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Selective and specific cleavage of the D1 and D2 proteins of Photosystem II by exposure to singlet oxygen: factors responsible for the susceptibility to cleavage of the proteins

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Abstract

Exposure of isolated Photosystem II (PS II) complexes to singlet oxygen ($^1O_2$) results in cleavage of the D1 protein to specific fragments as does illumination with strong light (Mishra, N.P. and Ghanotakis, D.F. (1994) Biochim. Biophys. Acta 1187, 296–300). We reexamined the effects of $^1O_2$, generated by the photosensitizing reaction of rose bengal, on proteins of the PS II complexes. It was found that the D1 protein and also the D2 protein were selectively cleaved into specific fragments as under strong illumination. This observation suggests that only the D1 and D2 proteins have amino acid sequences that are cleavable after attack by $^1O_2$. These two proteins were almost equally susceptible to cleavage by $^1O_2$. By contrast, when the PS II complexes that had been solubilized with SDS were exposed to $^1O_2$, no distinct fragments of the D2 protein were detected, while the D1 protein was cleaved to specific fragments, though the yield of fragments was about half of that obtained from the intact PS II complexes. These results imply that the conformation of proteins is crucial for the specific cleavage by $^1O_2$, and that only the D1 protein could retain the conformation required for the cleavage, though partly, after solubilization with SDS.

Keywords: Active oxygen; D1 protein; Photoinhibition; Photosynthesis; Photosystem II; Singlet oxygen

1. Introduction

Photosystem II (PS II) of oxygenic photosynthetic organisms is a complex assembly of membrane proteins that consists of at least 25 different proteins, and performs the photochemical reaction and the subsequent electron-transport reactions from water to plastoquinone molecules [1]. All of the redox components required for the photochemical and electron-transport reactions are bound to the reaction center complex, which consists of two homologous proteins, namely, the D1 and D2 proteins [2–4].

The D1 protein of the PS II reaction center has the highest turnover rate under illumination of all the proteins in the thylakoid membranes [5]. The half-time of the turnover of the D1 protein under light conditions suitable for plant growth ranges from several to 10 h, while it can be as little as about half an hour under illumination with strong light that causes photoinhibition of photosynthesis [6]. Under light conditions that do not cause the photoinhibition, the D1 protein is selectively degraded in vivo [5]. Under strong photoinhibitory illumination, by contrast, the D2 protein is also degraded both in vivo [7] and in vitro [8], albeit more slowly than the D1 protein.

The cleavage of the D1 protein under illumination occurs at specific sites within the protein. The cleavage site that gives rise to a major fragment of 22–24 kDa is located in the loop that connects the membrane-spanning

Abbreviations: Anti-D1, anti-$D1_C$, anti-D2 and anti-47, antibodies specific to the D1 protein, the C-terminal region of the D1 protein, the D2 protein, and the C-terminal region of the 47-kDa protein, respectively; Chl, chlorophyll; cyt, cytochrome; DABCO, 1,4-diazabicyclo[2.2.2]octane; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; P680, primary electron donor of Photosystem II; PAGE, polyacrylamide gel electrophoresis.

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helixes D and E (D–E loop) on the stromal side of the thylakoid membrane in vivo [9,10] and in vitro [8,11]. Under photoinhibitory illumination, cleavage also occurs at another site, generating a fragment(s) of about 16 kDa [8,10,11].

It is generally accepted that active oxygen species generated in PS II under illumination participates in the cleavage of the D1 protein [6,8]. However, the action of active oxygen species is still controversial, and two possibilities have been proposed. One possibility involves enzymatic cleavage by a protease(s) specific to the D1 protein after the protein is attacked by active oxygen. According to a recent model of this type [8,12], singlet oxygen ($^1O_2$) generated by the triplet state of P680 under illumination alters the conformation of the D1 protein and renders it susceptible to a serine-type protease(s) that is a component of PS II. As demonstrated previously [13], however, specific cleavage of the D1 protein occurs even in isolated PS II subcomplexes that lack the putative protease.

The second possibility is the direct cleavage by active oxygen species. Hydrogen peroxide (H_2O_2) [14,15], superoxide anion [16] and $^1O_2$ [17] are generated in PS II under illumination. On the basis of effectiveness of various scavengers, it has been proposed that these active oxygen species might directly cleave the D1 protein under illumination (e.g., [13,18–20]). In addition, it has been demonstrated that exposure of PS II to exogenous $^1O_2$ [19] and H_2O_2 [21] each lead to the cleavage of the D1 protein even in darkness, as is observed under photoinhibitory illumination.

The mechanism of the specific cleavage of the D1 protein by active oxygen species remains to be solved. In the case of the treatment with H_2O_2, oxygen radicals responsible for the cleavage (probably hydroxyl radicals, ·OH) are generated in the vicinity of the cleavage sites of the D1 protein: the radicals are generated by the reaction of H_2O_2 with the non-heme iron at the acceptor side of PS II [21], that is located on the stromal side of the thylakoid membrane [2,4]. Therefore, the cleavage of the D1 protein at specific sites could be explained by the site-specific generation of the radicals in this case. To examine if an intrinsic feature of the D1 protein contributes to the susceptibility to cleavage, it is necessary to investigate the effects of active oxygen species when the entire PS II or the entire D1 protein is uniformly exposed to active oxygen.

In this study, we investigated the effects of exogenous $^1O_2$ on proteins of PS II using isolated PS II complexes. We found that both the D1 and D2 proteins were selectively cleaved to specific fragments as observed under photoinhibitory illumination. It was also found that, when the PS II complexes were solubilized with SDS, only the D1 protein was cleaved to specific fragments by exogenous $^1O_2$. A possible mechanism of the cleavage and also the factors responsible for the selective and specific cleavage of the D1 and D2 proteins are discussed.

2. Materials and methods

PS II membranes were prepared from rice seedlings with Triton X-100 [13]. PS II complexes depleted of the major light-harvesting Chl complexes were prepared by treatment of the PS II membranes with *n*-heptyl β-D-thioglucoside by the method of Kashino et al. [22] with modifications [13]. Chl was determined by the method of Arnon [23].

The PS II complexes were exposed to $^1O_2$ by illuminating the complexes with green light in the presence of rose bengal, as follows. The PS II complexes were suspended in 1 mM *n*-dodecyl β-D-maltoside, 10 mM NaCl, 0.4 M sucrose and 50 mM MES-NaOH (pH 6.5; treatment medium) at 100 μg Chl/ml and allowed to stand in darkness at 25°C for 10 min. The suspension (100 μl) was placed in a plastic cuvette (1 × 1 × 1 cm^3), supplemented with 1/100 vol. of an aqueous solution of rose bengal, and then illuminated from above with green light at 200 μE m^{-2} s^{-1} and 25°C for 10 min with gentle stirring. Green light was obtained by passing light from a projector lamp through a 10-cm-thick layer of water, two heat-reflecting filters and an interference filter of the maximum transmittance at 550 nm (half-width 10 nm). At this wavelength rose bengal has maximum absorbance but PS II complexes have minimum absorbance. After the illumination, the suspension was immediately supplemented with 1/10 vol. of the treatment medium that contained 100 mM histidine and then the complexes were solubilized in 50 mM Na_2CO_3, 50 mM dithiothreitol, 2% SDS and 12% (w/v) sucrose. The solubilized sample was kept at −30°C prior to analysis.

The PS II complexes were treated with photoinhibitory light and with H_2O_2 as described previously [13,21]. The PS II complexes, suspended in the treatment medium at 100 μg Chl/ml, were illuminated with white light (8 mE m^{-2} s^{-1}) at 10°C for designated times or they were incubated with 10 mM H_2O_2 in the presence of 2 mM EDTA in darkness at 25°C for 30 min.

SDS-PAGE and subsequent immunoblotting were performed as described previously [13]. Antisera used for immunoblotting were anti-D1 raised against the entire D1 protein, anti-$D1_C$ raised against a synthetic peptide that corresponded to the residues 326–333 of the D1 protein, anti-D2 raised against the entire D2 protein [21], and anti-47 raised against a synthetic peptide that corresponded to the last 19 residues at the C-terminus of the 47-kDa protein of spinach (a generous gift from Dr. R. Barbato).

3. Results

Fig. 1 shows the changes in proteins of isolated PS II complexes on exposure to $^1O_2$. In this study, $^1O_2$ was generated by illuminating the PS II complexes with green light in the presence of rose bengal. It has been reported

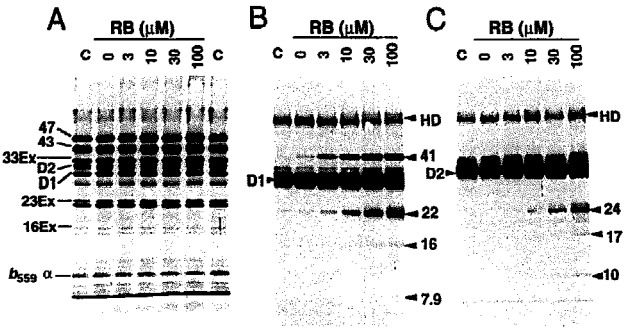

Fig. 1. Effects of $^1O_2$ on proteins of PS II complexes. PS II complexes were illuminated with green light in the presence of designated concentrations of rose bengal (RB) for 10 min. (A) Polypeptide profiles after staining with Coomassie brilliant blue R-250; (B and C) immunoblot profiles with anti-D1 and anti-D2, respectively. In B and C, samples of about 100 times the optimum amount for quantification of the intact D1 and D2 proteins were subjected to SDS-PAGE for the detection of fragments. Apparent molecular masses of fragments were estimated from their mobilities on the gel, with intrinsic proteins of PS II taken as molecular-mass markers. C denotes a control sample kept in darkness in the absence of rose bengal, and HD denotes the heterodimer of the D1 and D2 proteins.

[20] that exogenous $^1O_2$ can result in the formation of significant amounts of high-molecular-mass aggregates of proteins that fail to enter the gel during SDS-PAGE. For our detailed investigation of the effects of $^1O_2$ on individual proteins in PS II, we employed experimental conditions that did not form the aggregates of proteins. As seen in a Coomassie-stained gel (Fig. 1A), a small amount of the high-molecular-mass aggregates was detected in the upper part of the gel only at 100 μM rose bengal. Under these conditions, the bands of proteins were modified only slightly: the positions on the gel of bands of several proteins, namely, the D1 and D2 proteins, the 47- and 43-kDa proteins of the core antenna, and the extrinsic 33-kDa protein, were shifted slightly toward the origin, and the bands of the D1 and D2 proteins and of the α subunit of cyt b_{559} became fainter with increasing concentrations of rose bengal.

Immunoblots with anti-D1 and anti-D2 (Fig. 1B,C) revealed that exposure to $^1O_2$ led to cleavage of the D1 and D2 proteins to specific fragments. The D1 protein was cleaved to fragments of 22, 16 and 7.9 kDa. Although barely visible in Fig. 1B, another fragment of 9.3 kDa was also generated, albeit at a much lower level than the 7.9-kDa fragment (see Fig. 2). The D2 protein was cleaved to fragments of 24, 17 and 10 kDa. Concomitantly, a band of 41 kDa that cross-reacted with anti-D1, possibly a cross-linked adduct of the D1 protein and the α subunit of cyt b_{559} [24], was also generated. The amounts of fragments of the D1 and D2 proteins and the 41-kDa adduct increased with increased concentrations of rose bengal. The cleavage of the D1 and D2 proteins to specific fragments and the formation of the 41-kDa adduct were also observed in isolated thylakoids, PS II membranes and reaction center complexes on exposure to $^1O_2$ (data not shown).

The damage to the D1 and D2 proteins caused by

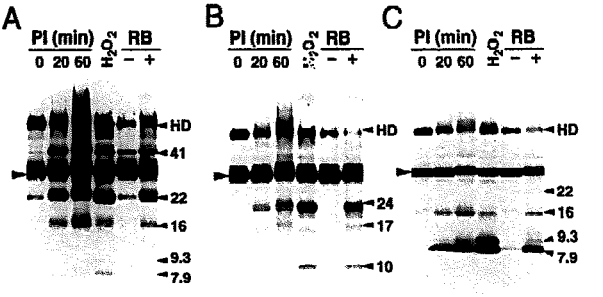

Fig. 2. Comparison of damage to the D1 and D2 proteins caused by photoinhibitory illumination, treatment with H_2O_2 and exposure to $^1O_2$. PS II complexes were illuminated with strong white light for designated times (PI), treated with 10 mM H_2O_2 in darkness for 30 min (H_2O_2), or illuminated with green light in the presence of 100 μM rose bengal (RB) for 10 min. Immunoblots with anti-D1 (A), anti-D2 (B) and anti-$D1_C$ (C) are shown. Arrowheads indicate the positions of the intact protein bands.

exposure to $^1O_2$ was compared with that caused by photoinhibitory illumination and with that caused by treatment with H_2O_2 (Fig. 2A,B). It was obvious that the overall pattern of the damage was quite similar in each case, although the mobility shifts of bands were marked and the high-molecular-mass aggregates accumulated in the case of photoinhibitory illumination. As discussed previously [13], the mobility shifts and aggregation resulted from the actions of active oxygen species (see Refs. [25,26]). No fragments of the 47-kDa protein were detected in each of the three different treatments by immunoblotting with anti-47 (data not shown).

If the marked mobility shifts of protein bands in the case of photoinhibitory illumination were taken into consideration, the sizes of fragments of the D1 and D2 proteins were almost the same in each of the three different treatments (Fig. 2A,B). This result suggests that the D1 and D2 proteins are cleaved at identical sites in each case. This possibility was confirmed by immunoblotting with anti-$D1_C$ specific to the C-terminal region of the D1 protein (residues 326–333).

We demonstrated previously that treatment with H_2O_2 results in cleavage of the D1 protein in two different regions, namely, one located between residues 250–280 in the D–E loop and another located within or immediately adjacent to the helix D [21]. The cleavage in the former region occurs at two different sites and gives rise to N-terminal fragments of 22 kDa and two different C-terminal fragments of 9.3 and 7.9 kDa, while the cleavage in the latter region gives rise to C-terminal fragments of 16 kDa [21]. As shown in Fig. 2C, the fragments generated by photoinhibitory illumination, by treatment with H_2O_2 and by exposure to $^1O_2$ each exhibited the same cross-reactivity with anti-$D1_C$; the 16-kDa fragment and the small fragments of 9.3 and 7.9 kDa cross-reacted with anti-$D1_C$, while the 22-kDa fragment did not. Thus, it appeared that the cleavage sites of the D1 protein were identical in each of the three different cases. As judged from the relative

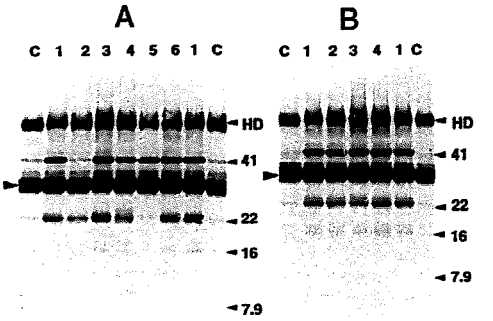

Fig. 3. Effects of active oxygen scavengers and inhibitors of serine-type proteases on damage to the D1 protein by $^1O_2$. PS II complexes were incubated with the designated additions in darkness for 5 min and then illuminated with green light for 10 min in the presence of 30 μM rose bengal. Immunoblots with anti-D1 are shown. C denotes the control sample as in Fig. 1. (A) Effects of scavengers. 1, No addition; 2, 100 mM histidine ($^1O_2$); 3, 10 mM DABCO ($^1O_2$); 4, 50 mM sodium azide (·OH, $^1O_2$); 5, 1 mM *n*-propyl gallate (·OH, RO·); 6, 50 mM D-mannitol (·OH). (B) Effects of protease inhibitors. 1, No addition; 2, 0.1 mM (4-amidinophenyl)methanesulfonyl fluoride (APMSF); 3, 1 mM phenylmethanesulfonyl fluoride (PMSF); 4, 0.1 mM N^{α}-tosyl-L-phenylalanine chloromethyl ketone (TPCK).

amounts of fragments, the cleavage of the D1 protein occurred predominantly in the D–E loop in each case. In the case of both photoinhibitory illumination and exposure to $^1O_2$, one of the two cleavage sites in this region seemed to be cleaved preferentially, since the 7.9-kDa fragment was much more abundant than the 9.3-kDa fragment.

As described above, exposure to $^1O_2$ resulted in the cleavage of the D1 and D2 proteins in the same way as photoinhibitory illumination. A marked difference from photoinhibitory illumination was that the D1 and D2 proteins were almost equally susceptible to cleavage. In the presence of 100 μM rose bengal, the amounts of the 22-kDa fragment of the D1 protein and of the 24-kDa

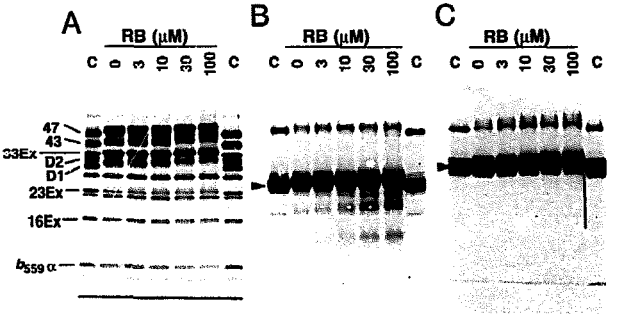

Fig. 4. Exposure to $^1O_2$ of PS II complexes that had been solubilized with SDS. PS II complexes were suspended in 1% SDS, 2 mM EDTA, 0.4 M sucrose and 50 mM MOPS-NaOH (pH 7.5), incubated in darkness at 25°C for 10 min, and then illuminated with green light in the presence of designated concentrations of rose bengal at 25°C for 10 min. (A) Polypeptide profiles; (B and C) immunoblot profiles with anti-D1 and anti-D2, respectively. C denotes the control sample as in Fig. 1. A vertical bar in C indicates a broad smear (see text).

fragment of the D2 protein were each equivalent to about 2% of those of the intact proteins in the control sample. This result contrasts with the observation under photoinhibitory illumination that the D1 protein is more susceptible to cleavage than the D2 protein [6,8,27].

Fig. 3A shows the effects of active oxygen scavengers on the damage to the D1 protein caused by exposure to $^1O_2$. Among three scavengers of $^1O_2$ tested (histidine, DABCO and azide), only histidine had a suppressive effect: the formation of the 41-kDa adduct and the cleavage of the D1 protein were both mitigated. Azide had no suppressive effect, and DABCO slightly enhanced the damage. The absence of suppressive effects of DABCO and azide has also been observed in the cleavage of the D1 protein under photoinhibitory illumination [13]. D-mannitol, a scavenger of ·OH, did not have any suppressive effect. By contrast, *n*-propyl gallate, a scavenger of ·OH and alkoxyl radical (RO·) [28], suppressed the damage. In this case, however, while the cleavage of the D1 protein was almost completely suppressed, the formation of the 41-kDa adduct was not at all affected. The cleavage of the D2 protein was also suppressed by *n*-propyl gallate (data not shown). These observations suggest that the cross-links between the D1 protein and the α subunit of cyt b_{559} might be caused by the direct action of $^1O_2$, while the cleavage of proteins involves some oxygen radicals, possibly alkoxyl radicals, generated by $^1O_2$.

Irreversible inhibitors of serine-type proteases had no effect on the cleavage of the D1 protein (Fig. 3B) and the D2 protein (data not shown). This result was consistent with previous observations by Mishra and Ghanotakis [19] and allowed us to rule out the involvement of serine-type proteases.

Fig. 4 shows the effects of exposure to $^1O_2$ of PS II complexes that had been solubilized with 1% SDS. In this experiment, the solubilization and subsequent exposure to $^1O_2$ were performed at pH 7.5, instead of pH 6.5, since incubation with SDS at pH 6.5 resulted in bleaching of Chl even in darkness. As seen in a Coomassie-stained gel (Fig. 4A), illumination with green light, even in the absence of rose bengal, caused the mobility shifts and the smearing of protein bands, as observed in the intact PS II complexes after exposure to $^1O_2$ (see Fig. 1A). This damage to proteins might have been caused by $^1O_2$ generated by photosensitizing reactions of Chl and its derivatives [29] that had been incorporated into SDS micelles. The presence of rose bengal during the illumination enhanced the damage. Almost all bands of proteins, with exception of that of about 22 kDa, became smeared and shifted toward the origin with increasing concentrations of rose bengal. The smearing and mobility shifts were more marked in the case of the intrinsic proteins of the PS II core, namely, the D1 and D2 proteins and the 47- and 43-kDa proteins.

An immunoblot with anti-D1 (Fig. 4B) revealed that specific fragments of the D1 protein of about 22–24 and 16–18 kDa were generated even in the solubilized PS II complexes. The amounts of these fragments increased and their positions on the gel shifted toward the origin with increasing concentrations of rose bengal. The yield of fragments was about half of that obtained from the intact PS II complexes. By contrast, no distinct fragments of the D2 protein were detected and there was only a broad smear that spread out over the gel region that corresponded to 15–35 kDa (Fig. 4C). Similar damage to the D1 and D2 proteins was also observed at pH 6.5 (data not shown). It seems unlikely that the D2 protein in the solubilized PS II complexes escaped attack by $^1O_2$, since the band of the D2 protein became smeared and shifted toward the origin in the same way as that of the D1 protein. Thus, it is suggested that, unlike the D1 protein, the D2 protein in a solubilized form can not be cleaved to specific fragments even when attacked by $^1O_2$.

The cleavage of the D1 protein in a solubilized form was suppressed by scavengers of $^1O_2$ and also by *n*-propyl gallate (data not shown). The suppression by *n*-propyl gallate suggests that the cleavage of the solubilized D1 protein also involves some oxygen radicals generated by $^1O_2$.

4. Discussion

Mishra and Ghanotakis [19] demonstrated previously that exposure of isolated PS II complexes to $^1O_2$ results in cleavage of the D1 protein to specific fragments in the same way as photoinhibitory illumination. We confirmed this observation and found that the D2 protein was also cleaved to specific fragments as under illumination (Fig. 1, Fig. 2). The cleavage sites of the D1 and D2 proteins appeared to be identical to those of cleavage by photoinhibitory illumination and by treatment with H_2O_2 (Fig. 2). The exposure to $^1O_2$ also resulted in the cross-links between the D1 protein and the α subunit of cyt b_{559} and the mobility shifts of bands of several proteins (Fig. 1), which are ascribable to the actions of active oxygen species as discussed previously [13].

It is unlikely that the cleavage of proteins was catalyzed by the putative protease since irreversible inhibitors of serine-type proteases had no suppressive effect on the cleavage (Fig. 3B). Therefore, we consider that the cleavage was caused solely by the action of active oxygen species, as in the case of both photoinhibitory illumination of isolated PS II subcomplexes [13,20] and treatment with H_2O_2 [21].

In general, $^1O_2$ reacts strongly with His residues and, to a lesser extent, with Trp and Met residues [30]. On the other hand, in the case of treatment with H_2O_2, amino acid residues that coordinate to the non-heme iron, the site at which toxic oxygen radicals are generated by reaction with H_2O_2, are the most probable targets [21]. Thus, the most likely candidates for the cleavage sites are His215 and His272 of the D1 protein and His215 and His269 of

the D2 protein, which participate in the binding of the non-heme iron [2,4]. This hypothesis is supported by the molecular masses of the fragments of the D1 and D2 proteins: cleavage at His215 would give rise to fragments of 16 and 17 kDa, and that at His272/269 would give rise to fragments of 22 and 24 kDa of the D1 and D2 proteins, respectively. The cleavage sites of the D1 protein in a solubilized form might also be His215 and His272, as judged from the molecular masses of the fragments (Fig. 4), though further studies are required to test this possibility.

From the effectiveness of active oxygen scavengers (Fig. 3A), it is suggested that some oxygen radical(s) generated by $^1O_2$ might be crucial for the cleavage. We propose tentatively that this radical is the alkoxyl radical since cleavage was almost completely suppressed by *n*-propyl gallate but was unaffected by either azide or D-mannitol.

The suppression by *n*-propyl gallate of the cleavage of proteins has also been observed in the photoinhibitory illumination of isolated PS II subcomplexes [13] and the treatment with H_2O_2 [21]. The active oxygen species that induce the cleavage are $^1O_2$ and ·OH in the case of photoinhibitory illumination [13,20] and oxygen radical (probably ·OH) in the case of treatment with H_2O_2 [21]. Thus, the suppressive effect of *n*-propyl gallate that is evident in each case of these different treatments implies that a similar oxygen radical is generated, irrespectively of whether the cleavage reaction is initiated by $^1O_2$ or ·OH.

In general, when peptide bonds are cleaved by ·OH [31,32], the radical first attacks an α-carbon and/or a side chain of an amino acid residue to generate intermediate derivatives of the residue, such as alkyl (R·), alkylperoxy (ROO·), and alkoxyl (RO·) radicals. It is quite possible that *n*-propyl gallate quenches any such radical derivative generated in the D1 and D2 proteins during the course of the potential cleavage reaction. It has been demonstrated that *n*-propyl gallate also effectively slows down the turnover of the D1 protein under illumination with weak light in vivo [18]. Thus, the cleavage reaction in vivo appears to involve similar oxygen radicals. We propose that ·OH and $^1O_2$, generated either exogenously or endogenously, each attack the same amino acid residues within the D1 and D2 proteins to generate the same intermediate radical derivatives of the residues during cleavage.

Hideg et al. [33] studied the active oxygen species generated during photoinhibitory illumination of thylakoids using spin-trap techniques with EPR spectrometry, and they detected a carbon-centered (alkyl or hydroxyalkyl) radical. They proposed that this radical might be crucial for the cleavage of the D1 protein and that it could be a histidine radical [33]. This carbon-centered radical could be one of the intermediate derivatives of target amino acid residues during cleavage of the D1 and D2 proteins.

Under photoinhibitory illumination, active oxygen species are generated at specific sites inside the PS II reaction center. $^1O_2$ is generated by the reaction of the triplet state of P680 with oxygen [17]. H_2O_2 is generated by autooxidation at the acceptor side [14] and is converted to ·OH by the reaction with the non-heme iron [21]. We proposed previously [21] that such site-specific generation of active oxygen species is responsible for the selective cleavage of the D1 and D2 proteins under illumination. In the case of exposure to $^1O_2$ in this study, the selective cleavage of the D1 and D2 proteins could be explained by the accessibility of $^1O_2$ to the cleavage sites within the proteins. Since rose bengal is a water-soluble molecule, it is likely that $^1O_2$ is generated for the most part in an aqueous phase outside the PS II complexes and preferentially attacks the outer surface of the complexes, resulting in cleavage of peptide bonds in surface-exposed domains of proteins. This hypothesis well explains the observation that the cleavage occurred predominantly in the D–E loops of the D1 and D2 proteins that are exposed to the outer aqueous phase. However, the accessibility of $^1O_2$ would not be an only factor responsible for the selective cleavage, since the 47-kDa protein was not cleaved even when a band of the protein exhibited a significant mobility shift on exposure to $^1O_2$ (data not shown). We consider that only the D1 and D2 proteins have amino acid sequences that are cleavable after attack by active oxygen. Attack by $^1O_2$ of amino acid residues that are different from those at the cleavable sites could result in oxidation and/or modification of the residues [30]. Such modification would be responsible for the observed smearing and mobility shifts in bands of proteins (Figs. 1 and 4).

Even at the cleavable sites, an attack by active oxygen does not always lead to cleavage of a peptide bond. As shown in Fig. 4, the D2 protein was not cleaved to specific fragments by $^1O_2$ when it had been solubilized with SDS. This observation implies that some conformation of the D2 protein is required for the specific cleavage by $^1O_2$, and that SDS distorts this conformation. By contrast, the D1 protein in a solubilized form was cleaved to specific fragments by $^1O_2$, but the extent of cleavage was about half of that of the protein in intact PS II complexes. It is unlikely that the cleavable sites of the D1 protein have higher reactivity toward $^1O_2$ than those of the D2 protein, since these proteins were almost equally susceptible to cleavage in intact PS II complexes. Therefore, it is suggested that the effects of SDS on the cleavage of the D1 protein also resulted from distortion of the conformation. Thus, we consider that the conformation of proteins is crucial for cleavage reactions at cleavable sites. The assembly of the D1 and D2 proteins into functional PS II probably increases the efficiency of cleavage reactions.

Provided that the conformation of proteins is crucial for cleavage, it is suggested that the entire D1 protein or the cleavable sites within the protein can retain the conformation required for cleavage even after solubilized with SDS.

Further studies are required to test this hypothesis, but it could be inferred that, even after the D1 protein is disassembled from PS II under illumination in vivo [34], the protein might retain the native conformation, though partly. This feature of the D1 protein might account for the selective cleavage of the protein observed under illumination in vivo.

This and previous studies [13,20,21] have demonstrated that active oxygen species can directly cleave the D1 and D2 proteins at specific sites, and proposed that this mechanism is responsible for the cleavage of these proteins under photoinhibitory illumination in isolated PS II subcomplexes. However, it is noted again that an attack by active oxygen at the cleavable sites does not always lead to cleavage of a peptide bond. Even when intact PS II complexes were exposed to $^1O_2$, a fraction of the D1 protein was cleaved, but simultaneously, other fractions were cross-linked to form the 41-kDa adduct or modified to exhibit slightly lower mobility during SDS-PAGE (Fig. 1). Thus, subtle changes in the conformation around the cleavable sites and/or the redox state of the prosthetic groups in the PS II reaction center appears to affect the fate of the proteins after attack by active oxygen species. We proposed previously [13,21] that the primary cleavage of the D1 and D2 proteins in vivo is also performed by active oxygen species generated inside PS II, and that further degradation of the fragments requires proteases that degrade abnormal proteins, such as the Clp protease [35]. Such proteases might also degrade the cross-linked adducts and modified proteins after they are released from PS II and migrate from the grana to stromal regions of the thylakoids. This pathway could be another mechanism for complete degradation of the D1 and D2 proteins in vivo.

Acknowledgements

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