it is unlikely that singlet oxygen is formed by the reaction between triplet state of P680 and oxygen under the present conditions.

Chen et al. demonstrated with NH₂OH-treated PS II membranes that superoxide is produced at the donor side of PS II in the donor-side photoinactivation and is involved in photoinactivation of Yz (Chen et al., 1992). The annihilation reaction between superoxide radicals and appropriate cation radicals can yield an appreciable amount of singlet oxygen (Mayada and Bard, 1973). In the donor-side photoinactivation, it is reported that various cation radicals such as Yz⁺, P680⁺, chlorophyll⁻ and the oxidized form of putative redox-active histidine, are produced at the donor side of PS II (Blubaugh and Cheniae, 1990, Blubaugh et al., 1991, Eckert et al., 1991, Ono and Inoue, 1991b). P680, Yz, which is tyrosine-161 of the D1 protein (Debus et al., 1988), and the putative redox-active histidine, which recently is suggested to be histidine-190 of the D1 protein (Kramer et al., 1994), are located at the luminal surface of the D1 protein (Fig. 22, Svensson et al., 1990). I speculate that such cation radicals are reactive with superoxide and that oxidation of superoxide by such cation radicals results in formation of singlet oxygen on the luminal surface of the D1 protein. Addition of SOD, however, had no effect in the degradation of the D1 protein (Fig. 19). Thus, singlet oxygen may be produced directly from oxygen through an unknown pathway, but not from superoxide, at alkaline pH.

As described above, it is presumed that singlet oxygen is produced at the luminal surface of the D1 protein. This idea is supported by ineffectiveness of histidine, a scavenger of singlet oxygen, on the photo-induced degradation of the D1 protein in Tris-treated thylakoid membranes. Histidine may be inaccessible to the luminal surface of PS II in thylakoid membranes (Fig. 20).

The degradation of the D1 protein was not observed here at neutral pH by the illumination with weak light (Fig. 21). For photo-induced degradation of the D1 protein in vitro, strong illumination of thylakoid membranes and of PS II preparations is required (Aro et al., 1993). Two conditions, i.e. alkaline pH and the presence of cations, may allow the degradation of the D1 protein, which is induced only under strong illumination in vitro. It is likely that the production of singlet oxygen is accelerated or that the D1 protein becomes susceptible to singlet oxygen, under these conditions.

Recently, Miyao, and Yamamoto and Akasaka suggested the involvement of active oxygen on the degradation of the D1 protein in the acceptor-side or donor-side photoinactivation (Miyao, 1994; Yamamoto and Akasaka, 1995). Singlet oxygen and hydroxyl radical are known to cause cross-linking and fragmentation of proteins. Hydroxyl radical can act directly to cleave the peptide bonds of proteins (Stadman, 1993), while singlet oxygen does indirectly, e.g., by generating alkoxyl radicals (Elstner, 1982). With respect to cleavage of the peptide bonds of the D1 protein, I favor the indirect cleavage by singlet oxygen rather than the proteolytic cleavage (Aro et al., 1993), although I have no direct evidence showing the generation of alkoxyl radicals.

Photo-induced formation of the C-terminal fragment of the D1 protein with a similar molecular mass as the 16 kDa fragment detected here was observed in the acceptor-side photoinactivation (Virgin et al., 1990). In that case, it is generally believed that singlet oxygen is involved in the degradation of the D1 protein although proteolytic cleavage is thought to be mainly responsible for the degradation (Vass et al., 1992). Barbato et al. also detected a 16 kDa degradation fragment of the D1 protein in the donor side photoinactivation (Barbato et al., 1992a). In the degradation of the D1 protein, which generates the 16 kDa fragment irrespective of the illumination conditions, singlet oxygen produced near the CD-loop of the D1 protein may be involved.

Summary

Degradation of the D1 protein by the illumination of Tris-treated thylakoids and PS II membranes with weak light (20 μE m⁻² s⁻¹) was studied in the presence of cations under alkaline pH conditions. It was estimated that the D1 protein is cleaved near the CD-loop of the D1 protein to generate a degradation fragment with a molecular mass of 16 kDa. Production of the 16 kDa fragment was completely inhibited under anaerobic conditions, and greatly suppressed by the addition of histidine, a scavenger of singlet oxygen. The effect of histidine was observed in Tris-treated PS II membranes but not in Tris-treated thylakoid membranes. These results suggest that singlet oxygen that is produced at luminal surface of the D1 protein is involved in the degradation of the D1 protein under the present conditions.
CONCLUSION

PS II in thylakoid membranes is a target for photoinhibition of photosynthesis. As PS II is the site of the primary photochemical reaction in photosynthesis, its inhibition leads to the suppression of the whole reaction of photosynthesis. The impairment of PS II during photoinhibition involves photoinactivation of electron transfer activity and specific degradation of the D1 protein. In the present study, I investigated the effects of the donor-side photoinhibition of PS II on the polypeptides of PS II.

Illumination of PS II membranes, which lack the oxygen-evolving complex, with weak light (20 μE m⁻² s⁻¹) induced partial deletion of several components of PS II, i.e., chlorophyll α-binding proteins CP43 and CP47 and the reaction-center D1 protein, and inactivation of electron transfer in PS II. The photo-induced deletion of the PS II components was due to cross-linking among these components. The cross-linking reactions were dependent on pH and temperature, but independent of oxygen. Two conditions, i.e., elevated pH and formation of cation radicals in the donor-side of PS II, were responsible for the cross-linking. The cross-linking reactions seemed to be regulated by divalent cations in PS II complexes.

Electrostatic interaction in PS II regulated the degradation of the D1 protein and generation of the 16 kDa fragment, under illumination. Probably the D1 protein is cleaved at the CD-loop of the D1 protein exposed to the lumen. From the effectiveness of scavengers of various active oxygen species, it is suggested that the formation of singlet oxygen in the luminal side of PS II complex is involved in the degradation of the D1 protein.

The degradation and cross-linking of the polypeptides in PS II are closely associated with the repair cycle of PS II under the photoinhibitory conditions. Involvement of oxygen and cations in regulation of these processes was demonstrated in the present work.

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