Release and Reactive-Oxygen-Mediated Damage of the Oxygen-Evolving Complex Subunits of PSII during Photoinhibition

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Oxygen-evolving complex (OEC) subunits are extrinsic proteins that are associated with the intrinsic core part of PSII at the lumenal surface, and participate in the oxygen-evolving reaction catalyzed by the Mn cluster. The three OEC subunits from higher plants have Mr of 33, 24 and 18 kDa (Yamamoto et al. 1981, Kuwabara and Murata 1982) and are designated OEC33, 24 and 18, respectively. Among the three subunits, OEC33 plays a central role in oxygen evolution, and stabilizes the catalytic Mn cluster. It helps the functional binding to PSII of OEC24 and 18, which are involved in the retention of Ca2+ (Hundal et al. 1990). The OEC subunits, together with the role of the reservoir pool of OEC subunits in the lumen, are discussed.
When the PSII membranes were illuminated with strong white light, the PSII activity, monitored by the activity of 2,6-dichloroindophenol (DCIP) photoreduction, was decreased with a half-time of about 30 min (Fig. 1). There was not much difference in the polypeptide profiles analyzed by SDS/urea-PAGE and Coomassie blue staining between the illuminated samples and the dark control, although some bands became smeared and faint after illumination for 3 h. The level of the D1 protein band of 32 kDa gradually decreased to reach two-thirds of the original level in 3 h. Concomitantly, the three OEC subunits released from the PSII membranes to the outer aqueous phase. The levels of the subunits released from PSII increased with the illumination time and reached about 30%, 30% and 15% of the total OEC33, 24 and 18, respectively, in 3 h illumination. No other protein components were released from the PSII membranes under illumination for up to 3 h. As reported previously (Hundal et al. 1990), the levels of the D1 protein and OEC33 in the PSII membranes decreased in parallel during the illumination. These results suggest that the release of OEC33 from PSII is more closely related to the photodamage to the D1 protein than to the inactivation of PSII electron transport. Since the OEC33 associated with PSII provides the high-affinity binding site for OEC24, and OEC24 together with OEC33 does the same for OEC18 (Miyao and Murata 1989), it is likely that the release of OEC33 from PSII was the main cause for the release of the other two subunits during the illumination. The low level of released OEC18 probably resulted from non-specific rebinding of the protein to the PSII membranes, which occurs at low ionic strength (Miyao and Murata 1983).

The Coomassie blue-stained bands of the OEC subunits released from PSII during the illumination appeared to be less sharp than those remaining bound to PSII (Fig. 1B), indicating some photodamage to the proteins. To confirm this possibility, the OEC subunits that remained bound to PSII and those released from PSII were compared by SDS/urea-PAGE and silver staining, the sensitivity of which is much higher than that of Coomassie blue staining. Fig. 2 clearly shows that, while the OEC33 and 24 in the membrane fraction appeared as sharp bands throughout the illumination, the proteins released from PSII showed smeared bands with significant tailing toward the origin. The smearing of the bands became more marked after longer illumination and was more prominent in OEC33 than in OEC24. In contrast, the bands of OEC18 in both the membrane and released protein fractions were not affected significantly. These results suggest that OEC33, and also OEC24 to a lesser extent, suffer photodamage during and/or after release from PSII.

The release and photodamage of the OEC subunits under photoinhibitory illumination were not unique to the isolated PSII membranes. When isolated thylakoid membranes were illuminated with photoinhibitory light, the levels of free OEC33 in the lumen, which were determined as a fraction...
polypeptide profiles after staining with silver are shown. Amounts of samples on the Chl basis were applied to SDS-PAGE. The original OEC fractions used for the reconstitution experiments. Equal subunits that had rebound to PSII (denoted by B). O represents the (denoted by UB). The pellet consisting of the reconstituted membrane was then centrifuged and the resultant supernatant was taken.

Fig. 3 Rebinding capability of OEC33 released from PSII during photoinhibitory illumination. PSII membranes were illuminated with photoinhibitory light for 3 h. The proteins released were separated from the membranes by centrifugation, dialyzed against 10 mM MES-NaOH (pH 6.5; denoted by Released) and used for reconstitution experiments with urea/NaCl-treated PSII membranes. For the control experiments, the intact OEC fraction was used (Intact). (A) Rebinding of the OEC subunits. Urea/NaCl-treated membranes were incubated with the intact or released OEC fraction at the designated molar ratios of OEC33 to PSII. The reconstituted membranes were collected by centrifugation and washed with medium A. (Left panel) Polypeptide profiles of the reconstituted membranes after staining with Coomassie blue. The molar ratios of the added OEC33 to PSII were 0, 0.25, 0.50, and 1.0 for lanes 1 through 4, respectively. (Right panel) Relationship between the amounts of added OEC33 and that rebound to PSII. The levels of OEC33 in the reconstituted samples were quantified from densitograms of the Coomassie blue-stained gel. Intact OEC33 (closed circles); OEC33 released during illumination (open circles). (B) Comparison between the OEC subunits that rebound to PSII and those that remained unbound after the reconstitution experiments. Urea/NaCl-treated membranes were incubated with the intact or released OEC fractions at a molar ratio of OEC33 to PSII of 0.5. The membrane suspension was then centrifuged and the resultant supernatant was taken as the OEC subunits that remained unbound to the membranes (denoted by UB). The pellet consisting of the reconstituted membranes was subjected to the urea/NaCl treatment to release the OEC subunits that had rebound to PSII (denoted by B). O represents the original OEC fractions used for the reconstitution experiments. Equal amounts of samples on the Chl I basis were applied to SDS-PAGE. The polypeptide profiles after staining with silver are shown.

To examine whether the photodamaged OEC subunits retained their function, reconstitution experiments were performed using urea/NaCl-treated PSII membranes depleted of all three OEC subunits but retaining the Mn cluster. As shown in Fig. 3A, the OEC33 released from PSII during illumination rebound to the depleted membranes, albeit to a lesser extent than the intact protein. Although not clear in the polypeptide profile, the released OEC24 and 18 also rebound to PSII (see Fig. 3B). Concomitant with the rebinding of the subunit proteins, the oxygen-evolving activity of the depleted membranes was partly restored (data not shown). Since the fraction of the released protein seemed to be a mixture of intact and photodamaged OEC subunits (Fig. 2), we examined which form of the protein was capable of binding to PSII by re-extracting the OEC subunits from the reconstituted membranes (Fig. 3B). It can clearly be seen that, even when the depleted membranes were reconstituted with the released protein fraction, the OEC33 that bound to the membranes produced a sharp band, while that in the unbound fraction produced a smeared band. These results suggest that the photodamaged OEC33 that exhibited the smeared band totally lost the capability of binding to PSII. The limited rebinding of the released OEC24 is ascribable to the reduced rebinding of OEC33 and also some photodamage to OEC24 itself.

OEC33 has many Lys and Arg residues, and it has been suggested that these positively charged residues are important for the interaction with PSII (Frankel and Bricker 1995, Miura et al. 1997). There was no difference in the digestion patterns after limited proteolysis with trypsin, which selectively cleaves peptide bonds at the C-terminal side of Lys and Arg residues, between the intact and released OEC33 (data not shown), suggesting that these residues in OEC33 were not significantly affected by the illumination.

Fig. 4 shows the effects of scavengers of reactive oxygen species (ROS) and chelating reagents on the release and photodamage of the OEC subunits during photoinhibitory illumination. None of the scavengers tested affected the release, while some of them suppressed the photodamage which was seen as the band smearing. Histidine (for singlet oxygen O$_2^\cdot$ and the hydroxyl radical OH$^\cdot$) and superoxide dismutase (SOD), Cyt c and Tiron (for superoxide anion O$_2^{-}$) almost completely suppressed the band smearing of OEC33 and 24. Mannitol (for OH$^\cdot$) slightly suppressed the smearing while 1,4-diazabicyclooctane (DABCO) (for O$_2^\cdot$) and catalase (for hydrogen peroxide H$_2$O$_2$) had no effect. The suppressive effects of the O$_2^\cdot$ scavengers suggest that O$_2^\cdot$ plays a key role in the photodamage to the OEC subunits. In general, O$_2^\cdot$ cannot directly damage proteins (see Davies 1987) but does so indirectly by generating highly reactive OH in the presence of metal ions: H$_2$O$_2$ generated by dismutation of O$_2^\cdot$ reacts with metal ions, such as...
Fe(II), Cu(I), and Mn(II), to generate •OH via the Fenton reaction (Halliwell and Gutteridge 1984). Consistent with this, the presence of a chelating reagent, EDTA or ethylene glycol-bis(β-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), during the illumination completely suppressed the band smearing. These results suggest that •OH generated from O$_2^\cdot^-$ is responsible for the photodamage to the OEC subunits. The absence of the suppressive effects of catalase might result from inactivation of the enzyme by O$_2^\cdot^-$, which likely reduces the heme of the enzyme.

Fig. 4 Effects of ROS scavengers (A) and chelating agents (B) on the damage to OEC subunits during photoinhibitory illumination of the PSII membranes. PSII membranes were illuminated with photoinhibitory light for 3 h in the presence of the designated additives. The OEC subunits released during illumination were analyzed by SDS-PAGE and silver staining. For the control experiment, the membranes were kept in darkness in the absence of additives (Dark). The concentrations of the additives were 5 mM for histidine, 2 mM for DABCO, 20 mM for mannitol, 0.1 mg ml$^{-1}$ for catalase from bovine liver, 50 U ml$^{-1}$ for SOD from bovine erythrocytes, 80 μM for Cyt c from equine heart, 2 mM for Tiron and 1 mM each for EDTA and EGTA. The asterisks indicate the positions of catalase (60 kDa), SOD (15 kDa), and Cyt c (12.4 kDa).

It is now evident that the release and photodamage of the OEC subunits are independent processes and only the latter involves ROS. This hypothesis was confirmed by experiments where the samples were exposed to exogenously generated •OH in darkness (Fig. 5). Exposure of the PSII membranes to •OH generated by the Fenton reaction selectively damaged OEC33 but did not induce any release of the OEC subunits from PSII (Fig. 5A). However, the damage to OEC33 observed here was different from that detected in the illuminated PSII samples. The exposure of OEC33 in PSII membranes to •OH gave rise to an additional distinct sharp band with slower migration during SDS/urea-PAGE (Fig. 5A, left), while the band of the free form became smeared by the exposure in the same manner as that induced by the photoinhibitory illumination (Fig. 5B). When proteins are damaged by •OH generated by the Fenton reaction, metal ions bound to amino acid residues play a key role and the radicals generated by the reaction with these metals preferentially oxidize residues at the metal-binding sites (Stadtman 1993). It is thus likely that the observed differences in the damage between the membrane-bound and free forms of OEC33 reflect differences in the accessibility of metal ions to such metal-binding residues. The OEC33 associated with PSII interacts with some intrinsic proteins as well as with the other two OEC subunits (Enami et al. 1989, Nield et al. 2002) and only a limited number of charged residues are exposed to the outer surface. In contrast, the free soluble form of OEC33 has a loosely folded structure (Lydakis-Simantiris et al. 1999), which would allow metal ions to bind to multiple sites within the protein. As compared with OEC33, damage to the free OEC24 and 18 caused by exposure to •OH was less marked (Fig. 5B). This result suggests that OEC33 has a higher capability of binding metal ions than the other two subunits.

To confirm the involvement of the Fenton reaction in damaging the OEC subunits during photoinhibition of PSII, the samples were exposed to •OH by incubation with the designated concentrations of H$_2$O$_2$ and 1 mM FeSO$_4$ at 25°C for 10 min. The suspension was then supplemented with 0.1 mg ml$^{-1}$ catalase and 2 mM EDTA to stop the Fenton reaction. (A) Exposure of the PSII membranes. After exposure for 10 min, the membranes and released proteins were separated by centrifugation. The OEC subunits that remained bound to PSII were released from the membranes by urea/NaCl treatment. Equal amounts of samples on the Chl basis were applied to SDS-PAGE. (B) Exposure of the intact OEC subunits. The intact OEC fraction was exposed to •OH for 10 min. The polypeptide profiles after staining with silver are shown. The arrowheads indicate the position of catalase.
Damage to OEC subunits in photoinhibition of PSII

A spot for OEC18 was not detected because of its basic pH. In the OEC fraction released from PSII by the illumination, additional spots were detected in close vicinities of those observed in the intact fraction. Several spots of 33 kDa were detected at slightly more acidic positions than that of intact OEC33 (indicated by arrow 1*), and some spots of 24 kDa were detected in positions close to that of the isoform/truncated form of OEC24 (indicated by arrow 2*). These spots of 33 and 24 kDa were cross-reacted with antibodies specific to OEC33 and 24, respectively (data not shown), and they were smeared toward the origin in the second electrophoresis. Thus, they probably represent the photodamaged forms of OEC33 and the truncated OEC24. The photodamaged form of OEC33 accounted for 30–50% of the total OEC33 protein. Even the spots corresponding to the original OEC33 and 24 seemed to include the damaged form since they were less sharp compared with the spots in the intact OEC fraction. These results indicate that the photodamage to OEC33 and 24 is not accompanied by a drastic change in the surface charge. The OEC subunits exposed to exogenously generated ‘OH exhibited almost the same profile of two-dimensional electrophoresis as that observed with the OEC subunits released from PSII during illumination. This observation strongly supports the involvement of the Fenton reaction in the photodamage to the OEC subunits.

The observations in this study clearly show that the OEC subunits are released from PSII and subsequently damaged by ROS under photoinhibitory illumination of PSII membranes. We confirmed that the same phenomena occurred in isolated thylakoid membranes (data not shown). The release of OEC33 correlated well with the photodamage to the D1 protein (Fig. 1), suggesting that structural alterations of the PSII reaction center complex are responsible for the release of OEC33. The release of the other two subunits would be a secondary event caused by the release of OEC33. The subsequent photodamage to the released subunits was mediated by ‘OH generated from O_2^- via the Fenton reaction (Fig. 4–6). OEC33 was the most susceptible to photodamage among the three, and once damaged, it lost the capability of binding to PSII (Fig. 3). The damage to OEC33 did not involve a drastic change of its surface charge (Fig. 6) or significant modification of surface-exposed Arg and Lys residues as judged from the digestion pattern with trypsin (data not shown). It seems likely that oxidation of amino acid residues renders OEC33 non-functional by affecting its flexible structure.

According to the chemistry of ROS (Halliwell and Gutteridge 1984, Stadtman 1993), H_2O_2 generated by dismutation of O_2^- reacts with metal ions bound to amino acid residues

Fig. 6 Comparison of damage to the OEC subunits caused by photoinhibitory illumination of the PSII membranes and by exogenously generated ‘OH. Profiles of two-dimensional gel electrophoresis after staining with silver are shown. (A) Intact OEC fraction. (B) OEC subunits released from the PSII membranes during photoinhibitory illumination for 3 h. (C) OEC subunits exposed to ‘OH by incubating with 3 mM H_2O_2 and 1 mM FeSO_4 for 10 min. The arrows numbered 1 and 2 indicate the spots of 33 and 24 kDa, respectively. The open arrowheads indicate the spots that were occasionally detected, the asterisks indicate the spots of the damaged OEC subunits, and the closed arrowhead in (C) indicates the spot of catalase. Under the present conditions, OEC18 was not focused because of its basic pH (9.2).
to generate 'OH. The most probable candidate for the metal ion involved in the protein damage here is Mn(II) released from the Mn cluster during the illumination (Virgin et al. 1988, Hundal et al. 1990). OEC33 is located in the immediate vicinity of the Mn cluster in PSII, and has a high capability of binding metal ions (Fig. 5B). Taken together, it seems quite possible that upon release from PSII, Mn(II) remains loosely bound to OEC33 and damages the protein in the presence of O$_2^-$. Actually, it was previously reported that OEC33 can be isolated in a Mn-carrying form when potassium ferricyanide and diaminodurene were present as oxidants (Yamamoto et al. 1984). Under illumination of PSII, O$_2^-$ is generated at the acceptor side by reduction of molecular oxygen (Chen et al. 1992, Ananyev et al. 1994), while its generation at the donor side has not yet been reported. In that case, O$_2^-$ generated on the stromal side of PSII may migrate to the lumenal surface. Passage of O$_2^-$ through protein matrices and membranes is not so unlikely, when the equilibrium between charged (O$_2^-$) and uncharged protonated (HO$_2^-$) forms is taken into consideration (see Asada 1999). These considerations are supported by previous studies, which demonstrated that the OEC subunits, presumably OEC33, participated in the SOD-like activity to scavenge O$_2^-$ generated at the acceptor side of PSII (Ananyev et al. 1994, Zhang et al. 2003).

It is generally accepted that the ROS generated inside PSII under illumination trigger the turnover of the D1 protein (Prášil et al. 1992, Aro et al. 1993). The present study suggests that they also participate in damaging the OEC subunits. Materials used in this study are isolated thylakoid and PSII membrane preparations that are lacking in the two ROS scavenging systems in the chloroplast, one located in the stroma and the other loosely bound to the stromal surface of the thylakoid membrane (Asada 1999). One might assume that, in vivo, the ROS generated inside PSII would be successfully scavenged by these systems, and that the photodamage to the OEC subunits, if any, could occur only at low temperatures where scavenging enzymes are inactive. However, PSII is located in the appressed granal region of the thylakoid membrane, which is separated from both the stroma and the non-appressed region of the thylakoid membrane where the scavenging systems are present. Therefore, it is more likely that the ROS generated inside PSII reacts with molecules inside and/or close to PSII rather than the scavenging systems located at a long distance away from PSII. We consider that the photodamage to the OEC subunits can occur in vivo to some extent, although it is more easily detectable in isolated thylakoid and PSII preparations.

During the course of photoinhibition of PSII, the OEC subunits and Mn are released from PSII in parallel (Hundal et al. 1990). We found that OEC33 has a high capability of binding metal ions and it possibly retains the released Mn(II) after liberation from PSII. Taken together, we propose that OEC33 can function as a temporary reservoir of Mn(II) and a mediator of the Fenton reaction. Although the binding of the metal ions increases a risk of OEC33 being oxidatively damaged, the intact free form in the lumen can replace the damaged protein. It is well known that the D1 protein has the highest turnover rate of all proteins in the thylakoid membrane under illumination (Mattoo et al. 1984). The D1 protein is encoded in the chloroplast genome and its transcription and translation are under the control of the physiological conditions inside the chloroplast (for a review, see Zhang and Aro 2002). By contrast, the OEC subunits are nuclear-encoded and their synthesis cannot be controlled promptly in response to changes in the chloroplast conditions. This would be the reason why the large reservoir pool of the OEC subunits is present in the lumen.

PSII membranes were prepared from market spinach according to the method of Kuwabara and Murata (1982). To isolate the OEC subunits, the membranes were treated with 0.8 M Tris (pH 9.3) at 2.0 (mg Chl) ml$^{-1}$ for 60 min and then centrifuged at 35,000 $\times$ g for 15 min. The resultant supernatant containing the OEC subunits was dialyzed against 10 mM MES-NaOH (pH 6.5) for 16 h and taken as the intact OEC fraction. For reconstitution experiments, the OEC subunits were removed from PSII by treatment of the PSII membranes with 2.6 M urea containing 200 mM NaCl as described previously (Miyao and Murata 1984b). The urea/NaCl-treated membranes were incubated with the designated amounts of the OEC subunits at 0.4 (mg Chl) ml$^{-1}$ for 60 min. The membranes were then collected by centrifugation at 35,000 $\times$ g for 15 min and washed once with 0.4 M sucrose, 10 mM NaCl and 40 mM MES-NaOH (pH 6.5; medium A) by recentrifugation and resuspension. Thylakoid membranes were prepared from intact chloroplasts that had been isolated from market spinach according to the method of Mullet and Chua (1983). All procedures were performed at 0–4°C under dim light. The samples were frozen in liquid nitrogen and stored at −80°C until use. Chl was determined by the method of Arnon (1949).

For photoinhibitory light treatment, untreated PSII membranes were suspended in medium A at 0.5 (mg Chl) ml$^{-1}$. The suspension was placed in a glass cuvette and illuminated with white light from a projector lamp at 4,000 µE m$^{-2}$ s$^{-1}$. During the illumination the suspension was stirred gently and the temperature was maintained at 25°C with circulating water under thermostatic control. To analyze the protein subunits released from PSII during the illumination, the suspension was centrifuged at 35,000 $\times$ g and 4°C for 15 min. The resultant pellet and supernatant were taken as the membrane and released protein fractions, respectively. To analyze the OEC subunits that remained bound to PSII after the illumination, they were released from the membrane fraction by the urea/NaCl treatment.

DCIP photoreduction was measured in medium A at 25°C (Miyao et al. 1995). SDS/urea-PAGE and the subsequent immunoblotting were performed essentially as described previously (Miyao 1994, Yamamoto and Akasaka 1995) using a separation gel containing 12% polyacrylamide and 6.0 M urea. The gel was stained with either Coomassie brilliant blue R-250 or silver (2D-Silver Stain II “DAIICHI”, Daichi, Japan), or
subjected to immunoblotting. Three different antisera raised against a synthetic polypeptide corresponding to residues 225–249 of the D1 protein of spinach, OEC33 and 24 isolated from the spinach PSII membranes were used. The intensities of the Coomassie blue-stained or immunoreactive bands were quantified in terms of peak areas on densitograms recorded with a TLC scanner (CS-9300PC; Shimadzu, Japan). The levels of the intact and photodamaged OEC33 were determined from the densitogram after Coomassie blue staining on the assumption that the photodamage did not affect the staining intensity of the protein. For two-dimensional gel electrophoresis, samples were first subjected to isoelectric focusing using Immobiline DryStrip pH 4–7 and a Multiphor II Electrophoresis Unit (Pharmacia LKB, Sweden) and then the polypeptides were separated by SDS/urea-PAGE.

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References


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