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Deletion of antenna chlorophyll-*a*-binding proteins CP43 and CP47 by Tris-treatment of PS II membranes in weak light: Evidence for a photo-degradative effect on the PS II components other than the reaction center-binding proteins

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Treatment of spinach Photosystem 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 sodium dodecyl sulfate/urea-polyacrylamide gel electrophoresis. D1 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 were 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 oxidizing side of PS II during the Tris-treatment of the PS II membranes in the weak light. Difficulty in detecting the degradation products of CP43 indicates the high efficiency of the breakdown process of the protein.

Introduction

Photosystem II (PS II) is a multi-subunit complex in the thylakoid membrane, consisting of the reaction center-binding proteins D1 and D2 [1,2], antenna chlorophyll a -binding proteins CP43 and CP47 [3], the extrinsic 33, 24 and 18 kDa proteins [4], alpha- and beta-subunits of cytochrome *b*-559 [5], I protein [6], and the other components of lower molecular weight (see Ref. 7 for a review). With irradiation of PS II, charge separation occurs between the primary electron donor P680 and the intermediary electron acceptor pheophytin [8], and the electron is transferred to the first stable electron acceptor Q_A [9] and then to the secondary electron acceptor Q_B [10]. On the oxidizing side of PS II, $P680^+$ is reduced by the secondary electron donor, Z, which is known to be tyrosine 161 of the D1 protein [11], and the oxidized Z is then reduced by Mn. Four atoms of Mn in PS II organize a cluster [12,13] and accumulate the oxidizing equivalents required for water oxidation.

One of the remarkable features of PS II is its high susceptibility to photo-inhibition [14,15]. When chloroplasts are illuminated with strong light, electron transport is inhibited and degradation of D1 protein is accelerated [16–18]. Accumulation of radical species at the Q_B site of D1 protein [16,17] has been shown to be related to the rapid turn-over of D1 protein [19,20]. However, it was also shown that the reaction of the oxidizing side of PS II is responsible for the inhibition of electron transport and degradation of D1 protein [21,22]. In agreement with that, it was recently shown that the turn-over of D1 and D2 increases under illumination with weak light in the hydroxylamine-washed chloroplasts [23]. Acceleration of the breakdown of D1 and D2 is also observed in Cl$^-$-depleted thylakoid membranes where transition from S_2 to S_3 in the S-cycle of oxygen evolution is reversibly inhibited [24]. Thus, it is probable that the removal of Mn from the catalytic site of water oxidation, or the blocking of oxidation of Mn, induces inevitable degradation of D1

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Abbreviations: PS II, Photosystem II; D1 and D2, reaction center-binding proteins of PS II; CP43 and CP47, antenna chlorophyll-binding proteins of PS II; P680, primary electron donor of PS II; Q_A, first stable electron acceptor of PS II; Q_B, secondary electron acceptor of PS II; Z, secondary electron donor of PS II; DCMU, 3-(3, 4-dichlorophenyl)-1, 1-dimethylurea; DPC, 1,5-diphenylcarbazide; SDS, sodium dodecyl sulfate; LDS, lithium dodecyl sulfate; LHC II, light-harvesting chlorophyll-protein complex associated with PS II.

and D2 proteins under the illumination. Light-induced acceleration of breakdown of D1 protein was observed not only with chloroplasts but also with oxygen-evolving PS II core preparations [25] and PS II reaction center preparations [26]. In the latter case, the degradation was shown to occur on the oxidizing side of PS II.

In the present study, we treated the PS II membranes from spinach chloroplasts with 0.8 M Tris under illumination with weak light, which is known to remove Mn and the extrinsic proteins from the PS II membranes [4]. 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 D1 and CP47 also occurred, but the extent was less, compared with the deletion of CP43 under the same conditions. These results first present evidence that the PS II components, other than the reaction center-binding proteins, are significantly affected under photoinhibitory conditions.

Materials and Methods

PS II membranes were prepared from spinach by the method of Kuwabara and Murata [27]. The membranes were washed twice with a solution containing 0.4 M sucrose, 40 mM Mes-NaOH and 10 mM NaCl, pH 6.5 (buffer A), suspended in a solution containing 30% ethylene glycol, 0.4 M sucrose, 20 mM Mes-NaOH, 10 mM NaCl and 5 mM $MgCl_2$, pH 6.5, and stored at −80°C before use. For Tris-treatment, the PS II membranes, washed once with buffer A, were 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 the illumination, white fluorescent lamps were used and the incident intensity was 20 $\mu E\ m^{-2}\ s^{-1}$ 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 pelleted by centrifugation at 16000 $\times g$ for 15 min and washed twice with buffer A in the dark. Treatment of the PS II membranes with 1 M $CaCl_2$ to remove the extrinsic 33 kDa protein was carried out in the light (20 $\mu E\ m^{-2}\ s^{-1}$) by the method of Ono and Inoue [28]. The $CaCl_2$-washed PS II membranes were recovered by the same centrifugation step used for the Tris-treatment, but without further washing, to avoid removal of Cl^- from the membranes. In the experiments where the effects of the reductants for PS II and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) were studied, each reagent was dissolved in 100% ethanol and added to the Tris-treated PS II membranes during the illumination. The reductants used here were 0.5 mM each of 1,5-diphenylcarbazide (DPC), sodium ascorbate, hydroxylamine-HCl, potassium fertocyanide and hydroquinone. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was done according to Laemmli [29] except 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. The proteins in the gel were stained by Coomassie brilliant blue R250 and quantified by measuring absorption at 565 nm with a dual-wavelength TLC scanner CS-930 (Shimadzu). The non-denaturing lithium dodecyl sulfate (LDS)-polyacrylamide gel electrophoresis was done according to the method of Camm and Green [30]. The PS II membranes were solubilized with a solution containing 0.9% (w/v) *n*-octyl-β-D-glucopyranoside, 10% (v/v) glycerol and 2 mM Tris-maleate (pH 7.0) to a concentration of chlorophyll of 0.6 mg ml^{-1}. After incubation for 10 min, the solution was centrifuged at 16000 $\times g$ for 10 min and the supernatant was subjected to electrophoresis. Western blotting was carried out as previously described [31]. Proteins of the PS II membranes after SDS/urea polyacrylamide gel electrophoresis were blotted onto poly(vinylidene difluoride) (PVDF) membranes 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 measuring beam of the TLC scanner was set at 585 nm. The antisera against D1 protein were kindly gifted by Dr. J.E. Mullet of Texas A&M University. Chlorophyll was determined in 80% acetone using the absorption coefficients reported by MacKinney [32].

Results and Discussion

The PS II membranes were treated with 0.8 M Tris-HCl in the light (20 $\mu E\ m^{-2}\ s^{-1}$), and the proteins in the membranes were analyzed by SDS/urea-polyacrylamide gel electrophoresis (Fig. 1). 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 [4]. Mn was also released from the membranes under these conditions. We 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 buffer A (pH 6.5) (Fig. 2). Above pH 8.5, the apoprotein 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

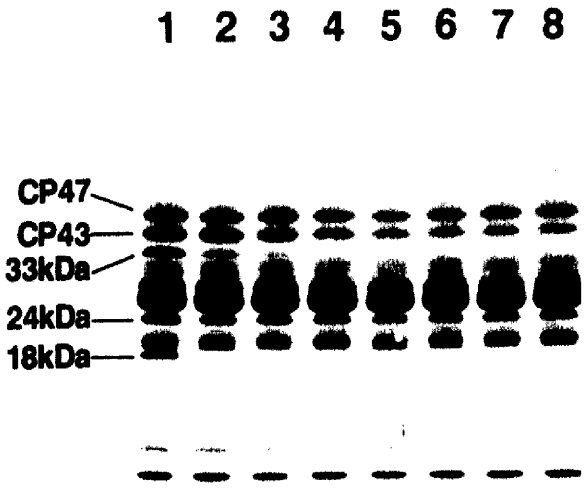

Fig. 1. SDS/urea-polyacrylamide gel electrophoresis showing the effect of Tris-treatment on the PS II membranes. Lane 1, the control PS II membranes, incubated with a solution containing 0.4 M sucrose, 40 mM Mes-NaOH and 10 mM NaCl, pH 6.5 (buffer A) for 30 min at 4°C, and illuminated with white fluorescent lamps (20 μE m^{-2} s^{-1}). Lanes 2–8, the PS II membranes treated with 0.8 M Tris-HCl for 30 min at 4°C under the same illumination condition as lane 1. The pH values of Tris-HCl were 7.0 (lane 2), 7.5 (lane 3), 8.0 (lane 4), 8.5 (lane 5), 9.0 (lane 6), 9.5 (lane 7), 10.0 (lane 8), respectively. Chlorophyll was adjusted to 0.5 mg/ml in each treatment.

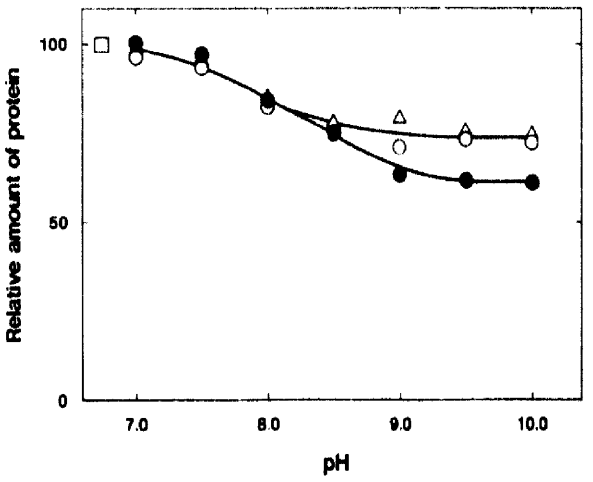

Fig. 2. Degradation of the PS II core components by Tris-treatment in the light, at various pH values. The concentration of Tris was 0.8 M, and the incident intensity was 20 μE m^{-2} s^{-1}. The amounts of the apoproteins of CP43 and CP47 were determined from the densitogram of the proteins shown in Fig. 1. D1 was determined by Western analysis with a specific antibody. ●, CP43; ○, CP47; △, D1 protein. As control, the amount of each protein in lane 1 of Fig. 1 is depicted (shown as □).

of D1 protein in parallel with the decrease in the apoproteins of CP43 and CP47, we determined D1 protein by Western blotting with a specific antibody (Fig. 2). The pattern of decrease in the amount of D1 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. 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-polyacrylamide gel electrophoresis of the PS II membranes (Fig. 3). 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 holo-complexes of CP43 and CP47, were reduced significantly after the Tris-treatment in the light. Other major protein components of the PS II membranes, such as LHC II and cytochrome *b* 559, were not affected at all under the same condition, judging from both the SDS/urea-polyacrylamide and non-denaturing LDS-polyacrylamide gel electrophoresis.

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 polypeptides and Mn from PS II by the treatment [4]. Usually Tris-treatment was carried out either in complete darkness or under dim light, and there is no extensive comparative study on the effects of both the conditions on the intrinsic membrane proteins of PS II. To see the effect of light during the Tris-treatment of CP43, we carried

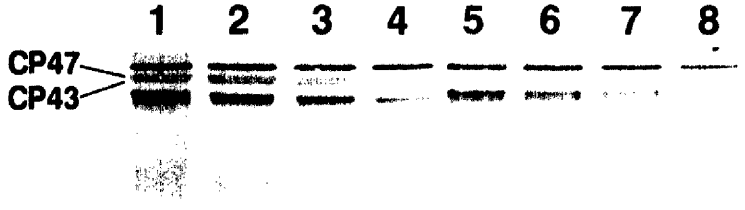

Fig. 3. A gel showing the non-denaturing lithium dodecyl sulfate polyacrylamide gel electrophoresis of the PS II membranes. The PS II membranes before (lane 1) and after Tris-treatment (lanes 2–8) under weak illumination were subjected to the electrophoresis and four green bands were detected in each lane. The numbers of the lane correspond to those in Fig. 1. The positions of CP43 and CP47 are indicated to the left of the gel. The bands above and below CP43 and CP47 correspond to the dimer and monomer of LHC II, respectively.

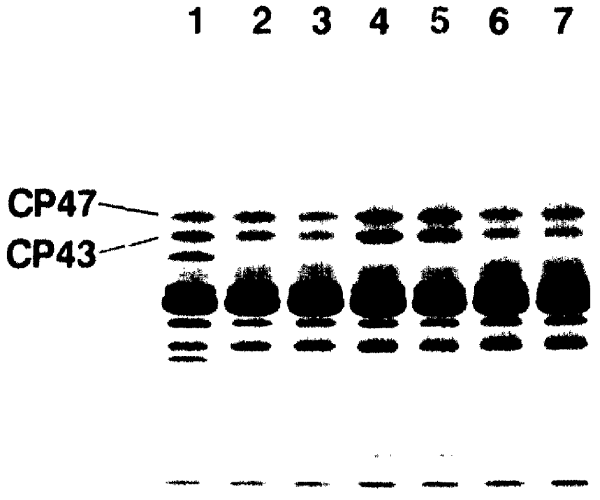

Fig. 4. Comparison of the effects of Tris-treatment on the PS II membranes in the light and in the dark. Lane 1, control PS II membranes incubated with buffer A in the light. Lane 2, PS II membranes treated with Tris-HCl (0.8 M, pH 9.0) in the dark. Lane 3, PS II membranes treated with Tris-HCl in the light. Lane 4, the same as lane 3 but with 0.5 mM 1,5-diphenylcarbazide. Lane 5, PS II membranes treated with 1 M $CaCl_2$ in the light. Other conditions are the same as those described in the legend to Fig. 1.

out the Tris-treatment (pH 9.0) in the dark and light (Fig. 4). Tris-treatment of the PS II membranes induced only slight degradation of PS II components in the dark (Fig. 4, lane 2). By contrast, decrease in the amounts of CP43 and CP47 was significant with the same treatment in the light (Fig. 4, lane 3). In the range of light intensity from 20 to 84 $\mu E\ m^{-2}\ s^{-1}$, no difference was observed in the effect of Tris (pH 9.0) on CP43 (Fig. 4; lanes 3, 6 and 7), suggesting that the incident intensity used here (20 $\mu E\ m^{-2}\ s^{-1}$) is saturating for the effect. The deletion of CP43 observed here is in contrast with the degradation of D1 that occurs under illumination with much stronger light [16–18]. Removal of the three extrinsic proteins was also attained by $CaCl_2$-treatment under the illumination (20 $\mu E\ m^{-2}\ s^{-1}$), but in this case, the deletion of CP43 was not so significant (Fig. 4, lane 5). In the $CaCl_2$-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 [33]. These results suggest that the destructive effect of light on CP43 appears when the functioning of Mn atoms is hindered. Treatment of the PS II membrane 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, we assume that excitation of PS II induces oxidizing radicals at the donor side of PS II where Mn is effectively removed. To examine this possibility, we studied the effect of reductants for PS II on the deletion of CP43 during the Tris-treatment of the PS II membranes in the light (Fig. 4 and Table I). It is expected that the reductant blocks the formation of oxidizing radicals and, therefore, the deletion of CP43 does not occur. When 0.5 mM 1,5-diphenylcarbazide (DPC) was added to the PS II membranes in the light (20 $\mu E\ m^{-2}\ s^{-1}$), apparently no significant decrease was detected in the amount of CP43 during the Tris-treatment. The other reductants, including hydroquinone, sodium ascorbate, hydroxylamine and potassium ferrocyanide, had the same effect as DPC. The addition of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) in the light eliminated th effect of Tris-treatment on CP43 (Table I), which suggests the involvement of electron transport of PS II in the formation of the radicals.

The oxidized form of the reaction-center chlorophyll P680, and of the secondary electron donor Z, are candidates for the oxidizing radicals produced in the Tris-treated PS II membranes. It was reported that $P680^+$ and Z^+ are strongly oxidizing enough to oxidize the nearby amino acid residues or redox components [22,34]. When P680 and Z 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 I), 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

TABLE I

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

The incident intensity was 20 $\mu E\ m^{-2}\ s^{-1}$. Buffer A contained 0.4 M sucrose, 40 mM Mes-NaOH and 10 mM NaCl (pH 6.5). The concentration of each reductant was 0.5 mM. DCMU was added at a concentration of 20 μM.

Conditions	Amount of CP43 (%)
Incubated with buffer A in the dark	100
Treated with Tris in the dark	87.0
Treated with Tris under light irradiation	63.6
+ DPC	98.3
+ sodium ascorbate	91.7
+ NH_2OH	91.8
+ potassium ferrocyanide	94.1
+ hydroquinone	92.0
+ DCMU	100

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 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 degradation of CP43 among the proteins of PS II observed here, it is suggested that a portion of CP43 polypeptide is located closely to P680 and/or Z 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 Z, which affect the surrounding polypeptides non-specifically. Illumination of the PS II membranes in the Tris-treatment induced deletion of CP43 as well as D1, and CP47 (Figs. 1–3). 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 is probably 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 the Tris-treatment, with the SDS/urea polyacrylamide gel electrophoresis and Western blotting analysis with a specific antibody against CP43 (data not shown). We also tried to find a degradation product in the soluble fraction after the Tris-treatment of the PS II membranes by protein sequencing of the minor proteins detectable in SDS/urea polyacrylamide gel electrophoresis, 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 [35], which suggests 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.

Difficulty in finding the degradation products has also been met with the D1 protein. A single membrane-bound fragment of 23.5 kDa, corresponding to the N-terminal region was identified as a degradation product of D1 in a pulse-chase experiment [36], but degradation products have not been detected in vitro. Quite recently, proteolytic fragments of D1 protein, having molecular weights of 21000, 16000 and 10000, were identified immunologically in vitro [37]. Similar degradation products of D1 were also reported in study of photoinhibition with the reaction-center complex of PS II [26]. The mechanism of degradation is under the debate. It was shown that D1 protein is degraded by enzymatic proteolysis and not by a direct photocleavage reaction [37]. On the other hand, an autoproteolytic process, without participation of exogenous proteinases, was suggested with the reaction center preparation [26].

Weak-light photoinhibition of PS II has been reported [23,38,39], but no evidence was presented for the deletion or degradation of the PS II-related proteins during the the photoinhibition process. The data presented here are the first to show the significant degradation of CP43, CP47 and D1, 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.

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